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Herpetozoa 37: 1–10 (2024)
DOI 10.3897/herpetozoa.37.e111749

Reproductive effort of *Plestiodon copei* (Squamata, Scincidae), a highland viviparous lizard from Central Mexico

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https://zoobank.org/373AA447-7987-4745-B7C8-29ABC892B395

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Academic editor: Johannes Foufopoulos ♦ **Received** 28 August 2023 ♦ **Accepted** 14 December 2023 ♦ **Published** 8 January 2024

Abstract

Female reproductive effort is defined as the proportion of total energy or resources devoted to reproduction. In reptiles, there is frequently high inter- and intra-population variation related to several factors, such as food availability, climatic conditions, age and size, all of which, in turn, also influence survival and future reproduction. The present study is the first reproductive effort analysis of a population of the high-mountain scincid lizard *Plestiodon copei* in central Mexico, focusing on relative litter mass (RLM), investment per capita (INV) and productivity (PROD). We also compared the reproductive efficacy of *P. copei* to those of other Mexican congeners. We collected 24 gravid females of *P. copei* over a 4-year period and recorded a total of 90 neonates born in captivity. We found significant variation in neonatal mass amongst individual females and across years. We recorded an average litter size of 3.75 and an average litter mass of 1.25 g, which were positively correlated with both the size and total mass of the reproductive females. The RLM, INV and PROD values (0.301, 0.428 g and 1.236 g × year^{-1}, respectively) for *P. copei* were greater than those for other Mexican species in the *P. brevirostris* group (0.290, 0.412 g and 1.135 g × year^{-1}), revealing that females of *P. copei* from Tenango invest a comparatively high amount of their resources in reproduction.

Key Words

life history, litter mass, litter size, reproductive investment, skink lizard, Trans-Mexican Volcanic Belt

Introduction

Reproductive investment is a central aspect of the theory of life history evolution, which states that an individual must allocate an optimal proportion of its available energy to reproduction which, in turn, will be reflected in statistics, such as in neonate/egg size and mass, litter/clutch size and mass and reproductive effort (Stearns 1992; Schwarzkopf 1993; Shine 2005). In ectothermic organisms, reproductive investment is strongly affected by both physiological and ecological constraints. In reptiles, the reproductive investment of females is strongly influenced by body size and pelvic opening (Congdon and Gibbons 1987; Sinervo and Licht 1991; Rodríguez-Romero et al. 2002, 2004, 2005; Ramírez-Bautista et al. 2016, 2017; Suárez et al. 2018). However, local environmental conditions also affect the reproductive investment of females and can lead to significant variations in the number and mass of eggs or

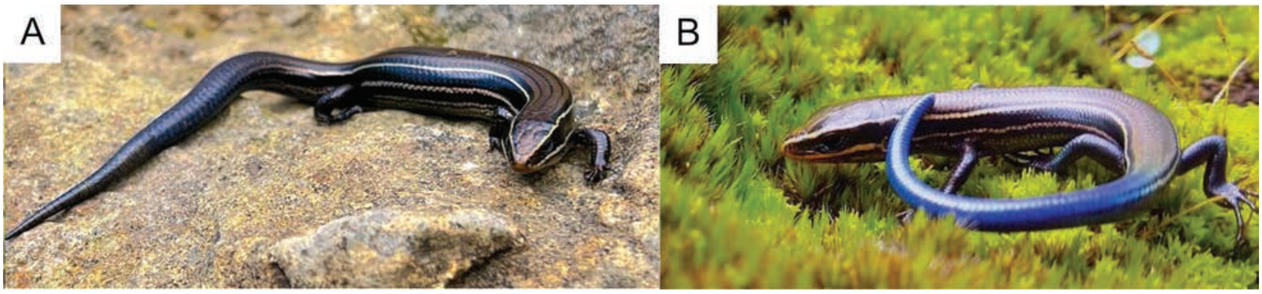

Figure 1. **A**) Adult female and **B**) neonate of *Plestiodon copei*. Photo Credits **A:** H. Sánchez-Sánchez; **B:** JC. Alvarado-Avilés.

neonates (Stearns 1992; Roitberg et al. 2013; Muñoz 2015; Suárez et al. 2018). The metabolic rate, food intake and environmental temperature are also related to reproductive investment, whereby lizard species inhabiting temperate environments may accelerate their sexual maturation and reproduce at smaller sizes, leading to smaller clutches/litters (Warne and Charnov 2008; Meiri et al. 2012).

Female lizards can also exhibit remarkable inter- and intraspecific variations in their reproductive life history traits (Cruz-Elizalde and Ramírez-Bautista 1998; López-Alcaide et al. 2020) due to environmental factors associated with temperature and precipitation regimes, as well as evolutionary history factors, such as phylogenetic constraints and adaptations to adverse environmental conditions, such as cold temperatures in high-elevation forests (Vitt and Price 1982; De Marco 1989; Rohr 1997; Abell 1999; Shine 2005; Castro-Franco et al. 2011).

In the mountainous regions of Mexico, several studies have analysed the reproductive strategies utilised in different genera of lizards, such as *Barisia* (Guillette and Casas-Andreu 1987), *Phrynosoma* (Zamudio and Parra-Olea 2000; Suárez et al. 2018), *Sceloporus* (Feria-Ortiz et al. 2001; Rodríguez-Romero et al. 2002, 2004, 2005; Bastiaans et al. 2013; Ramírez-Bautista et al. 2016; Maciel-Mata 2017; López-Alcaide et al. 2020) and *Plestiodon* (Guillette 1983; Vitt and Cooper 1986; Ramírez-Bautista et al. 1996, 1998; Ramírez-Bautista and Arizmendi 2004; Feria-Ortiz et al. 2007; López et al. 2008; García 2009; Chávez 2012; Laguna 2014; Morales 2014; Muñoz 2015; Bañuelos et al. 2016), in which both the characteristics of females and their progeny were evaluated. Some studies on the reproduction of sympatric or phylogenetically related species of the genus *Plestiodon* indicate that inter- and intra-population variations in reproductive patterns are related to environmental heterogeneity and can lead to local adaptations (Mathies and Andrews 1995; Wapstra and O'Reilly 2001; Rodríguez-Romero et al. 2002, 2004, 2005; Shine 2005; Chávez 2012; Ramírez-Bautista et al. 2016; Maciel-Mata 2017).

To estimate the reproductive investment of female reptiles, several indices have been developed that take into consideration the characteristics of both females and their progeny. Relative clutch mass/relative litter mass (RCM/RLM = fraction of female body mass devoted to reproduction) is the most frequently used index for estimating reproductive effort in lizards for both oviparous and viviparous species (Tinkle and Hadley 1975; Vitt and Congdon 1978; Vitt and Price 1982; Shine and Schwarzkopf 1992; Suárez et al. 2018; Padilla-Pérez et al. 2022). Other indices that have been estimated for several lizard species include the productivity index (PROD = total mass of offspring produced in one year) and the per capita reproductive investment index (INV = investment per capita in the offspring) (Meiri et al. 2012; Rutschmann et al. 2016).

Plestiodon copei (Fig. 1) is an endemic and high-elevation specialist skink lizard distributed in temperate pine and pine-oak forests (i.e. elevation range ca. 2466–3966 m) in the Trans-Mexican Volcanic Belt (TMVB) (Alvarado-Avilés et al. 2020). *Plestiodon copei* is a species with asynchronous gonadal activity and an autumn reproductive period (Guillette 1983; Ramírez-Bautista et al. 1996). This species produces one litter per year and apparently displays parental care, but little is known about the reproductive investment of the females of the species. The present study aimed to characterise the reproductive strategies adopted by females of *P. copei* in a mountainous region of Central Mexico by estimating the reproductive effort under extreme environmental conditions that can lead to different adaptive responses relative to those of other species of the same genus.

Materials and methods

We collected 24 late-pregnant females of *P. copei* from March–early April 2014 to 2017 near Tenango del Valle (19°05'54.74"N, 99°38'26.43"W) at an elevation of ca. 3030 m in the TMVB of central Mexico. Gravid females of *P. copei* were identified *in situ* by their greater abdominal volume. All females were captured in a landscape dominated by pine forests (*Pinus teocote, P. montezumae* and *P. rudis*) and grasslands (*Muhlenbergia macroura*) and surrounded by agricultural fields (Alvarado-Avilés et al. 2020).

We transported the collected females to the Laboratory of Genetic and Molecular Evolution (Universidad Autónoma del Estado de México) and individually housed them in plastic boxes (400 × 220 × 180 mm) with natural substrate (e.g. soil, gravel, mulch) and refuge objects (e.g. rocks and bark) obtained from the capture site. All females were maintained according to the thermal conditions described by García (2009), were fed live food every two days (crickets and mealworms) and were supplied with water *ad libitum*.

Reproductive effort estimation

We recorded the snout–vent length (SVL) and the total mass (TM = female body mass before birth) of each gravid female after capture. Females were checked at least once per day for 2 to 3 weeks and weighed daily until parturition. After birth, the following data were immediately recorded: absolute mass (AM = female body mass after birth), litter size (LS = number of neonates produced by a female), litter mass (LM = sum of the masses of all neonates produced by a female) and mean litter mass (MLM = average mass of the neonates of each litter).

The reproductive effort of *P. copei* females was estimated as follows: (a) relative litter mass; RLM = LM/AM; (b) reproductive investment per capita; INV = (TM−AM)/LS; and (c) estimated productivity; PROD = (MLM × LS) × year^{-1} (Tinkle and Hadley 1975; Vitt and Congdon 1978; Vitt and Price 1982; Rodríguez-Romero et al. 2002, 2004, 2005; Castro et al. 2011; Meiri et al. 2012; Bastiaans et al. 2013; Muñoz 2015; Rutschmann et al. 2016; Suárez et al. 2018). After all the measurements were made, both the females and neonates were released at the place where the female was captured. We also estimated the reproductive effort of other Mexican *Plestiodon* species by examining published data. Recently, taxonomic changes were considered for undescribed species (Pavón-Vázquez et al. 2018).

Data analysis

We checked all variables for normality using the Shapiro–Wilk test and for the presence of outliers with box plots in SPSS ver. 24 (IBM Corporation 2016). To estimate whether the variations in litter characteristics could be explained by female traits, we performed linear regression analysis on the LS, LM, RLM, INV and PROD variables, using the SVL, TM and AM of the females as independent variables. To evaluate whether female mass affects reproductive characteristics, we conducted two regression analyses using LS and MLM as dependent variables and the residuals of the regression of AM on SVL (a measure of robustness) as independent variables (Castro-Franco et al. 2011). We estimated the coefficient of variation (CV = standard deviation/mean × 100%), a standardised measure of the dispersion of a frequency distribution (Sokal and Rohlf 2012; Hutchings 2021) of SVL, LS, LM, MLM and the three reproductive effort indices; values whose CVs were $\leq 5\%$ were considered invariant and those whose CVs were $> 5\%$ were considered variable, as described by Castro-Franco et al. (2011) and Suárez et al. (2018) in studies on reproductive effort in Mexican lizard species. Finally, after checking the homogeneity of the variances with Levene's tests and not assuming the same variances, we analysed whether the mass of neonates at birth differed between litters and the annual variation in mean litter mass using Welch's ANOVA and Games–Howell post hoc tests in SPSS ver. 24.

Results

Births

Twenty-four gravid females were captured over a 4-yr period. The SVL ranged from 51.8 mm to 69.0 mm and the mean size was 62.711 mm (SD = 4.141 mm, CV = 6.6%, n = 24). In captivity, the females gave birth to a total of 90 neonates (range = 2−5 neonates per litter, mean = 3.750, SD = 0.897, CV = 23.9%) from mid-April to the last week of June. LMs ranged from 0.659 g to 1.844 g (mean = 1.250 g, SD = 0.310, CV = 31.2%, n = 24), while MLMs ranged from 0.256 g to 0.405 g (mean = 0.330 g, SD = 0.033, CV = 10.0%, n = 24).

Reproductive effort

For *P. copei*, the estimated range for RLM was 0.129−0.530 (mean = 0.301, SD = 0.094, CV = 31.2%, n = 24), the range for INV was 0.190−0.694 g (mean = 0.428 g, SD = 0.103, CV = 24.1%, n = 24) and the range for PROD was 0.659−1.844 g × year^{-1} (mean = 1.236 g × year^{-1}, SD = 0.326, CV = 26.4%, n = 24).

Female characteristics and their effects on reproductive output

Correlation analyses revealed significant positive correlations between female SVL and LS ($r = 0.427$, $R^2 = 0.182$, $F_{1,23} = 4.903$, $p = 0.037$) and LM ($r = 0.458$, $R^2 = 0.210$, $F_{1,23} = 5.851$, $p = 0.024$) (Fig. 2A, B). Similarly, we found significant positive correlations between female TM and both litter traits (LS: $r = 0.616$, $R^2 = 0.379$, $F_{1,23} = 13.451$, $p = 0.001$; LM: $r = 0.527$, $R^2 = 0.278$, $F_{1,23} = 8.455$, $p = 0.008$) (Fig. 2C, D). Analyses between the SVL of females and the three estimated reproductive investment indices revealed a significant correlation only for PROD ($r = 0.433$, $R^2 = 0.188$, $F_{1,23} = 5.082$, $p = 0.034$), but not for INV ($r = 0.386$, $R^2 = 0.149$, $F_{1,23} = 3.885$, $p = 0.062$) or RLM ($r = 0.253$, $R^2 = 0.064$, $F_{1,23} = 1.502$, $p = 0.233$). Additionally, a negative correlation between female AM and RLM was observed ($r = -0.291$, $R^2 = 0.540$, $F_{1,23} = 9.041$, $p = 0.006$). We also observed no significant relationships between LS ($r = 0.206$, $R^2 = 0.042$, $F_{1,23} = 0.976$, $p = 0.334$) or LM ($r = 0.024$, $R^2 = 0.001$, $F_{1,23} = 0.013$, $p = 0.912$) and the TM length of females. Finally, when comparing the masses of the neonates, we observed significant variations amongst both litters (Fig. 3) ($F_{23,20.871} = 5.765$, $p < 0.001$) (Suppl. material 1), and years ($F_{3,44.102} = 6.530$, $p < 0.001$). The MLM varied amongst years and was greater in 2014 (mean = 0.3604 g) than in other years (2015 = 0.3136 g; 2016 = 0.3301 g and 2017 = 0.3266 g) (Fig. 4).

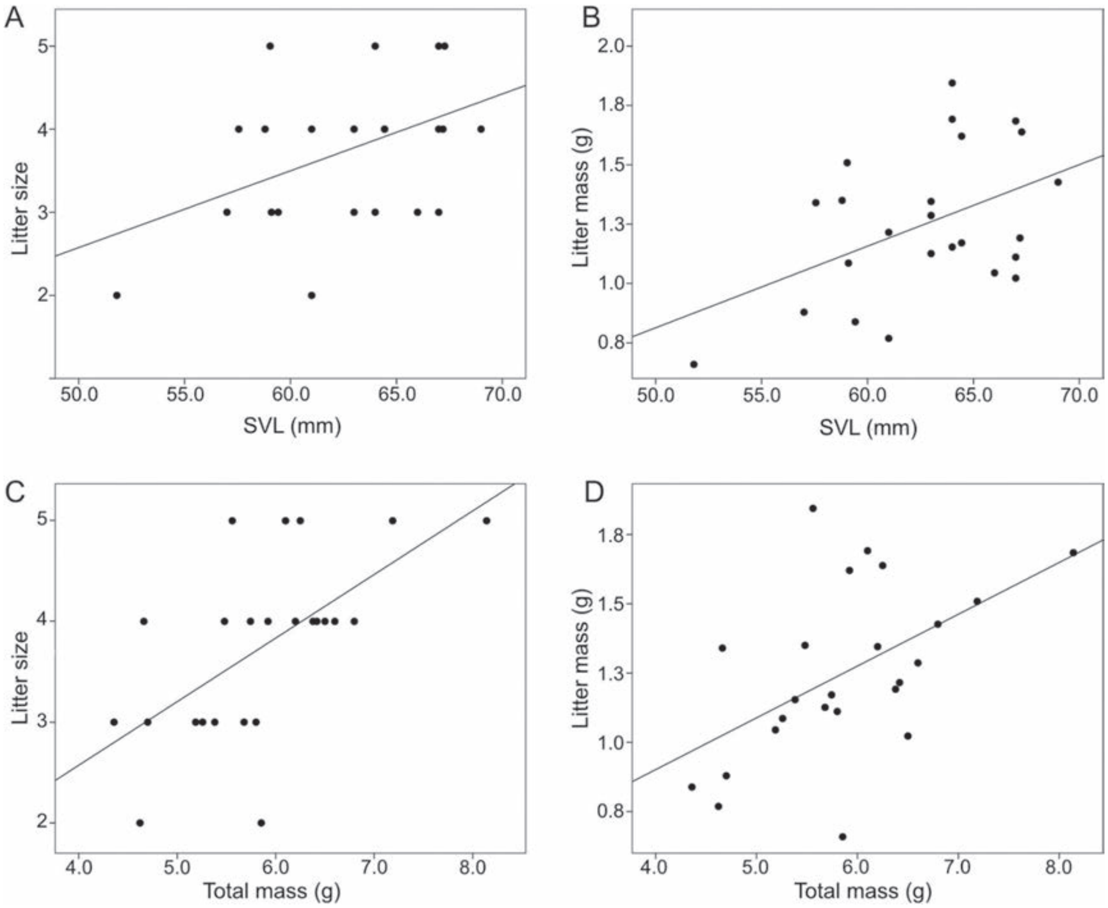

Figure 2. Relationships between individual *Plestiodon copei* female size (SVL) and (**A**) litter size, (**B**) litter mass. Additionally, relationships between individual female total mass and (**C**) litter size and (**D**) litter mass.

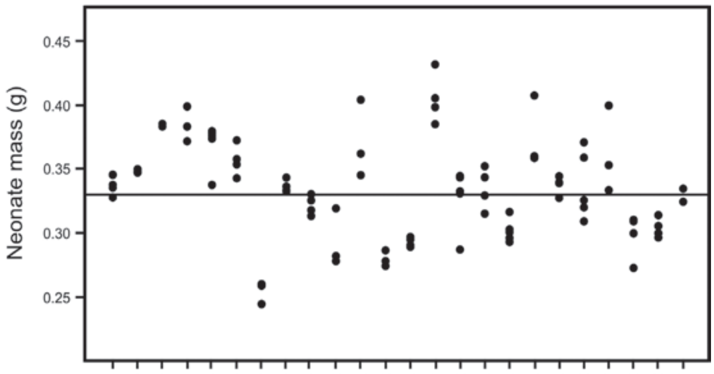

Figure 3. Within-litter variation in neonate mass (g) of *Plestiodon copei*. The horizontal line shows estimated mean litter mass (MLM) for the species.

Discussion

In several lizard species, reproductive investment has been shown to be associated with phenotypic and physiological traits, both of which are optimised by natural selection (Tinkle and Handley 1973; Vitt 1974; Rodríguez-Romero et al. 2002). The reproductive effort indices estimated in this study for *Plestiodon copei* showed that females invest a high amount of their resources in the development of their offspring and that the size of the female correlates with the size and aggregate weight of the offspring. In addition, Castro-Franco et al. (2011) discussed the pattern between the estimated values of RCM/RLM and their coefficients of variation (CVs) in several lizard species. They concluded that high RCM/RLM values and CV values above 20% could indicate a recurrent pattern for lizard species inhabiting extreme environments. In the case of *P. copei*, this

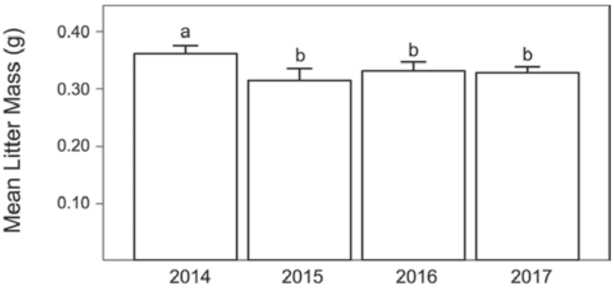

Figure 4. Annual variation in mean litter mass (g) of *Plestiodon copei*. Values are means ± SE. Letters denote differences between years, based on Games-Howell post-hoc test.

reproductive effort is associated with its habitat at high elevations within the TMVB. This condition has also been documented for other viviparous high-mountain lizard species within the TMVB, such as *Sceloporus bicanthalis* (Rodríguez-Romero et al. 2002), *Phrynosoma orbiculare* (Suárez et al. 2018) and Mexican skink lizards, such as *Plestiodon* sp. from Tlaxcala and *P. dugessi* (Muñoz 2015), all of which have high RLM values and > 20% variation. In the specific case of *P. copei*, the CVs of the three indices used to estimate reproductive effort were > 26%, also indicating high reproductive investment for the population studied.

The gravid females varied in both SVL and TM; these variables, in turn, showed significant correlations with litter size and litter mass. Ford and Siegel (1989) showed that several phenotypic traits can vary within reptile populations in response to different biotic and abiotic factors and that phenotypic plasticity can be expected in life-history traits, as might be the case in *P. copei*. The reproductive investment of *P. copei* females is strongly influenced by maternal size; specifically, in *P. copei*, litter size increases with increasing maternal body size. Similar results were reported by Guillette (1983) and Ramírez-Bautista et al. (1996) for viviparous or oviparous lizard species sympatric with *P. copei* within the TMVB (Rodríguez-Romero et al. 2002, 2004, 2005; Suárez et al. 2018).

The timing of birth in captivity for these study animals was consistent with the birth period reported by García (2009) for *P. copei*. Similarly, the birth months of *P. copei* were the same as those of other *Plestiodon* species inhabiting the TMVB (e.g. *P. dugesii*, *P. indubitus*, *P. lynxe* and *Plestiodon* sp. PT) (López et al. 2008; Chávez 2012; Lagunas 2014; Muñoz 2015). The date of parturition of lizards is typically associated with an abundance of food resources, which favours the growth and survival of neonates (Olsson and Shine 1998; Ramírez-Bautista et al. 1998, 2004; Chávez 2012; Lagunas 2014; Muñoz 2015), increasing their probability of reaching sexual maturity earlier (Chávez 2012).

The minimum gravid female sizes reported here indicate that Tenango females can reach sexual maturity at relatively small body sizes (SVL = 51.8 mm), as the SVL of the smallest pregnant female previously reported was 56.0 mm (Ramírez-Bautista et al. 1996); this finding indicates a relatively fast life history for the Tenango population. Ramírez-Bautista et al. (1996) also reported the growth rates of *P. copei* in the first year of life; by comparing the SVL of the smallest gravid female from Tenango, we can confirm that at least some *P. copei* females from Tenango become sexually mature in the year after birth. Variation in the body size of sexually mature females has been associated with the specific climatic conditions (temperature and precipitation) to which a population is exposed (Wapstra and O'Reilly 2001; Arribas and Galán 2005; Gutiérrez et al. 2010), as well as a combination of biotic (predation, foraging capacity, food availability and quality) and abiotic factors (climatic conditions) (Du et al. 2005, 2014; Roitberg et al. 2013; Hosseinian et al. 2014; Cruz-Elizalde and Ramírez-Bautista 2016; Maciel-Mata 2017; Wang et al. 2017; López-Alcaide et al. 2020). Based on the records collected for 59 years from meteorological stations near each population, the mean annual temperature in our study area was greater (Tenango = 13.4 °C) than those in other *P. copei* population sites reported by other authors (Cuajimalpa: 10.1 °C; Zoquiapan = 11.6 °C; Ajusco = 10.0 °C) (Suppl. material 2), suggesting that warmer temperatures in Tenango may favour *P. copei* females reaching sexual maturity at a smaller size (Kubisch et al. 2012; Cabezas-Cartes et al. 2018).

It has been shown that, particularly for ectothermic species living at high elevations, temperature is an important factor shaping life history strategies, as well as determining growth rate and age and size at sexual maturity (Atkinson and Sibly 1997; Angilletta et al. 2006; Amat and Meiri 2018; Padilla-Pérez and Angilleta Jr. 2022). For example, populations living in relatively warmer environments, such as the Tenango population compared to those of Cuajimalpa, Zoquiapan and Ajusco, are expected to have faster gonadal maturation. This would allow sexual maturity to be reached at a smaller size relative to populations living in colder regions, where sexual maturity is delayed and, in turn, reflected in the larger average body size of the gravid females (Wapstra et al. 2001; Morrison and Hero 2003; Angilletta Jr. et al. 2006; Meiri et al. 2013; Padilla-Pérez and Angilletta Jr. 2022).

The effects of temperature on determining the size of females of *P. copei* at sexual maturity are similar to those of other high-mountain lizards, such as *Mediodactylus heterocercus* and *Darevskia derjugini*. In all three lizard species, sexually mature females with smaller minimum body sizes were encountered under relatively warm temperature conditions, while sexually mature females with larger minimum sizes were found in comparatively cooler environments. (Altunışık and Eksilmez 2020; Altunışık et al. 2022). This variation in reproductive strategy amongst and within lizard species (i.e. the existence of females with early reproduction and small body size versus females with later reproduction at larger sizes) has already been recognised by earlier herpetologists within the fast–slow continuum hypothesis of life history theory (Adolph and Porter 1996; Shine 2005; Rojas-González et al. 2008; Pérez-Mendoza and Zúñiga-Vega 2014; Cruz-Elizalde and Ramírez-Bautista 2016; Boretto et al. 2018). The fast–slow continuum hypothesis proposes that organisms inhabiting colder environments and higher altitudes adopt a slow reproductive strategy, resulting in delayed maturation, as well as comparatively smaller litters. Conversely, in warmer climates and at lower altitudes, a rapid reproductive strategy is observed, marked by early maturation and larger litters (Promilsow and Harvey 1990; Shine 2005; Li et al. 2014; Pérez-Mendoza and Zúñiga-Vega 2014). Comparing these conditions between the populations of *P. copei* in Tenango and San Lorenzo Acopilco, we observe that the Tenango population follows a faster strategy, while the San Lorenzo Acopilco population follows a slower strategy. Thus, in Tenango, which is a population living at relatively warm temperatures at lower elevations, natural selection favours sexually mature females with smaller body sizes and larger litters. Conversely, in San Lorenzo Acopilco, which has higher elevations and colder temperatures, natural selection favours larger gravid females, but smaller litters (García 2009). Moreover, the possibility that a fast life history strategy may lead to a decrease in adult female survival has also been documented (Tinkle et al. 1970; Stearns 1992; Rohr 1997; Shine 2005; Bestion et al. 2015; Boretto et al. 2017).

The mass of the offspring is another reproductive characteristic of *P. copei* that, if influenced by environmental temperature, suggests a rapid life history strategy for Tenango animals. According to the PROD index, the total mass of offspring produced in a year is greater in Tenango than in San Lorenzo Acopilco. This pattern is similar to that observed in another temperate-adapted skink species, *Eulamprus tympanum* (Rohr 1997); in populations living at higher environmental temperatures and lower elevations, the total offspring mass is significantly greater than that in populations living at colder temperatures and higher elevations. In conclusion, female lizards living in colder environments, as in the case of San Lorenzo Acopilco, evolve a slow strategy characterised by a combination of sexually mature females with larger body sizes and lower offspring masses (Adolph and Porter 1996; Rohr 1997; Badyaev and Ghalambor 2001; Pérez-Mendoza and Zúñiga-Vega 2014; López-Alcaide et al. 2020).

Our analysis of the masses of individual *P. copei* neonates revealed marked differences both within and between litters (see Fig. 3), which have been attributed to the availability of resources in the environment (Bleu et al. 2013; Wang et al. 2017) or predation pressures (Shine and Downes 1999; Bestion et al. 2014) to which the females are exposed during gestation. However, other factors could also explain this observed variation, such as sperm storage by females or multiple paternity events (Bateson et al. 2011; Chávez 2012; Muñoz 2015). Such factors have been suggested to be important in species of the genus *Plestiodon* (Ramírez-Bautista et al. 1996; Bateson et al. 2011) and may matter for *P. copei* as well, although this has not yet been demonstrated for this species.

Moreover, the differences in MLM observed between years suggest that there are other factors that could influence the reproductive investment of *P. copei* females in Tenango. Possible factors include differences in the SVL of females or in environmental conditions, such as temperature variation and food availability (Ballinger 1977; Ji et al. 2007; Warner et al. 2007; Bleu et al. 2013; Cruz-Elizalde and Ramírez-Bautista 2016; Guo et al. 2022), which could be responsible for the heavier litters produced in 2014 compared to those in the three subsequent years. First, we ruled out the possibility that the greater MLM in 2014 was due to female size variation because the female SVL did not significantly differ across the four years. Second, data from the nearest meteorological station showed that there was no significant variation in environmental temperature that might account for the heavier litters in 2014 than in 2015–2017.

However, the lower MLMs recorded since 2015 could be attributed to reduced food availability, as the collection site showed repeated signs of human-caused grass fires due to traditional agricultural and grazing activities that allowed fires to spread into the forest. This type of vegetation disturbance has been shown – due to the lethal surface temperatures caused by fire – to negatively impact the abundance of ground-dwelling beetles which, in turn, are an important food source for *P. copei* (Nunes et al. 2006; Elia et al. 2012). The quantity and quality of the diet of females strongly influence their reproductive output (Selman and Huston 1996; Warner et al. 2007; Lovern and Adams 2008). Low feeding during fire years reduced MLM not only during that year, but also in subsequent years, as food resources for *P. copei* females decreased (Ballinger 1997; Warner et al. 2015). Bleu et al. (2013) documented that food availability is correlated with reproductive investment in *Zootoca vivipara*. Furthermore, in *Vipera aspis*, the size of the neonate is influenced by the food intake of the female immediately before ovulation and litter size depends on both long-term reserves and recent food intake (Bonnet et al. 2001). Thus, if human-caused grass fires in Tenango indeed resulted in a significant decrease in prey items consumed by females prior to fertilisation and during pregnancy, this could explain why MLM decreased in 2015–2017; however, this hypothesis needs to be tested in future studies.

In conclusion, by comparing the RLM, INV and PROD indices used to evaluate reproductive effort, we observed that *P. copei* had slightly greater values than the mean estimated for species in the *P. brevirostris* group (Table 1). The high reproductive effort values obtained for *P. copei* might be a result of inhabiting areas with extreme environmental conditions (e.g. high altitude) within the TMVB. Elevation is considered an important factor influencing the evolution of life history traits, especially in *P. copei*, as it is the most important abiotic factor determining its geographic distribution within the TMVB (Alvarado-Avilés et al. 2020). For example, colder temperatures and limited food availability at higher altitudes have been shown to lead to greater reproductive effort in high-altitude species, such as *P. copei* (Rohr 1997; Badyaev and Ghalambor 2001). Overall, we observed that the RLM obtained for *P. copei* was similar to the estimated values for other species of the Scincidae family (Vitt and Price 1982; Qualls and Shine 1997). However, when we examined data from only the species *Plestiodon* from Mexico, we observed that the RLM estimated for *P. copei* was slightly greater than those reported for *P. dugesii* and *Plestiodon* sp. PT from Jalisco and Tlaxcala, which live

Table 1. Reproductive effort estimations for several Mexican species of *Plestiodon*.

Species	Location	RCM / RLM	INV (g)	PROD (g × year^{-1})	Reference
Plestiodon bilineatus (n = 1)	Valparaíso, Zacatecas	–	–	1.820	Bañuelos et al. (2016)
Plestiodon sp. PT* (n = 7)	Volcán La Malinche, Tlaxcala	0.231	0.432	0.850	Muñoz (2015)
Plestiodon sp. PT*(n = 17)	Chilchota, Puebla	0.334	0.325	1.168	Chávez (2012)
Plestiodon sp. OX* (n = 1)	Santa Inés del Monte, Oaxaca	–	0.515	1.240	López et al. (2008)
P. indubitus (n = 1)	Sur de Tres Marías, Morelos	–	–	0.640	López et al. (2008)
P. copei (n = 3)	San Lorenzo Acopilco, CDMX	–	–	1.020	García (2009)
P. copei (n = 24)	Tenango, Estado de México	0.301	0.428	1.236	This study
P. dicei (n = 1)	Pablillo, Nuevo León	–	–	1.212	Feria-Ortiz et al. (2007)
P. dicei (n = 1)	San Antonio de las Alazanas, Coahuila	–	–	1.164	Feria-Ortiz et al. (2007)
P. dugesii (n = 21)	Mazamitla, Jalisco	0.292	0.362	1.001	Muñoz (2015)
Averages		0.290	0.412	1.135	

* Undescribed *Plestiodon* species reported by Pavón-Vázquez et al. (2018): *Plestiodon* sp. PT from Puebla and Tlaxcala; *Plestiodon* sp. OX from Oaxaca.

at lower elevations (Muñoz 2015). Conversely, the RLM obtained for *P. copei* was slightly lower than that estimated for the population of *Plestiodon* sp. PT from Puebla (Chávez 2012). We also observed that the PROD index calculated for *P. copei* from Tenango was greater than that (0.830) estimated by Meiri et al. (2012) for this species.

The observations presented here suggest that the reproductive strategy of *P. copei* from Tenango may have been shaped by the prevailing extreme environmental conditions (Castro-Franco et al. 2011; Bestion et al. 2015; Muñoz 2015), although it will be necessary to evaluate this hypothesis in future studies using both larger sample sizes and additional study sites. Furthermore, it will also be important to evaluate the effects of global warming and habitat loss on the reproductive strategies and sex ratio of this specialist skink.

Acknowledgments

We thank to E. Carbajal and S. H. Cuadros for their assistance in the field. Collecting permits (SGPA/DGVS/08371/14) were provided by the Secretaría de Medio Ambiente y Recursos Naturales (SEMARNAT). We also thank the anonymous reviewers for comments and suggestions to the manuscript.

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Supplementary material 1

Games-Howell post-hoc multiple comparisons

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Data type: xlsx

Link: https://doi.org/10.3897/herpetozoa.36.e97705.suppl1

Supplementary material 2

Mean monthly temperature based on 59 years of records (1951–2010) for four populations of *Plestiodon copei*

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Data type: docx

Link: https://doi.org/10.3897/herpetozoa.36.e97705.suppl2

ÖGH Herpetozoa
Austrian Herpetological Society

Herpetozoa 37: 11–20 (2024)
DOI 10.3897/herpetozoa.37.e114263

The validity of *Rana bannanica* Rao & Yang, 1997 (Anura, Ranidae)

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Academic editor: Günter Gollmann ♦ **Received** 16 October 2023 ♦ **Accepted** 26 December 2023 ♦ **Published** 11 January 2024

Abstract

One specimen of frog was collected from Mohan Town, Mengla County, Xishuangbanna Prefecture, Yunnan Province, China, which is the type locality of *Rana bannanica* Rao & Yang, 1997, currently considered as a junior synonym of *Hylarana milleti* (Smith, 1921). This specimen well agrees with the original description of *R. bannanica*. In regard to morphology, *R. bannanica* and *H. milleti* are, indeed, very similar, but can still be distinguished from each other. Phylogenetic analysis, based on mitochondrial gene sequences, showed that this specimen and *H. milleti* are different species. Hence, we remove *R. bannanica* from the synonymy of *H. milleti*.

Key Words

16S, COI, cytb, *Hylarana milleti*, resurrection, synonym, systematics, taxonomy

Introduction

The systematics and taxonomy of Golden-backed frogs of the genus *Hylarana* Tschudi, 1838 have long been chaotic (Dubois 1992; Oliver et al. 2015; Chan et al. 2020; Dubois et al. 2021; Reilly et al. 2022). Previously, it had been divided into 10 genera, namely *Hylarana*, *Hydrophylax* Fitzinger, 1843, *Amnirana* Dubois, 1992, *Chalcorana* Dubois, 1992, *Humerana* Dubois,1992, *Papurana* Dubois, 1992, *Pulchrana* Dubois, 1992, *Sylvirana* Dubois, 1992, *Abavorana* Oliver, Prendini, Kraus & Raxworthy, 2015, and *Indosylvirana* Oliver, Prendini, Kraus & Raxworthy, 2015 (Oliver et al. 2015; Chan et al. 2020). Later, except for *Abavorana*, the other nine genera were placed back into *Hylarana*, relegating those ranks as subgenera (Dubois et al. 2021; Reilly et al. 2022). At present, the website Amphibian Species of the World (https://amphibiansoftheworld.amnh.org/) also adopts this viewpoint (Frost 2023).

Rana bannanica Rao & Yang, 1997 is a poorly-known ranid species described in 1997 from Xishuangbanna Prefecture, Yunnan Province, China and its type locality is in Mohan Town, Mengla County, Xishuangbanna Prefecture, Yunnan Province, China, close to the border with Laos (Rao and Yang 1997). Since this species was described, there have been no formal collection reports of this species from its type locality.

Rana milleti Smith, 1921 was described from the Langbian Plateau, Lam Dong Province, central-southern

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Vietnam and, subsequently, this species was reported from Thailand and Cambodia (Chuaynkern et al. 2004; Stuart and Emmett 2006). Thereafter, Ohler (2007) proposed *R. bannanica* as a junior synonym of *R. milleti* by morphological data, based on several specimens collected from Phongsaly, Laos, as she considered that these specimens resemble both *R. bannanica* and *R. milleti*.

Currently, *Rana milleti* is regarded as *Hylarana milleti* and considered to be distributed in Vietnam, Thailand, Cambodia, Laos, southern China and peninsular Myanmar and *R. bannanica* is considered to be a synonym of *H. milleti* (Ohler 2007; Chan et al. 2020; Poyarkov et al. 2021; Zug 2022).

During our field surveys in Yunnan Province, China and in southern Vietnam from 2019 to 2023, we collected one frog specimen from the type locality of *Rana bannanica*, Xishuangbanna and six specimens of *Hylarana milleti* from its type locality, Langbian Plateau, Lam Dong Province (Fig. 1). We collected molecular sequence data of these specimens in order to reconstruct a molecular phylogeny and evaluate the phylogenetic position of them. Our phylogenetic analysis of molecular data demonstrated that the specimen from Xishuangbanna and the specimens from Langbian Plateau are not the same species. Thus, we resurrect *R. bannanica* and re-assess its taxonomic status.

Materials and methods

The specimen from China was collected from Manzhuang Village, Mohan Town, Mengla County, Xishuangbanna Prefecture, Yunnan Province and the specimens from Vietnam were collected from Da Lat and Di Linh, Lam Dong Province. Liver or muscular tissue samples were preserved in 99% ethanol for molecular analysis. The specimen from China was deposited in Kunming Institute of Zoology, Chinese Academy of Sciences (KIZ) and the specimens from Vietnam were deposited in Zoological Museum of Moscow University (ZMMU).

Measurements were taken with a digital calliper to the nearest 0.1 mm. We measured: snout-vent length (SVL), head length (HL), head width (HW), snout length (SL), eye diameter (ED), interorbital distance (IND), tympanum diameter (TD), forearm and hand length (FHL), total arm length (ARM), tibia length (TL), tarsus and foot length (TFL), foot length (FL) and total leg length (LEG).

Total genomic DNA was extracted from liver or muscular tissues. A fragment of the 16S rRNA (16S) gene was amplified and sequenced using the primers L2188: 5’–AAAGTGGGCCTAAAAGCAGCCA–3’ and 16H1: 5’–CTCCGGTCTGAACTCAGATCAC-

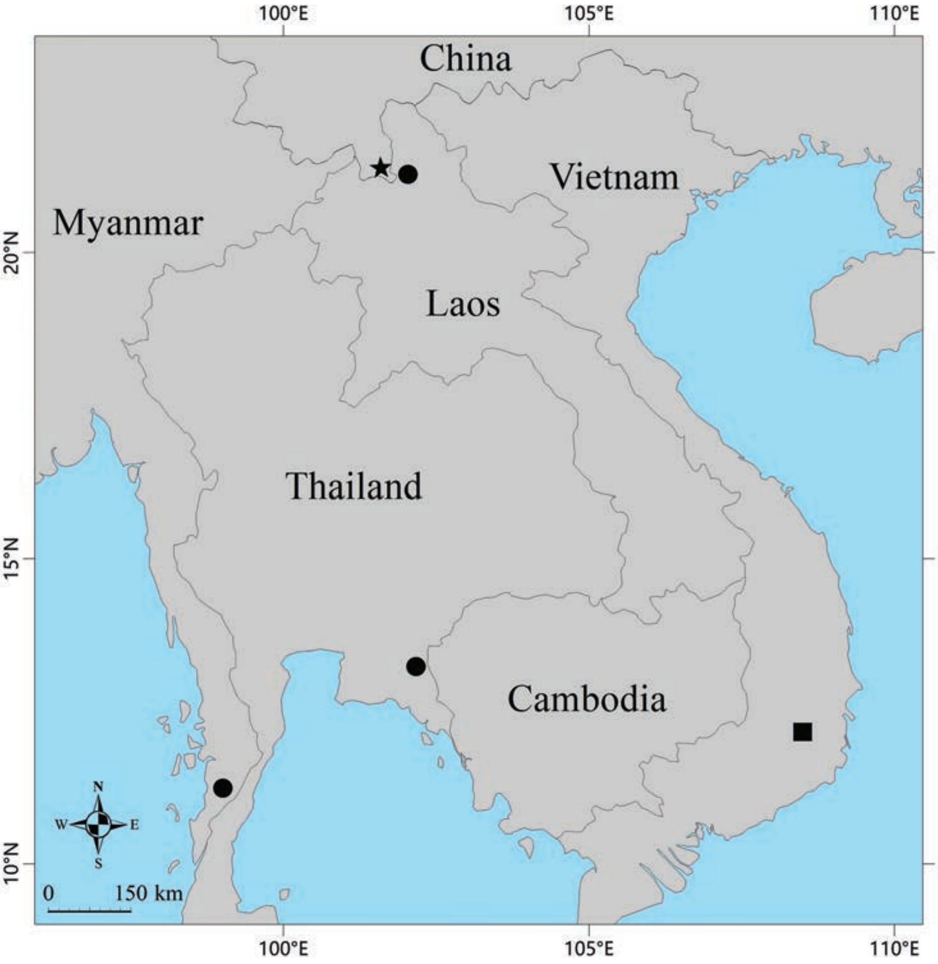

Figure 1. Map showing the type locality (black star) of *Hylarana bannanica* in Xishaungbanna, Yunnan, China and the type locality (black square) of *H. milleti* in Langbian Plateau, Lam Dong, Vietnam and other confirmed distributions (black dots) of *H. bannanica* in Phongsaly, Laos, Chanthaburi, Thailand and Tanintharyi, Myanmar, respectively, based on molecular data.

GTAGG–3’ (Hedges 1994; Matsui et al. 2006), a fragment of the cytochrome c oxidase subunit I (COI) gene was amplified and sequenced using the primers Chmf4: 5’–TYTCWACWAAYCAYAAAGAYATCGG–3’ and Chmr4: 5’–ACYTCRGGRTGRCCRAARAATCA–3’ (Che et al. 2012) and a fragment of the cytochrome b (cytb) gene was amplified and sequenced using the primers cytb-c: 5’–CTACTGGTTGTCCTCCGATTCATGT–3’ and CB-J-10933: 5’–TATGTTCTACCATGAGGACAAATATC–3’ (Bossuyt and Milinkovitch 2000). All new sequences have been deposited in GenBank. Other sequences were obtained from GenBank (Table 1).

Sequences were aligned using MAFFT 7.471 (Katoh and Standley 2013) with default parameters. Phylogenetic analyses were constructed, based on the concatenated sequences of 16S, COI and cytb genes. The best fit models were selected using the corrected Akaike Information Criterion (AICc) in ModelFinder which was implemented in IQ-TREE 1.6.12 (Kalyaanamoorthy et al. 2017). Bayesian Inference (BI) was performed in MrBayes 3.2.7 (Ronquist et al. 2012), based on the GTR+F+I+G4 substitution model for all partitions and the Markov chains were run for 5,000,000 generations and sampled every 100 generations. Maximum Likelihood (ML) analysis was performed in IQ-TREE 1.6.12 (Nguyen et al. 2015), based on the GTR+F+R4 substitution model for 16S, the TIM2+F+I+G4 substitution model for COI and the TVM+F+I+G4 substitution model for cytb and branch support was assessed using 1,000 ultrafast bootstrap replicates.

Table 1. Sequences used in this study.

Species	Voucher	16S	COI	cytb
Abavorana luctuosa	FMNH 273219	KR264088	–	KR264180
Hylarana albolabris	MVZ 234147	JX564871	JX564871	JX564871
Hylarana amnicola	USNM 584214	KY080042	KY079510	–
Hylarana annamitica	AMNH A-161290	MG606336	–	–
Hylarana arfaki	BPBM19463	KR264048	–	KR264139
Hylarana asperrima	AMNH 117606	MG552350	–	–
Hylarana attigua	FMNH 258259	MG606313	–	–
Hylarana aurantiaca	BNHS 5813	KM068913	KM069020	KM069128
Hylarana aurata	RG6548	KR264101	–	KR264192
Hylarana bahuvistara	BNHS 5921	KT281144	–	–
Hylarana bannanica	KIZ2019BN	PP034378	PP050559	PP035221
Hylarana bannanica	0281Y	KR827825	KR087730	–
Hylarana bannanica	2004.0373	KR827824	KR087729	–
Hylarana bannanica	2004.0375	KR827823	KR087728	–
Hylarana bannanica	USNM 594329	MT608886	MT608217	–
Hylarana bannanica	USNM 594330	MT608885	MT608216	–
Hylarana baramica	BNP024	DQ835348	–	–
Hylarana caesari	BNHS 5842	KM068915	KM069022	KM069130
Hylarana centropeninsularis	DWNP1189	EU604198	–	–
Hylarana chalconota	MVZ 239431	KR264095	–	KR264187
Hylarana cubitalis	2005.0228	KR827781	KR087688	–
Hylarana daemeli	BPBM36025	KR264057	–	KR264148
Hylarana darlingi	PEMA6989	KR264121	–	KR264208
Hylarana doni	BNHS 5819	KM068928	KM069035	KM069143
Hylarana elberti	USNM 579404	OL960068	–	–
Hylarana erythraea	USNM 587708	MT608883	MT608214	–
Hylarana eschatia	USNM 587721	MT608717	MT608044	–
Hylarana faber	2003.0419	KR827801	KR087706	–
Hylarana flavescens	BNHS 5845	KM068931	KM069038	KM069146
Hylarana florensis	MVZ 292869	OL960058	–	–
Hylarana fonensis	ZMBMH0145	MG552337	–	–
Hylarana galamensis	MVZ 245225	KR264096	–	KR264188
Hylarana garritor	BPBM15488	KR264042	–	KR264133
Hylarana glandulosa	KUHE 53618	AB719223	–	–
Hylarana gracilis	DZ 1173	KM068939	KM069046	KM069154
Hylarana grandocula	PNM 7588	KP298046	–	–
Hylarana guentheri	LC2018092202	MN248533	MN248533	MN248533
Hylarana humeralis	USNM 583171	MG935823	MG935527	–
Hylarana indica	BNHS 5856	KM068951	KM069058	KM069165
Hylarana intermedia	BNHS 5831	KM068960	KM069067	KM069174
Hylarana jimiensis	BPBM22832	KR264053	–	KR264144
Hylarana krefftii	–	KM247362	KM247362	KM247362
Hylarana lacrima	USNM 583124	MG935997	MG935703	–
Hylarana lateralis	MBM-JBS19852	MG935831	MG935537	–

Species	Voucher	16S	COI	cytb
Hylarana laterimaculata	KUHE 17594	AB719229	–	–
Hylarana latouchii	LSU20200422001ZL	MT702387	MT702387	MT702387
Hylarana leptoglossa	ZHRAB	OR058745	OR058745	OR058745
Hylarana lepus	USNM 584215	KY080044	KY079512	–
Hylarana longipes	CAS 254205	MG552472	–	–
Hylarana macrodactyla	0095Y	KR827808	KR087713	–
Hylarana magna	BNHS 5857	KM068964	KM069071	KM069178
Hylarana malabarica	BNHS 5880	KM068968	KM069075	KM069182
Hylarana malayana	USNM 587709	MT609321	MT608642	–
Hylarana maosonensis	K742	KR827809	KR087714	–
Hylarana megalonesa	FMNH 268981	KR264085	–	KR264176
Hylarana melanomenta	ELR165	KF477693	–	–
Hylarana milleti	ZMMU NAP-12372	PP034384	–	–
Hylarana milleti	ZMMU NAP-12373	PP034383	–	–
Hylarana milleti	ZMMU NAP-12374	PP034382	–	–
Hylarana milleti	ZMMU NAP-12862	PP034381	–	–
Hylarana milleti	ZMMU NAP-12863	PP034380	–	–
Hylarana milleti	ZMMU NAP-12864	PP034379	–	–
Hylarana milleti	DL35	OR095101	–	–
Hylarana milleti	DL38	OR095092	–	–
Hylarana milleti	DL40	OR095093	–	–
Hylarana milleti	DL51	OR095094	–	–
Hylarana milleti	ROM 34429	KR264108	–	KR264199
Hylarana cf. *milleti*	ROM 7240	AF206490	–	–
Hylarana milneana	BPBM15749	KR264044	–	KR264135
Hylarana miopus	0313Y	KR827778	KR087686	–
Hylarana montana	BNHS 5866	KM068978	KM069085	KM069192
Hylarana montosa	NCSM 76398	MG606619	–	–
Hylarana mortenseni	FMNH 263303	KR264076	–	KR264166
Hylarana nigrovittata	USNM 583176	MG936001	MG935707	–
Hylarana papua	LSUMZ97639	KR264091	–	KR264183
Hylarana parvaccola	FMNH 268599	EF487450	–	–
Hylarana raniceps	FMNH 267958	DQ650408	–	–
Hylarana roberti	CAS 243913	MG606593	–	–
Hylarana rufipes	FMNH268575	KR264081	–	KR264172
Hylarana serendipi	DZ 1145	KM068980	KM069087	KM069194
Hylarana siberu	BJE203	KF477742	–	–
Hylarana signata	FMNH 273117	KR264086	–	KR264178
Hylarana similis	KU 306511	KF477775	–	–
Hylarana spinulosa	MVZ 236683	KR264093	–	KR264185
Hylarana sreeni	BNHS 5872	KM068994	KM069102	KM069208
Hylarana supragrisea	BPBM24218	KR264055	–	KR264146
Hylarana taipehensis	USNM 595412	OM387135	OM420406	–
Hylarana temporalis	DZ 1092	KM068995	KM069103	KM069209
Hylarana tytleri	USNM 583137	MG935833	MG935539	–
Hylarana urbis	BNHS 5841	KM069008	KM069116	KM069221
Hylarana volkerjane	RG7636	KR264104	–	KR264195
Hylarana waliesa	BPBM16384	KR264046	–	KR264137

Results

Morphologically, the specimen from the type locality of *Rana bannanica* well agrees with the original description of *R. bannanica* in that all measurements and the relative lengths of body parts of this specimen are within the ranges of those in the type series of *R. bannanica* (n = 8) (Table 2). Therefore, we consider this specimen to belong to the species described by Rao and Yang (1997). However, the ratio of head width to head length in males of *R. bannanica* (n = 9) is relatively smaller than that in males of *H. milleti* (n = 5) (HW/HL 0.31–0.35 vs. 0.35–0.36), the ratio of snout length to snout-vent length in males of *R. bannanica* is relatively larger than that in males of *H. milleti* (SL/SVL 0.17–0.18 vs. 0.15–0.17) and the ratio of total leg length to snout-vent length in males of *R. bannanica* is relatively smaller than that in males of *H. milleti* (LEG/SVL 1.53–1.69 vs. 1.70–1.78) (Table 2). In addition, *R. bannanica* differs from *H. milleti* by having relatively larger discs on tips of fingers and toes, having relatively larger outer metatarsal tubercle, having tarsal folds and having vocal sacs in males.

In phylogenetic analyses of mtDNA sequences, BI and ML methods yielded similar results. The newly-generated sequences of *Hylarana milleti*, as well as the sequences of the specimens of *H. milleti* from Lam Dong and Gia Lai

Table 2. Morphological comparison between the type and topotypic specimens of *Hylarana bannanica* and the type specimens of *H. milleti*. Data for the type specimens of *H. bannanica* and *H. milleti* were obtained from the original descriptions by Rao and Yang (1997) and Smith (1921), respectively.

	Hylarana bannanica	***Hylarana bannanica***	***Hylarana milleti***	***Hylarana milleti***
	n = 8	**n = 1 (KIZ2019BN)**	**n = 5**	**n = 5**
	♂	♂	♂	♀
	Holotype and paratypes	**Topotype**	**Syntypes**	**Syntypes**
SVL	38.0–43.0	40.4	36–39	45–48
HL	10.5–17.0	16.2	14–14.5	16–17
HW	12.0–14.0	13.5	12.5–14	15–16
HL/SVL	0.28–0.44	0.40	0.36–0.39	0.33–0.37
HW/SVL	0.31–0.35	0.33	0.35–0.36	0.31–0.35
HW/HL	0.76–1.14	0.83	0.89–1.00	0.94
SL	7.0–7.5	6.8	6	7–8
SL/HL	0.41–0.67	0.42	0.41–0.43	0.41–0.47
SL/SVL	0.17–0.18	0.17	0.15–0.17	0.15–0.17
ED	/	5.0	4–4.5	5
ED/HL	/	0.31	0.29–0.32	0.29–0.31
ED/SVL	/	0.12	0.10–0.13	0.10–0.11
IND	3.8–4.2	3.9	3–4	4–5
TD	4.1–5.0	4.1	3.4–4	4
TD/HL	0.25–0.43	0.25	0.24–0.29	0.24–0.25
TD/SVL	0.10–0.12	0.10	0.09–0.11	0.08–0.09
FHL	18–20	19.2	/	/
ARM	/	25.2	21–24	27–29
TL	19.8–23.0	21.2	20–21	25–27
TL/SVL	0.52–0.56	0.52	0.54–0.56	0.53–0.60
TFL	29.0–33.0	32.7	/	/
FL	20.0–23.0	22.9	20–21	25–27
FL/SVL	0.51–0.58	0.57	0.54–0.56	0.53–0.58
LEG	58–67	68.2	62–67	79–85
LEG/SVL	1.53–1.69	1.69	1.70–1.78	1.49–1.80

provinces in Vietnam and the newly-generated sequence of the specimen from the type locality of *Rana bannanica,* as well as the sequences of the specimens previously identified under the name *H. milleti* from Phongsaly Province in Laos, Chanthaburi Province in Thailand and Tanintharyi Region in Myanmar, formed two distant, separate clades within the genus *Hylarana* (Fig. 2). Clearly, the specimens previously considered to be *H. milleti* from China, Laos, Thailand and Myanmar and the specimens of *H. milleti* from Vietnam belong to different species, although their phylogenetic positions within the genus have not been resolved. Therefore, we formally remove *Rana bannanica* Rao & Yang, 1997 from the synonym of *Hylarana milleti* Smith, 1921.

Hylarana bannanica (Rao & Yang, 1997)

Figs 3, 4, 5A

Rana bannanica Rao & Yang, 1997.

Type material. ***Holotype.*** KIZ 94001, adult male. ***Paratypes.*** KIZ 94002–KIZ 94008, seven adult males.

Type locality. Mohan Town, Mengla County, Xishuangbanna Prefecture, Yunnan Province, China.

Specimen examined. KIZ2019BN, adult male, collected by Shuo Liu from Manzhuang Village, Mohan Town, Mengla County, Xishuangbanna Prefecture, Yunnan Province, China (21°21'51"N, 101°37'17"E, 760 m elevation), on 7 May 2019.

Description of the newly-collected topotypic specimen. SVL 40.4 mm; head width less than head length (HW/HL 0.83); snout pointed, projecting beyond lower jaw; nostril slightly closer to snout than to eye; canthus rostralis distinct; loreal region vertical, slightly concave; snout length greater than eye diameter; pineal body indistinct; tympanum relatively large (TD/ED 0.82), rounded; vomerine teeth distinct; choanae close to vomerine teeth; tongue oval, posterior notch U-shaped; vocal sac openings small, on floor of each corner of mouth; vocal sacs present.

Forelimbs relatively slender; relative lengths of fingers III > I ≥ IV > II; all finger tips expanded into small discs; webbing on fingers absent; subarticular tubercles distinct, oval and prominent; supernumerary tubercles below base of fingers II–IV present, small and rounded; thenar tubercle oval, large; median metacarpal tubercle oval, prominent; outer metacarpal tubercle elongated, indistinct; grey nuptial pad on finger I indistinct.

Hind limbs medium long; relative lengths of toes IV > V > III > II > I; all toe tips expanded into small discs, discs on toes slightly larger than on fingers; webbing on toes undeveloped; subarticular tubercles distinct, oval, prominent; inner metatarsal tubercle oval; outer metatarsal tubercle rounded; tarsal fold present.

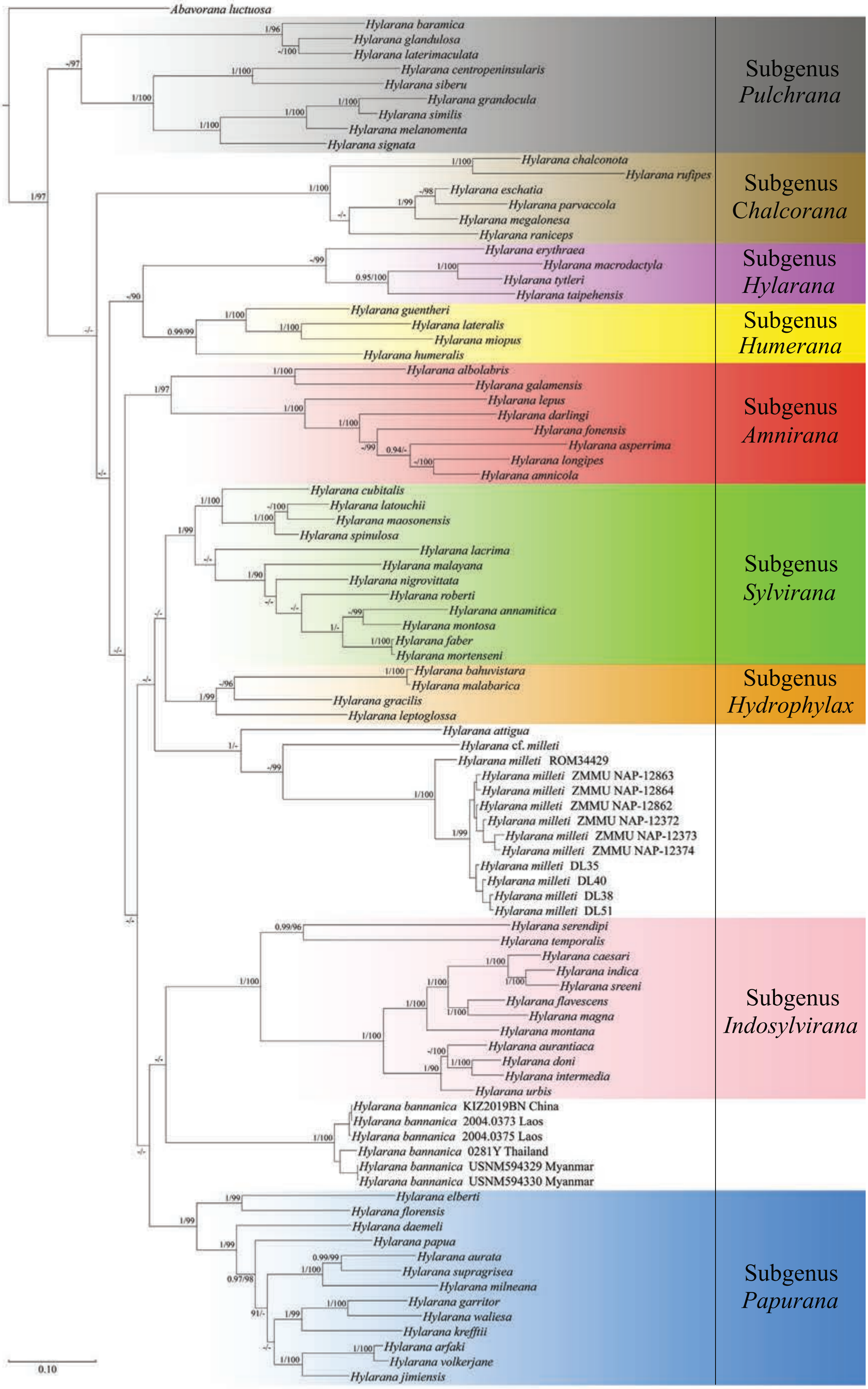

Figure 2. Bayesian phylogenetic tree of *Hylarana* inferred from the concatenated 16S, COI and cytb sequences. Numbers before slashes indicate Bayesian posterior probabilities (≥ 0.90) and numbers after slashes indicate ML bootstrap supports (≥ 90).

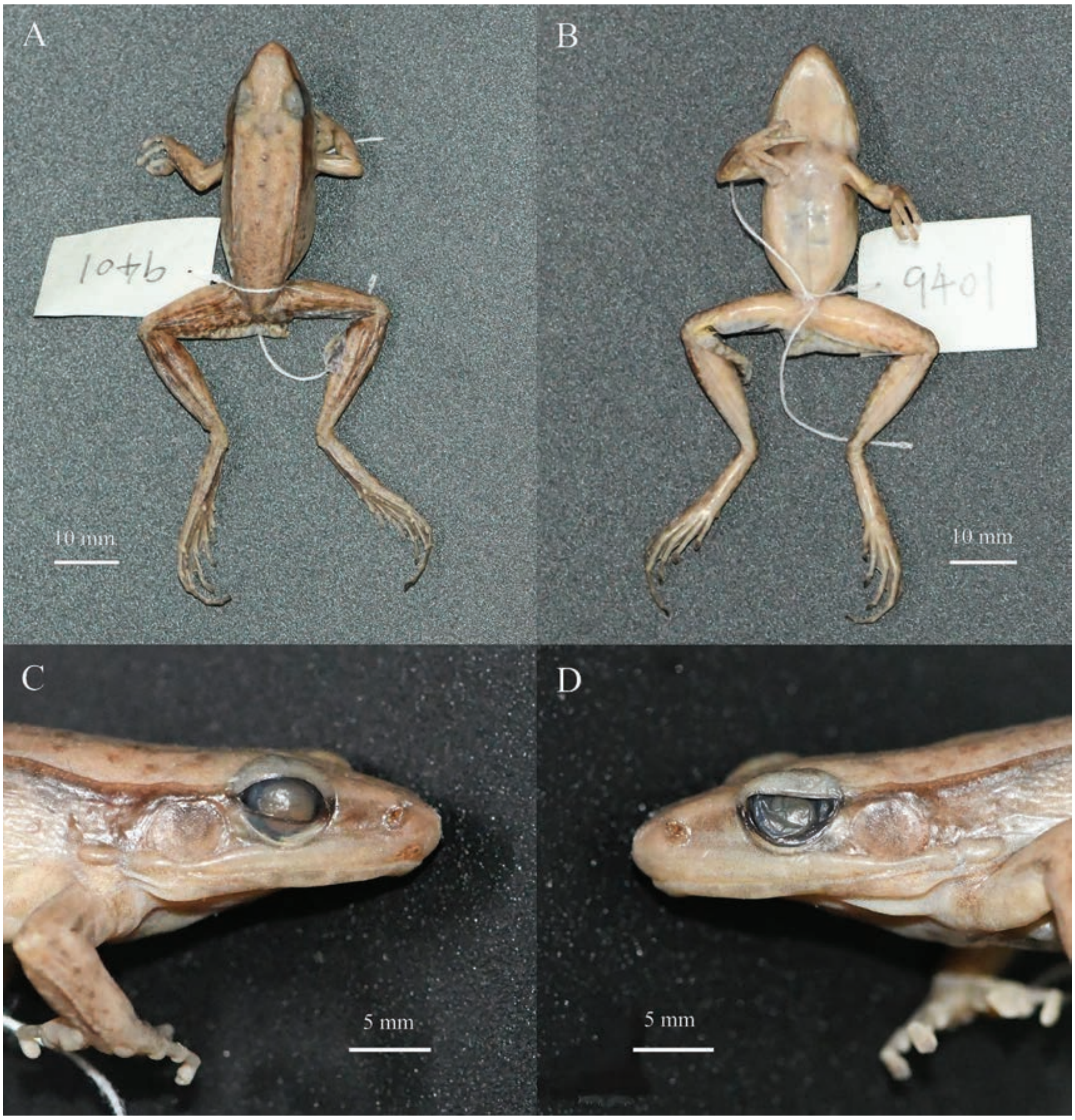

Figure 3. The holotype (KIZ 9401) of *Hylarana bannanica* in preservative. **A.** Dorsal view; **B.** Ventral view; **C.** Close-up view of the right side of the head; **D** Close-up view of the left side of the head. Photos by Dingqi Rao.

Dorsal skin slightly rough, upper lateral skin granular, lower lateral and ventral skin smooth; dorsolateral folds distinct, supratympanic fold absent; one white gland above base of forelimb behind tympanum on each side; some tiny tubercles form indistinct skin ridges on dorsal tibia.

In preservative, dorsum light brown, lateral side greyish-brown, ventral side of head and limbs yellowish-white, belly greyish-white, iris black, pupil white.

Distribution. Currently, *Hylarana bannanica* is known to be distributed in Xishuangbanna Prefecture, Yunnan Province, China, as well as northern Laos (Phongsaly Province), Thailand (Chanthaburi, Ubon Ratchathani, Amnat Charoen, Sa Kaeo, Prachin Buri, Nakhon Ratchasima and Chachoengsao provinces), southwest Cambodia (Cardamon Mountains) and peninsular Myanmar (Taninthary Region), based on data from Rao and Yang (1997), Chuaynkern et al. (2004), Stuart and Emmett (2006), Yang and Rao (2008), Poyarkov et al. (2021), Zug and Mulcahy (2020), Zug (2022) and this study.

Conservation status. Further research is required to clarify the extent of the distribution, population size, trends and conservation status of the species. *Hylarana bannanica* is distributed over a large area including many protected areas. Across its range, the species seems not to be rare. Thus, we tentatively suggest *Hylarana bannanica* be considered a Least Concern (LC) species, following the IUCN's Red List Categories (IUCN Standards and Petitions Committee 2019).

Discussion

Dubois (1992) partitioned the genus *Rana* into many sections, one of which is the section *Hylarana*, which contains two subsections, namely subsection *Hydrophylax* and subsection *Hylarana*. He treated *Hydrophylax* as a subgenus and put it together with the newly-erected subgenera *Amnirana*, *Humerana*, *Papurana*, *Pulchrana* and *Sylvirana*

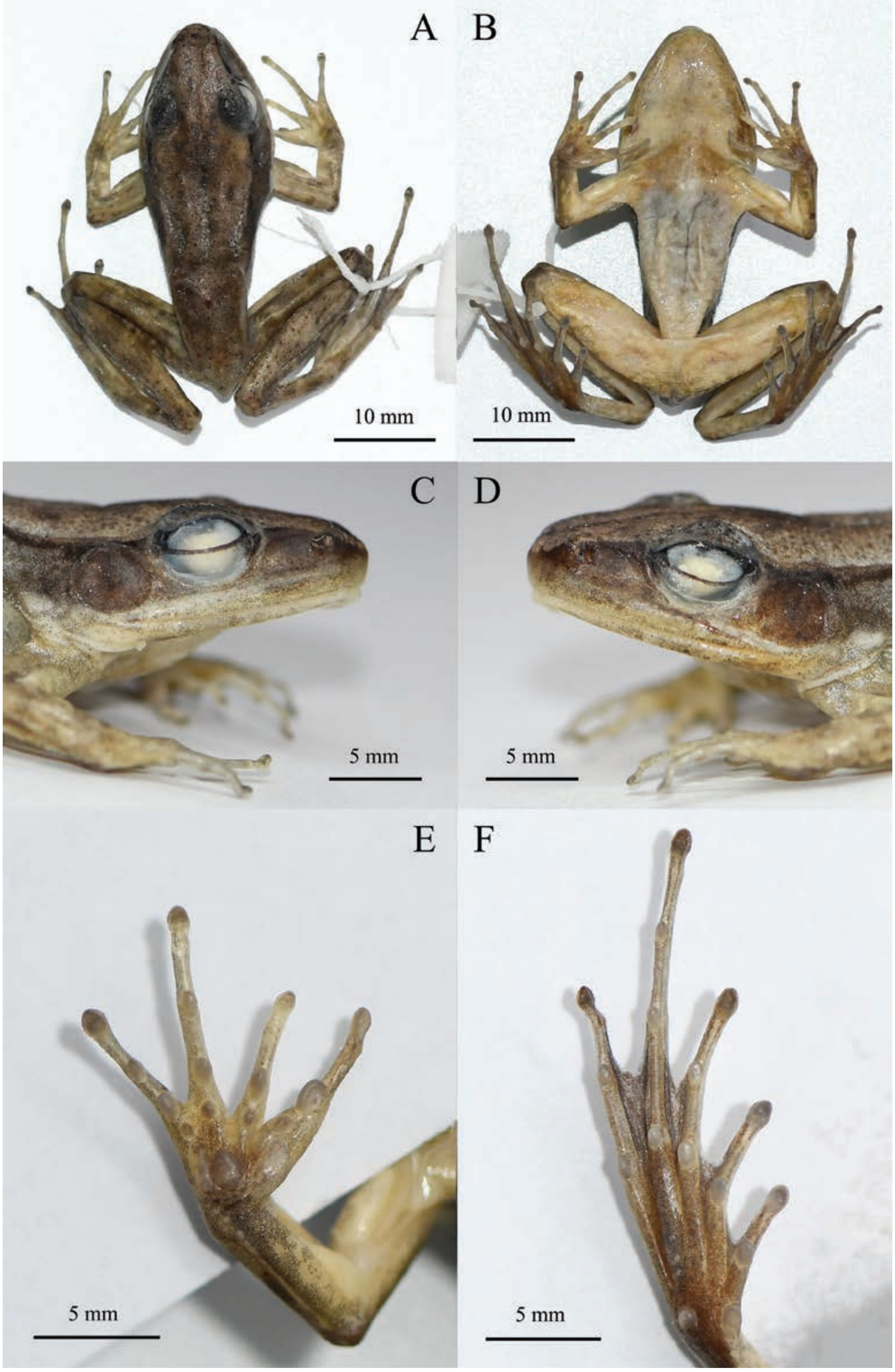

Figure 4. The topotypic specimen (KIZ2019BN) of *Hylarana bannanica* in preservative. **A** Dorsal view; **B** Ventral view; **C** Close-up view of the right side of the head; **D** Close-up view of the left side of the head; **E** Close-up view of the left palm; **F** Close-up view of the left sole. Photos by Shuo Liu.

in the subsection *Hydrophylax* and treated *Clinotarsus*, *Glandirana*, *Hylarana*, *Odorrana* and *Pterorana* as subgenera and put them together with the newly-erected subgenera *Chalcorana*, *Eburana*, *Nasirana*, *Sanguirana* and *Tylerana* in the subsection *Hylarana* and explicitly stated that these arrangements were preliminary hypotheses (Dubois 1992). Oliver et al. (2015) revised *Hylarana* sensu lato by utilising an inclusive molecular phylogeny, elevated *Amnirana*, *Chalcorana*, *Hydrophylax*, *Hylarana*, *Humerana*, *Papurana*, *Pulchrana* and *Sylvirana* to genus rank and erected two new genera, namely *Abavorana* and *Indosylvirana*. Chan et al. (2020) performed a comprehensive analysis of *Hylarana*

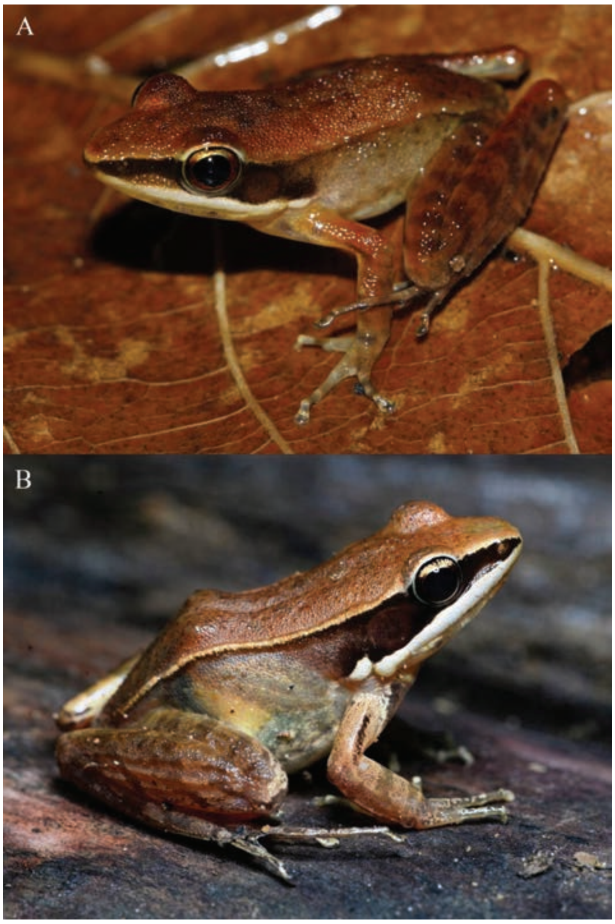

Figure 5. Comparison between *Hylarana bannanica* (**A** from Chanthaburi, Thailand) and *H. milleti* (**B** from Bidoup-Nui Ba, Lam Dong, Vietnam) in life. Photos by Parinya Pawangkhanant (**A**) and Nikolay A. Poyarkov (**B**).

sensu lato and also recognised the ten genera *Abavorana*, *Amnirana*, *Chalcorana*, *Hydrophylax*, *Hylarana*, *Humerana*, *Indosylvirana*, *Papurana*, *Pulchrana* and *Sylvirana* in it. Later, Dubois et al. (2021) found *Chalcorana* to be paraphyletic with *Pulchrana* and *Amnirana* and *Sylvirana*, *Papurana*, *Hydrophylax*, *Hylarana* and *Indosylvirana* to be polyphyletic; therefore, they placed all of the genera within *Hylarana* sensu lato, except for *Abavorana*, into the synonymy of *Hylarana*. However, we consider that these synonymous genera may be valid and need to be resurrected, at least for some of them. For example, Dubois et al. (2021) recovered *Sylvirana* to be polyphyletic due to *Sylvirana guentheri* as the sister taxon of *Humerana miopus*. In our phylogenetic analysis, *Hylarana guentheri* was also nested in the subgenus *Humerana* with strong support. Nevertheless, we cannot say directly that *Sylvirana* or *Humerana* is invalid, unless *Hylarana guentheri* is the type species of *Sylvirana*. *Hylarana guentheri* is a very common species in southern China and northern-central Vietnam and we have collected specimens of this species multiple times. This species has a large body size, which is different from most species of the subgenus *Sylvirana* and consistent with most species of the subgenus *Humerana*. Since *Hylarana guentheri* is not the type species of *Sylvirana*, therefore, we consider that *Hylarana guentheri* should be moved to *Humerana* from *Sylvirana*, and *Sylvirana* and *Humerana* are still valid, instead of synonymising them. Anyway, we still adopt the current arrangement (Dubois et al. 2021; Frost 2023) of *Hylarana* sensu lato for the time being; further morphological and molecular studies are required to elucidate the division of genera within *Hylarana* sensu lato.

Hylarana bannanica was described from Xishuangbanna, Yunnan, China, in 1997 and no new specimens from its type locality have been reported since then. The specimen (KIZ2019BN) we collected is the first rediscovery from the type locality of this species since it was described more than 20 years ago. Unfortunately, this specimen was misidentified as a subadult of the local common species *H. cubitalis* (Smith, 1917) and was not taken seriously when it was collected, so that the habitat information at the collection site and photos of this specimen in life were not recorded. In the subsequent process of specimen sorting, we discovered that this specimen is an adult male due to the presence of nuptial pad and vocal sacs, but without a large oval gland on the inner side of each elbow, which shows it is obviously not *H. cubitalis*, so we conducted a detailed study on this specimen. This experience reminds us that it is necessary to have sufficient taxonomic competence when doing fieldwork and that those who visit different locations should be aware of what species are distributed or what species may be distributed there.

In previous studies (e.g. Frost et al. (2006); Oliver et al. (2015); Chan et al. (2020); Dubois et al. (2021); Reilly et al. (2022)), *Hylarana milleti* (Fig. 5B) was once placed in the genera or subgenera *Sylvirana*, *Indosylvirana* and *Papurana*, respectively. In our phylogenetic analysis, it is evident that *H. milleti* and *H. bannanica* are not conspecific, but their phylogenetic positions within the genus have not been resolved. At present, we cannot determine which subgenus these two species belong to respectively. The systematic relationship within this genus needs to be further resolved.

Acknowledgements

We thank Parinya Pawangkhanant (AUP) for providing the photo of *Hylarana bannanica* from Thailand. We thank George Zug (USNM) for his help in this study. Thanks to the forest rangers of Xishuangbanna National Nature Reserve for their help in the field. We also thank the editors and reviewers for their working on the manuscript. This work was supported by the Science-Technology Basic Condition Platform from the Ministry of Science and Technology of the People's Republic of China (Grant No. 2005DKA21402), the project of the second comprehensive scientific investigation of Xishuangbanna National Nature Reserve, the project of Ministry of Ecology and Environment of China: investigation and assessment of amphibians and reptiles in Jinghong City, Menghai County and Mengla County, in part the Russian Science Foundation to N.A. Poyarkov (Grant No. 22-14-00037, molecular analyses) and in part the Rufford Foundation to T.V. Nguyen (Grant No. 39897-1; data analysis).

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ÖGH Herpetozoa
Austrian Herpetological Society

Herpetozoa 37: 21–23 (2024)
DOI 10.3897/herpetozoa.37.e115750

First record of *Bombina variegata* (Linnaeus, 1758) at the southern part of Euboea Island, Greece

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Academic editor: Günter Gollmann ♦ **Received** 14 November 2023 ♦ **Accepted** 24 December 2023 ♦ **Published** 12 January 2024

Abstract

The herpetofauna of Greek islands is fairly well known. However, new records enhance our knowledge of the insular distribution of reptiles and amphibians. Here, we report a new addition to the herpetofauna of Euboea Island: a dense population of the yellow-bellied toad (*Bombina variegata*) was found in Ochi Mountain, at the south part of the Island.

Key Words

Anura, distribution, islands, Mediterranean

The anuran genus *Bombina* comprises six species that mainly occur in Europe and parts of northeast Asia (Hofman et al. 2007; Fijarczyk et al. 2011). The yellow-bellied toad, *Bombina variegata* (Linnaeus, 1758), is a small-bodied (snout vent length up to 56 mm) amphibian that feeds on invertebrates, mainly insects (Ghiurca and Zaharia 2005; Bisa et al. 2007). The species has a wide distribution in central and southeast Europe (Gasc et al. 1997; Sillero et al. 2014). In Greece, the yellow-bellied toad occurs in the central and northern part of the country, in altitudes varying from 600 to 2,000 m (Valakos et al. 2008; Pafilis and Maragou 2020). Recently, a new population was reported from Paros Island (Troidl and Troidl 2019) that was presumably introduced to the Island (Tzoras et al. 2023).

On 23 September 2022, we visited the southern part of Euboea Island during a herpetological survey (under the national monitoring project of Greek herpetofauna). Around noon, close to the summit of Ochi Mountain (38.0654°N, 24.4582°E, 945 m a.s.l.), we discovered a network of small ponds that were supplied with water from a mountainous fountain (Fig. 1). Within these ponds, we counted over 80 individuals of *B. variegata*, adults and juveniles. The weather was good with partially clouded sky and air temperature around 18 °C. We captured one adult individual (Fig. 2, SVL: 62 mm, body weight: 4.9 g) that was later deposited in the Herpetological Collection of the Zoological Museum of the National and Kapodistrian University of Athens (ZMUA 4000) under a special permit issued by the Ministry of Environment and Energy (ΥΠΕΝ/ΔΔΔ/79427/2548). The site is designated as a Special Area of Conservation (GR2420001) of the Natura 2000 network of protected areas.

Though Euboea is an island, because of its close proximity to mainland Greece – nowadays it is separated by a channel (shortest distance: 29.3 m) – it shares the fate, in terms of herpetological research, of the largely undiscovered mainland (Pafilis and Maragou 2013). Indeed, islands have always attracted scientific research since the time of the founding fathers of Greek herpetology (Bedriaga 1883; Boettger 1888; Werner 1930), especially the Aegean Islands that harbour many endemic taxa have

Figure 1. The fountain, just below the summit, that waters the pond network.

Figure 2. Adult *Bombina variegata* (Linnaeus, 1758) (ZMUA 4000) from south Euboea, Greece.

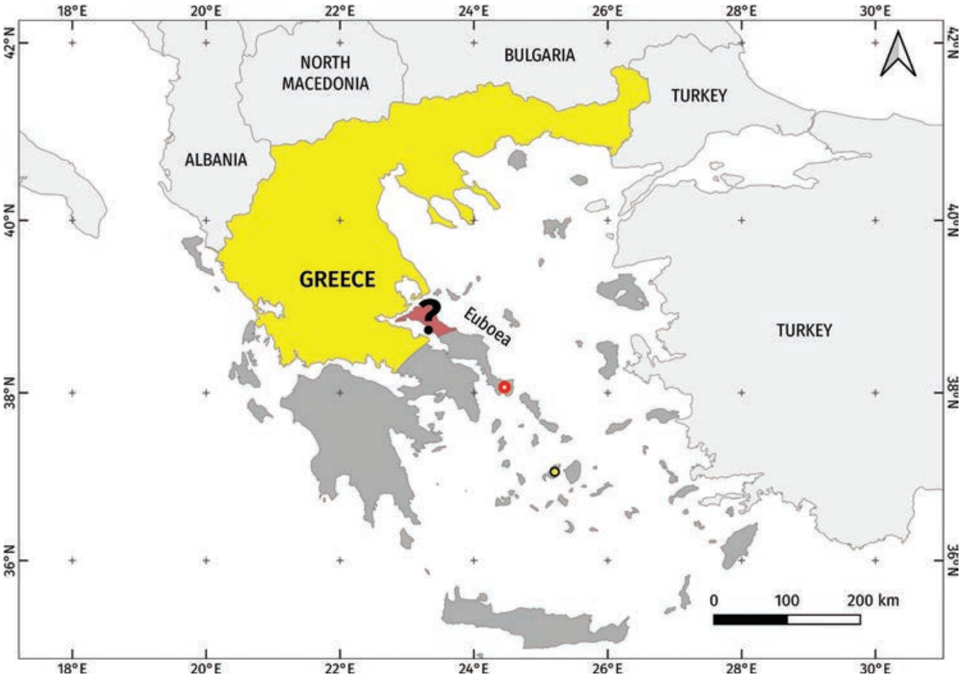

Figure 3. The distribution of *Bombina variegata* in Greece (yellow shading), including the new record from south Euboea Island reported here (red point). The occurrence of the species on north Euboea (red shading) is dubious.

been thoroughly surveyed (Pafilis 2010; Lymberakis et al. 2018). To the contrary, few studies focus on the mainland Greece and, thus, our knowledge on the continental herpetofauna remains poor (e.g. Annousis et al. 2021; Christopoulos 2022). As such, new entries in the herpetofauna of Euboea have been added during the last few years (Christopoulos et al. 2019; Pafilis et al. 2020; Strachinis 2021). We believe that the presence of the yellow-bellied toad in Euboea should be attributed to the long-standing connection with mainland Greece, from which it became isolated only recently, some 5,500 years ago (Mariolakos and Bantekas 2002).

The presence of *Bombina variegata* has never been plotted in distribution maps or been reported from the Island (Boettger 1891; Werner 1938; Valakos et al. 2008; Sillero et al. 2014; Pafilis and Maragou 2020). The only exception comes from the recent work by Tzoras et al. (2023), that probably echoes the unclear distribution of the species in Greece, as depicted in the coarsely detailed map (50 km × 50 km grid) of the European Atlas (Gasc et al. 1997), where it is hard to tell whether the yellow-bellied toad occurs on Euboea or not (Fig. 3). However, this is a typical flaw in small scale maps when zooming in on specific regions. However, even in this case, the map includes exclusively the northern part of the Island. We do believe that the species also occurs in the central and northern part of Euboea. Further targeted fieldwork will bring together the missing pieces of this small biogeographical puzzle.

Acknowledgements

The project “Monitoring and Assessing the Conservation Status of Amphibians and Reptiles (except Marine) of E.U. and national interest in Greece” funded by the E.U. and national resources was assigned by the Ministry of Environment and Energy to ATEPE Ecosystem Management Ltd with the Hellenic Herpetological Society as the scientific collaborator.

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ÖGH Herpetozoa
Austrian Herpetological Society

Herpetozoa 37: 25–42 (2024)
DOI 10.3897/herpetozoa.37.e112093

An updated checklist of the amphibians and reptiles of Nayarit, Mexico with conservation status and comparison with adjoining States

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Academic editor: Bibiana Rojas ♦ **Received** 3 September 2023 ♦ **Accepted** 24 January 2024 ♦ **Published** 12 February 2024

Abstract

Herein, we present an updated list of the species of amphibians and reptiles that inhabit Nayarit, Mexico. In the years since the publication of a previous list in 2016, threats to amphibians and reptiles in Nayarit have continued unabated and efforts to more fully catalogue the richness of Nayarit's herpetofauna have continued. Nayarit harbours 162 native species of amphibians and reptiles, representing 35 families and 85 genera. These include 37 species of amphibians (35 anurans and two salamanders) and 125 species of reptiles (one crocodile, 44 lizards, 69 snakes and 11 turtles). Of the amphibian and reptile species in Nayarit, 102 are endemic to Mexico and two endemic to Nayarit. The ecoregion with the highest richness of amphibians and reptiles in Nayarit is the Trans-Mexican Volcanic Belt followed closely by the Sierra Madre Occidental and then the Sierra Madre del Sur, the Pacific Lowlands and the Nayarit Islands. Just 5.8% of the species of amphibians and reptiles in Nayarit is IUCN-listed as Vulnerable, Endangered or Critically Endangered, 11.1% are placed in a protected category by SEMARNAT and nearly a third are categorised as high risk by the Environmental Vulnerability Score. The herpetofauna of Nayarit overlaps the most with Jalisco (88.9%). One of the main conclusions of our updated list of the amphibian and reptile species of Nayarit is that our understanding of the full complement of species is not complete, especially the reptiles.

Key Words

ecoregions, endemic species, Environmental Vulnerability Score, herpetofauna, IUCN, species status

Introduction

Woolrich-Piña et al. (2016) published a then up-to-date checklist of the amphibians and reptiles of Nayarit, Mexico, including assessments of the conservation status and threats for these taxa. In the years since that publication, several economic and social factors have changed in the State of Nayarit. In particular, several of the threats to amphibians and reptiles in Nayarit identified by Woolrich-Piña et al. (2016) have continued or even increased in extent since they published their checklist. Agricultural land cover in parts of Nayarit increased from 39% to 50% from 1999 to 2019 (Luja et al. 2022; see also Navidad Murrieta et al. (2023)). Additional continuing threats include agricultural practices, such as water consumption for irrigation and the runoff of fertilisers and

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pesticides (Loc-Barragán 2016; Ahumada Nájera et al. 2020; Loc-Barragán et al. 2020); urban expansion in the Municipality of Tepic in the Trans-Mexican Volcanic Belt (Avalos Jiménez et al. 2018, 2022) and Compostela in the Sierra Madre del Sur; tourist developments in Bahia de Bandera in Sierra Madre del Sur; highway and road construction in Jala, Sierra Madre del Sur and Huajicori in the Sierra Madre Occidental; construction of dams in the Municipality of Ruiz in the Sierra Madre Occidental (see Hernández-Guzmán et al. (2019) for a discussion of potential hydrologic impacts); and resource extraction in Huajicori in the Sierra Madre Occidental. In addition, five physiographic provinces are now recognised in Nayarit: Sierra Madre Occidental (SMO), Trans-Mexican Volcanic Belt (TMVB), Sierra Madre del Sur (SMS), Pacific Lowlands (PL) and Mexican Pacific Island System (MPIS) (Figs 1, 2) (Morán-Zenteno et al. 2018; Morrone 2019) rather than the four provinces used by Woolrich-Piña et al. (2016), so the species lists for the physiographic province need to be updated. There have also been several taxonomic updates, new records for the State and descriptions of new species (Duellman et al. 2016; Hansen and Salmon 2017; Campbell et al. 2018; Ramírez-Reyes and Flores-Villela 2018; Jadin et al. 2020; Loc-Barragán et al. 2020; McCraine et al. 2020; Reyes-Velasco et al. 2020; Wallach 2020; Ramírez-Reyes et al. 2021; Flores-Villela et al. 2022; Pérez-Ramos and Luja Molina 2022; Frost 2023; Uetz et al. 2023), which have increased the numbers of amphibians and reptiles known from Nayarit.

Given the dynamic nature of the threats to amphibians and reptiles and the dynamic nature of our understanding of the herpetofauna of Nayarit (e.g. taxonomy, increased survey activity), we here update the list of amphibians and reptiles found in Nayarit. Additionally, we present an updated summary of the conservation status. We also examine the number of species shared with adjacent States, which was not considered in Woolrich-Piña et al. (2016). We view this effort as a way to better understand how the herpetofauna is changing (i.e. this is intended as a means of summarising the state of our improving, but incomplete, understanding of the herpetofauna of Nayarit).

Physiographic characteristics of Nayarit

According to Morán-Zendero et al. (2018) and Morrone (2019) (see also Luja et al. (2014)), Nayarit contains five physiographic provinces: Sierra Madre Occidental; Trans-Mexican Volcanic Belt; Pacific Lowlands; Sierra Madre del Sur; and the Nayarit Islands (see Figs 2, 3). This differs from Woolrich-Piña et al. (2016) who used a different categorisation of physiographic regions and recognised only three (Coastal Plain, Sierra Madre Occidental, Transvolcanic Belt) on the mainland and also included the Nayarit Islands. Here, we provide some basic information of the five physiographic provinces that we use in this updated list of the herpetofauna of Nayarit, based on information in INEGI (2018a) and Blanco y Correa et al. (2021).

The Sierra Madre Occidental Province covers 57.2% of Nayarit, including the entire eastern part of the State. In

Figure 1. Map of Mexico with the State of Nayarit highlighted in green (modified from INEGI 2018b).

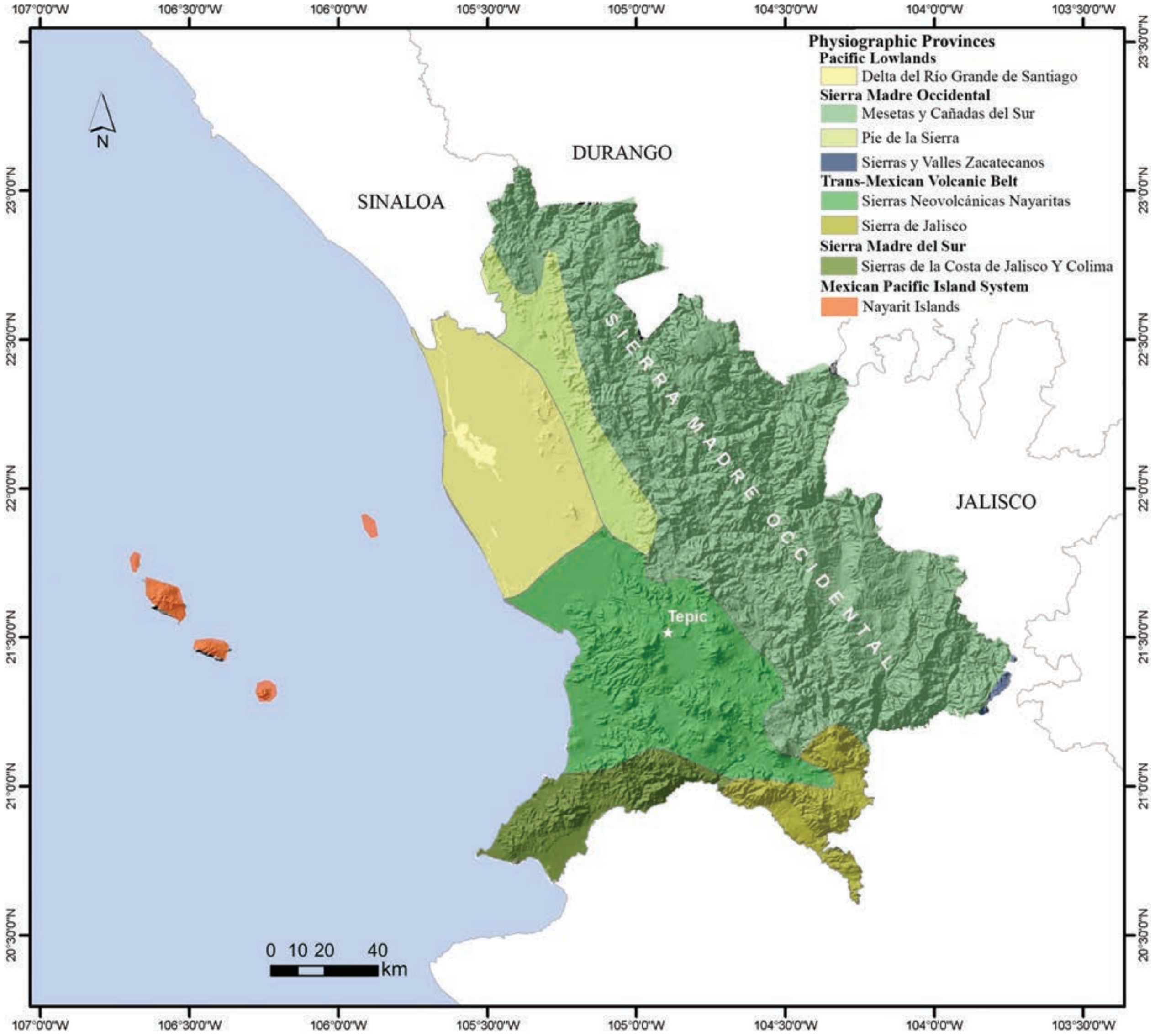

Figure 2. Physiographic provinces of the State of Nayarit, Mexico (modified from Cervantes-Zamora et al. 1990).

Nayarit, this Province being made up of three sub-provinces: Mesetas and Cañadas del Sur (50.01%); Pie de la Sierra (6.99%); and Sierras y Valles Zacatecanos (0.25%). The Trans-Mexican Volcanic Belt covers 20.03% of Nayarit, including the central portion of Nayarit from the coast to the south-eastern border of the State. In Nayarit, two sub-provinces of the Trans-Mexican Volcanic Belt are found: Sierras Neovolcánicas Nayaritas (18.34%); and Sierras de Jalisco (1.69%). The Pacific Lowlands Province encompasses 15.11% of Nayarit's territory in the north-western part of the State. In Nayarit, this Province is made up of the Delta del Río Grande de Santiago sub-province, which is an extensive plain covering the coastal area of northern Nayarit. The Sierra Madre del Sur is found in 7.61% of Nayarit in the extreme southern Nayarit. In Nayarit, it consists of the Sierras de la Costa de Jalisco and Colima sub-province. The Mexican Pacific Island System is represented in Nayarit by the Nayarit Islands sub-province, made up of the archipelagoes of Islas Marías (San Juanito, María Madre, María Magdalena and María Cleófas), Islas Marietas (Redonda, Larga, Corbeteña, Ampolla, Morro and Cuates) and Isla Isabel. These islands encompass 0.93% of the State territory and are located 115 km to the west of the San Blas coast and 10 km to the west-southwest of Punta Mita (Figs 2, 3).

Most of the State is covered by warm weather types (INEGI 2018a). The main types of this climate in the State are Warm Subhumid, present in 60.61% of Nayarit, along the coast and in the lower areas of the valleys of the Huaynamota and San Pedro Rivers, with important ramifications that extend to the east over the Sierra Madre Occidental and towards the south of the State. The Semi-warm Subhumid, present in 30.97% of the State, spreading in a strip that goes from the extreme northeast to the south of the State, including areas near Tepic and alternating with the Warm Subhumid in parts of medium altitude of the Sierra Madre Occidental. Another type of climate present in Nayarit is the Temperate Subhumid, covering only 6.16% of the State, restricted to small, very localised areas, scattered in the upper parts of the mountains. Semi-arid cli-

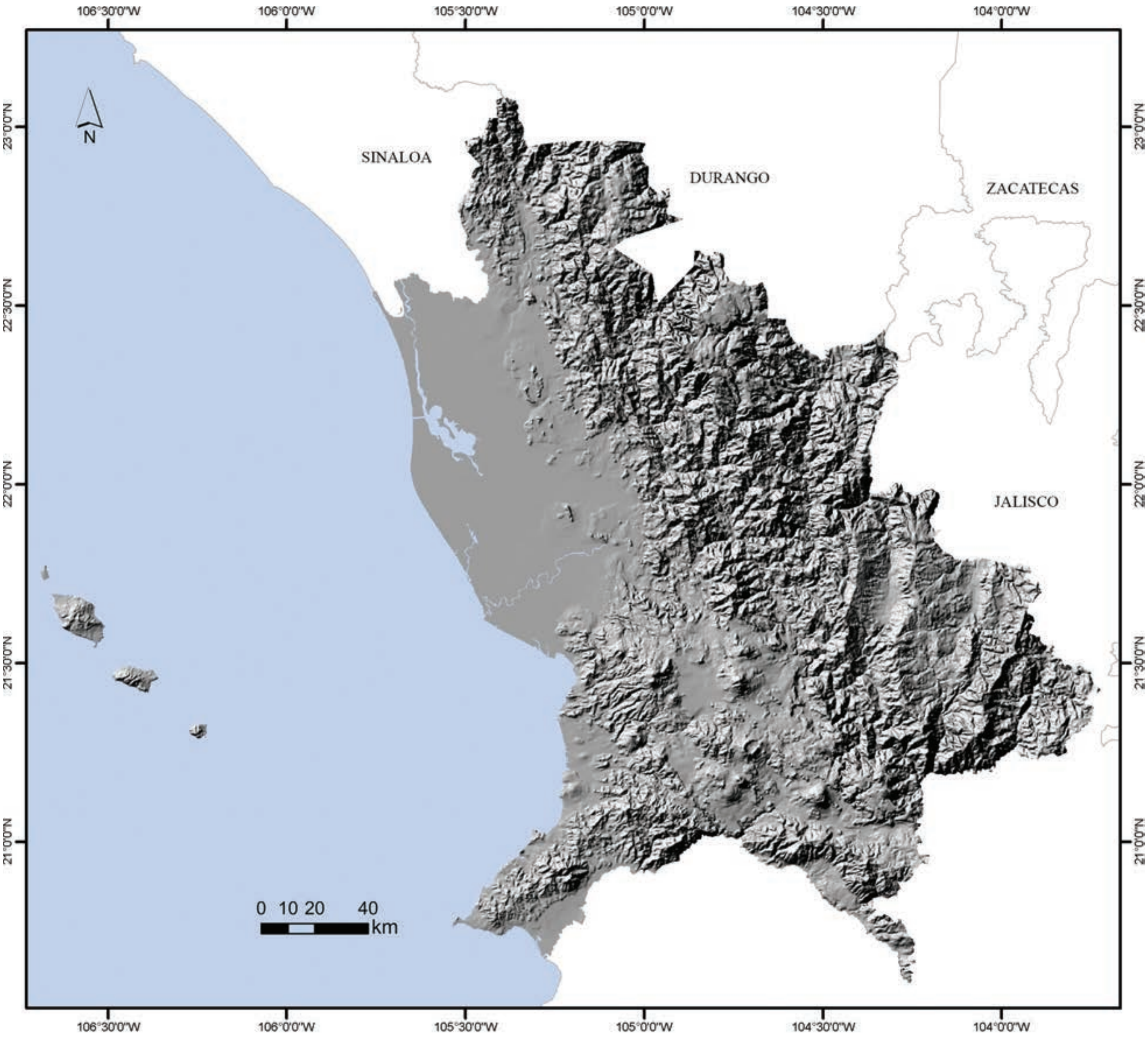

Figure 3. Topographical map of the State of Nayarit, Mexico (INEGI 2009).

mate is present in only 1.7% of the State's surface, in the extreme south of Nayarit (Fig. 4). Warm Humid, present in 0.56% of the State's territory is found in the central part of the State, as well as in a small region in the north.

Methods

We updated the list of amphibians and reptiles for Nayarit using our fieldwork (e.g. Loc-Barragán and Lazcano (2018); Loc-Barragán et al. (2018, 2019, 2020); Woolrich-Piña et al. (2021)), a thorough examination of the literature and records from VertNet.org. We follow Frost (2023) and AmphibiaWeb (2023) for amphibian names and Uetz et al. (2023) for reptile names; however, we did not include *Rhinocheilus antonii* Dugès, 1886, since we regard this taxon as a subspecies of *R. lecontei*, based on Manier (2004).

We created species accumulation curves for the total herpetofauna, as well as amphibians and reptiles separately, using the year of the first observation for each species in Nayarit. Generating species accumulation curves using this approach provides a reasonable estimate of herpetofaunal richness (see Raxworthy et al. (2012)). In addition, we recorded the conservation status and population trends of each species, based on the IUCN Red List 2022-2 (IUCN 2022), SEMARNAT (2019) and Environmental Vulnerability Scores from Wilson et al. (2013a, 2013b) and Johnson et al. (2015).

We determined the number of species shared with the four States that neighbour Nayarit using recent state lists: Jalisco (Cruz-Sáenz 2017); Durango (Lemos-Espinal et al. 2018); Sinaloa (Lemos-Espinal and Smith 2020) and Zacatecas (J. Sigala, personal communication), as well as updated State lists and a comprehensive checklist for the country of Mexico used in Smith and Lemos-Espinal (2022) and Lemos-Espinal and Smith (2023).

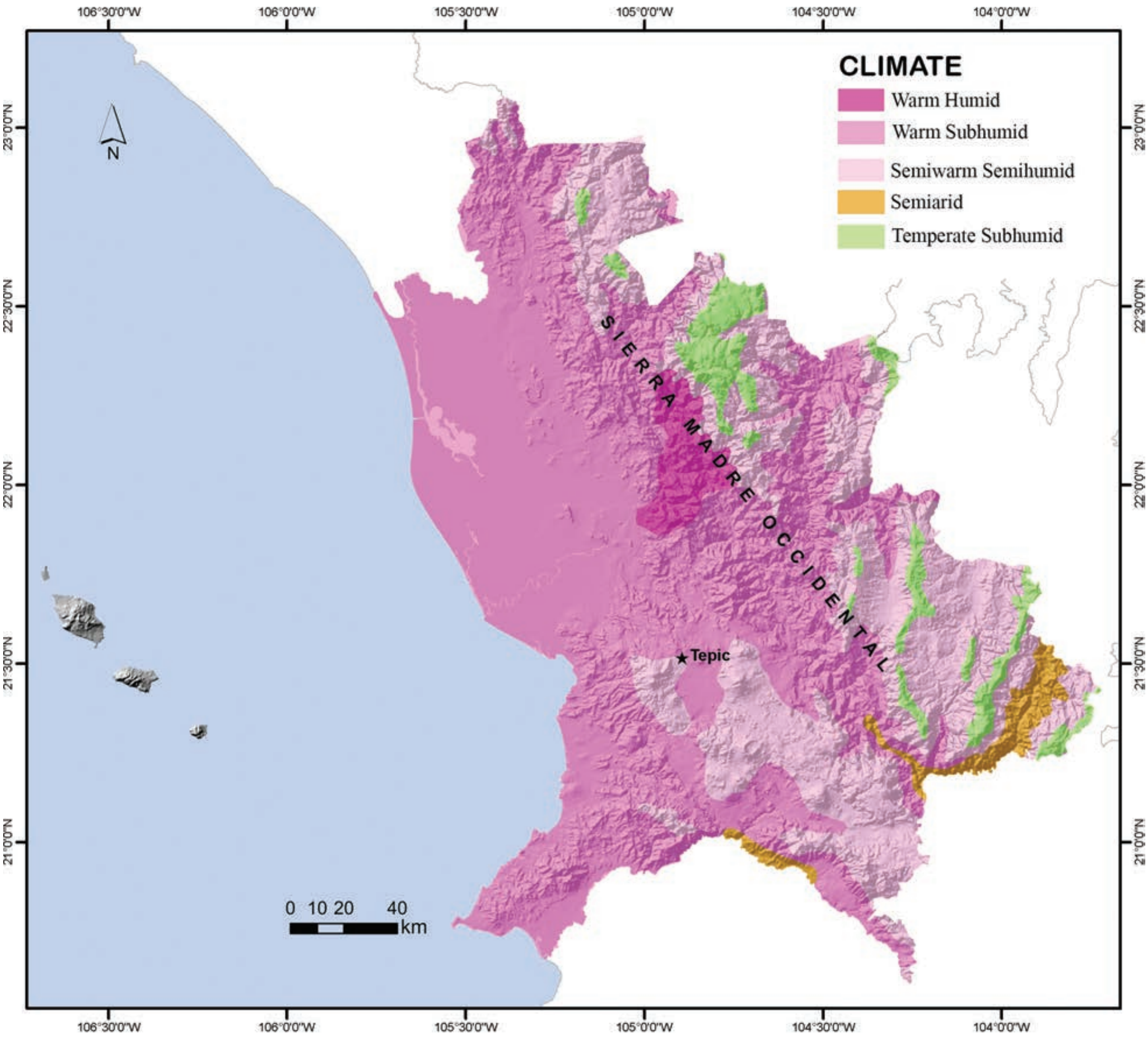

Figure 4. Climate map of the State of Nayarit, Mexico (modified from García – Comisión Nacional para el Conocimiento y Uso de la Biodiversidad 1998).

Results and discussion

Nayarit harbours 162 native species of amphibians and reptiles, representing 35 families (11 amphibian families [nine anurans and two salamanders] and 24 reptile families [one crocodilian, ten lizards, eight snakes and five turtles]) and 85 genera (20 amphibians and 65 reptiles) (Tables 1, 2). These include 37 species of amphibians (35 anurans and two salamanders) and 125 reptiles (one crocodile, 45 lizards, 69 snakes and 11 turtles). A species of *Plestiodon*, previously reported as *P. brevirostris*, occurs in Nayarit and, here, we report this undescribed species as *Plestiodon* aff. *brevirostris*. This is an increase of nine species from Woolrich-Piña et al. (2016), with an increase of one anuran, seven squamates and one turtle.

Two of the 162 native species are endemic to Nayarit: *Phyllodactylus cleofasensis* and *Thamnophis rossmani*. The most speciose amphibian family is Hylidae Rafinesque, 1815 and the most speciose reptile family is Colubridae Oppel, 1811 (Table 1). Additionally, eight species of amphibians and reptiles have been introduced to Nayarit: *Rana catesbeiana*, *Scinax staufferi*, *Anolis sagrei*, *Gehyra mutilata*, *Hemidactylus frenatus*, *Hemidactylus turcicus*, *Lepidodactylus lugubris* and *Indotyphlops braminus*.

We compiled a list of 28 species (nine amphibians, 19 reptiles: Table 3) that potentially occur in Nayarit, based on their distribution along the border with Sinaloa, Durango, Zacatecas and Jalisco. We used distributional records in Vertnet.org for the four neighbouring States and Nayarit to generate this list. As more herpetological work is done near the borders with the neighbouring States, we believe that these "likely to occur" species will be recorded for Nayarit.

Table 1. Native amphibians and reptiles of the State of Nayarit with distributional and conservation status. Ecoregion (ECR): (1 = Pacific Lowlands; 2 = Sierra Madre Occidental; 3 = Trans-Mexican Volcanic Belt; 4 = Sierra Madre del Sur; 5 = Nayarit Islands) according to INEGI 2018 and Blanco y Correa et al. 2021. IUCN Status: (DD = Data Deficient; LC = Least Concern, VU = Vulnerable, NT = Near Threatened; EN = Endangered; CR = Critically Endangered; NE = not evaluated), as well as the population trend, according to the IUCN Red List (The IUCN Red List of Threatened Species, Version 2022-2 (www.iucnredlist.org; accessed 25 August 2023); conservation status in Mexico according to SEMARNAT (2019) (Mex): (P = in danger of extinction, A = threatened, Pr = subject to special protection, NL – not listed); Environmental Vulnerability Score: (EVS – the higher the score the greater the vulnerability: low (L) vulnerability species (EVS of 3–9); medium (M) vulnerability species (EVS of 10–13); and high (H) vulnerability species (EVS of 14–20; NE = not evaluated) from Wilson et al. (2013a, b) and Johnson et al. (2015); Global Distribution: 0 = Endemic to Nayarit; 1 = Endemic to Mexico; 2 = Shared between the US and Mexico; 3 = widely distributed from Mexico to Central or South America; 4 = widely distributed from the US to Central or South America; 5 = circumglobal distribution; 6 = Pacific and Indian Oceans. Date in which the first record appeared (1st); and Source of the first record.

	ECR	IUCN	EVS	Mex	Global	1st	Source
CLASS AMPHIBIA							
ORDER ANURA							
Family Bufonidae							
Anaxyrus kelloggi (Taylor, 1938)	1,2	LC (=)	H (14)	NL	1	1897	USNM 47865
Incilius marmoreus (Wiegmann, 1833)	1,4	LC (=)	M (11)	NL	1	1949	KU KUH 27718
Incilius mazatlanensis (Taylor, 1940)	1,2,3,4,5	LC (=)	M (12)	NL	1	1897	USNM46949
Incilius occidentalis (Camerano, 1879)	2,3	LC (=)	M (11)	NL	1	1897	USNM47171
Rhinella horribilis (Wiegmann, 1833)	1,2,3,4	NE	L (3)	NL	4	1894	CAS Herp 3219
Family Craugastoridae							
Craugastor augusti (Dugès, 1879)	2,3,4	LC (=)	L (8)	NL	2	1950	KU KUH 29776
Craugastor hobartsmithi (Taylor, 1937)	3,4	LC (=)	H (15)	NL	1	1956	CAS 17448–49
Craugastor occidentalis (Taylor, 1941)	1,2,3,4	LC (=)	M (13)	NL	1	1934	FMNH 106835
Craugastor pygmaeus (Taylor, 1937)	2,3,4	LC (?)	L (9)	NL	3	1963	CAS Herp 99231
Craugastor vocalis (Taylor, 1940)	2,3,4	LC (↓)	M (13)	NL	1	1950	KU KUH 28141
Family Eleutherodactylidae							
Eleutherodactylus modestus (Taylor, 1942)	4	LC (=)	H (16)	Pr	1	2020	ITSZ 1025
Eleutherodactylus jamesdixoni Devitt, Tseng, Taylor-Adair, Koganti, Timugura & Cannatella, 2023	2,3,4	NE	NE	NL	1	1954	KU KUH 37832
Eleutherodactylus pallidus (Duellman, 1958)	1,2,3,4,5	LC (=)	H (17)	Pr	1	1955	UIMNH Herps 71981
Eleutherodactylus teretistes (Duellman, 1958)	3	VU (?)	H (16)	NL	1	1956	UMMZ115451
Family Hylidae							
Dryophytes arenicolor (Cope, 1886)	2,3	LC (=)	L (7)	NL	2	1954	KU KUH 37739
Dryophytes eximius (Baird, 1854)	1,2,3,4	LC (=)	M (10)	NL	1	1894	CAS Herp 3160
Exerodonta smaragdina (Taylor, 1940)	2,3,4	LC (↓)	M (12)	Pr	1	1957	NHM-LACM: Herps839
Sarcohyla hapsa Campbell, Brodie, Caviedes-Solis, Nieto-Montes de Oca, Luja, Flores-Villela, García-Vázquez, Sarker & Wostl, 2018	2,3	LC (?)	NE	NL	1	2018	Campbell et al. (2018)
Smilisca baudinii (Duméril & Bibron, 1841)	1,2,3,4,5	LC (=)	L (3)	NL	4	1894	CAS Herp 314I+I25
Smilisca fodiens (Boulenger, 1882)	1,2,3,4	LC (=)	L (8)	NL	2	1897	USNM 47442
Tlalocohyla smithii (Boulenger, 1902)	1,2,3,4	LC (=)	M (11)	NL	1	1934	FMNH 105999
Trachycephalus vermiculatus (Cope, 1877)	1,4	NE	L (4)	NL	3	1962	KU KUH 74339
Triprion spatulatus Günther, 1882	1,4	LC (=)	M (13)	NL	1	1970	UTEP: Herp:6624
Family Leptodactylidae							
Leptodactylus melanonotus (Hallowell)	1,3,4	LC (=)	L (6)	NL	3	1932	UIMNH 32738
Family Microhylidae							
Gastrophryne mazatlanensis (Taylor, 1943)	1	NE	L (8)	NL	2	1959	UIMNH 81980
Hypopachus ustus (Cope, 1866)	1,4	LC (=)	L (7)	Pr	3	1981	USNM 238117
Hypopachus variolosus (Cope, 1866)	1,2,5	LC (=)	L (4)	NL	4	1934	FMNH 75789
Family Phyllomedusidae							
Agalychnis dacnicolor (Cope, 1864)	1,2,3,4	LC (↓)	M (13)	NL	1	1934	FMNH 98250
Family Ranidae							
Rana cora Pérez-Ramos & Luja Molina, 2022	1	NE	NE	NL	1	1955	CAS Herp 94259
Rana magnaocularis Frost & Bagnara, 1976	2,3,4	LC (?)	M (12)	NL	1	1949	UMMZ102125
Rana megapoda Taylor, 1942	3	NT (↓)	H (14)	Pr	1	1934	UIMNH 32081
Rana psilonota Webb, 2001	2,3	LC (?)	H (14)	NL	1	2003	MZFC 17290
Rana pustulosa Boulenger, 1883	2,3,4	LC (=)	L (9)	Pr	1	1934	FMNH 110851-852
Family Scaphiopodidae							
Scaphiopus couchi Baird, 1854	1	LC (=)	L (3)	NL	2	1897	USNM 47864
Spea multiplicata (Cope, 1863)	2,3	LC (=)	L (6)	NL	2	2014	Luja-Molina et al. (2014)

	ECR	IUCN	EVS	Mex	Global	1st	Source
ORDER CAUDATA							
Family Ambystomatidae							
Ambystoma rosaceum Taylor, 1941	2	LC (?)	H (14)	Pr	1	2003	Canseco-Márquez et al. (2007)
Family Plethodontidae							
Isthmura bellii (Gray, 1850)	2	LC (?)	M (12)	A	1	1905	Gadow (1905)
CLASS REPTILIA							
ORDER CROCODYLIA							
Family Crocodylidae							
Crocodylus acutus (Cuvier, 1807)	1,5	VU (↑)	H (14)	Pr	4	1956	UPS PSM Herp-07724
ORDER SQUAMATA							
SUBORDER LACERTILIA							
Family Anguidae							
Barisia imbricata (Wiegmann, 1828)	2	LC (?)	H (14)	Pr	1	2016	Woolrich-Piña et al. (2021)
Elgaria kingii Gray, 1838	2,3	LC (=)	M (10)	Pr	2	1975	MSB-UNM: Herp:31877
Gerrhonotus liocephalus Wiegmann, 1828	3,5	LC (=)	L (6)	Pr	4	2014	MZUAN F0010
Family Corytophanidae							
Basiliscus vittatus Wiegmann, 1828	4	LC (=)	L (7)	NL	3	1976	USNM 238043
Family Dactyloidae							
Anolis nebulosus (Wiegmann, 1834)	1,2,3,4,5	LC (=)	M (13)	NL	1	1881	NHM-LACM: 81.10.1.85
Anolis sagrei Duméril & Bibron, 1837		IN	IN	IN	IN		
Family Eublepharidae							
Coleonyx elegans Gray, 1845	4	LC (=)	L (9)	A	3	2016	Woolrich-Piña et al. (2021)
Family Helodermatidae							
Heloderma horridum (Wiegmann, 1829)	1,2,3,4	LC (↓)	M (11)	A	1	1889	MCZ Herp R-6935
Family Iguanidae							
Ctenosaura pectinata (Wiegmann, 1834)	1,2,3,4,5	LC (↓)	H (15)	A	1	1885	USNM 14078
Iguana iguana (Linnaeus, 1758)	1,4	LC (?)	M (12)	Pr	3	1894	CAS Herp 3342
Family Phrynosomatidae							
Holbrookia elegans Bocourt, 1874	1	LC (=)	M (13)	NL	2	1954	NHM-LACM: Herps95207
Phrynosoma orbiculare (Linnaeus, 1766)	2	LC (=)	M (12)	A	1	2008	Reyes-Velasco et al. (2012)
Sceloporus albiventris Smith, 1939	2,3,4	NE	H (16)	NL	1	1894	USNM Amphibians and Reptiles 58813
Sceloporus asper Boulenger, 1897	3	LC (↓)	H (14)	Pr	1	1894	CAS Herp 3214
Sceloporus bulleri Boulenger, 1894	2	LC (=)	H (15)	NL	1	2020	Loc-Barragán and Woolrich-Piña (2020)
Sceloporus clarkii Baird & Girard, 1852	1,5	LC (=)	M (10)	NL	2	1894	CAS Herp 3328
Sceloporus dugesii Bocourt, 1874	2,3	LC (=)	M (13)	NL	1	1949	UIMNH 6469
Sceloporus grammicus Wiegmann, 1828	2	LC (=)	L (9)	Pr	2	2016	Woolrich-Piña et al. (2021)
Sceloporus heterolepis Boulenger, 1895	2	LC (?)	H (14)	NL	1	2015	Loc-Barragán et al. (2016)
Sceloporus horridus Wiegmann, 1834	3,4	LC (=)	M (11)	NL	1	1892	NHM-LACM: 92.2.8.24
Sceloporus huichol Flores-Villela, Smith, Campillo-García, Martínez-Méndez & Campbell, 2022	2,3	NE	NE	NL	1	2022	Flores-Villela et al. (2022)
Sceloporus jarrovii Cope, 1875	2	LC (=)	M (11)	NL	2	1892	NHM-LACM: 92.2.8.23
Sceloporus melanorhinus Bocourt, 1876	3,4	LC (=)	L (9)	NL	3	1897	USNM 64667
Sceloporus nelsoni Cochran, 1923	1,2,3,4	LC (=)	M (13)	NL	1	1934	FMNH 106436-438
Sceloporus poinsettii Baird & Girard, 1852	2	LC (=)	M (12)	NL	2	2014	Luja-Molina and Grünwald (2015)
Sceloporus scalaris Wiegmann, 1828	2	LC (=)	M (12)	NL	1	1970	Webb (1982)
Sceloporus shannonorum Langebartel, 1959	2,3	DD (?)	H (15)	NL	1	1959	NHM-LACM: Herps97384
Sceloporus spinosus Weigmann, 1828	1	LC (=)	M (12)	NL	1	ND	AMNH 15518
Sceloporus unicanthalis Smith, 1937	2,3	NE	H (16)	NL	1	ND	USNM 46626
Sceloporus utiformis Cope, 1864	1,2,3,4	LC (=)	H (15)	NL	1	1894	CAS Herp 3233
Urosaurus bicarinatus (Duméril, 1856)	1,4	LC (=)	M (12)	NL	1	1954	KUH 37737
Urosaurus ornatus (Baird & Girard, 1852)	2,5	LC (=)	M (10)	NL	2	1881	NHM-LACM: N/N
Family Phyllodactylidae							
Phyllodactylus cleofasensis Ramírez-Reyes, Barraza-Soltero, Nolasco-Luna, Flores-Villela & Escobedo-Galván, 2021	5	NE	NE	NL	0	2021	Ramírez-Reyes et al. (2021)
Phyllodactylus lanei Smith, 1935	4	LC (=)	H (15)	NL	1	1934	FMNH 94958
Phyllodactylus saxatilis Dixon, 1964	2,3,4	NE	NE	NL	1	1881	NHM-LACM: 81.10.1.90
Family Scincidae							
Marisora aquilonaria McCranie, Matthews & Hedges, 2020	1	NE	NE	NL	1	1992	Casas-Andreu (1992)
Plestiodon aff. *brevirostris* (Günther, 1860)	3	LC (=)	M (11)	NL	1	1970	NHM-LACM: Herps65135
Plestiodon callicephalus (Bocourt, 1879)	1,2,3	LC (=)	M (12)	NL	2	1959	NHM-LACM: Herps99505

	ECR	IUCN	EVS	Mex	Global	1st	Source
Plestiodon lynxe (Wiegmann, 1834)	2,3	LC (=)	M (10)	Pr	1	2007	Canseco-Márquez et al. (2007)
Plestiodon parviauriculatus (Taylor, 1933)	2	DD (?)	H (15)	Pr	1	2020	Loc-Barragán et al. (2020)
Plestiodon parvulus (Taylor, 1933)	1,2,4	DD (?)	H (15)	NL	1	1910	USNM 56903
Family Teiidae							
Aspidoscelis communis (Cope, 1878)	4,5	LC (=)	H (14)	Pr	1	1881	NHM-LACM: 81.10.1.88
Aspidoscelis costatus (Cope, 1878)	1,2,3,4	LC (=)	M (11)	Pr	1	1859	NHM-LACM: Herps 53169
Aspidoscelis lineattissimus (Cope, 1878)	4	LC (=)	H (14)	Pr	1	1894	CAS Herp 3344
Holcosus sinister (Wiegmann, 1834)	3,4	NE	M (13)	NL	1	1956	UAZ 21333
SUBORDER SERPENTES							
Family Boidae							
Boa sigma (Smith, 1943)	1,2,3,4,5	NE	M (10)	NL	1	1897	USNM 46484
Family Colubridae							
Conopsis nasus (Günther, 1858)	2	LC (=)	M (11)	NL	1	1963	UAZ 24127
Drymarchon melanurus (Duméril, Bibron & Duméril, 1854)	1,2,3,4,5	LC (=)	L (6)	NL	4	1925	CAS Herp 58993
Drymobius margaritiferus (Schlegel, 1837)	1,3,4	LC (=)	L (6)	NL	4	1913	USNM 51480
Gyalopion quadrangulare (Günther, 1893)	1	LC (=)	M (11)	Pr	2	1956	UAZ 20734
Lampropeltis greeri Webb, 1961	2	NE	NE	NL	1	2010	UTADC 6833
Lampropeltis polyzona Cope, 1860	1,2,3,4,5	LC (?)	M (11)	NL	1	1881	NHM-LACM:81.10.1.97
Leptophis diplotropis (Günther, 1872)	1,3,4,5	LC (=)	H (14)	A	1	1881	NHM-LACM: 81.10.1.104
Masticophis bilineatus Jan, 1863	2,5	LC (=)	M (11)	NL	2	1897	USNM 46417
Masticophis flagellum Shaw, 1802	2	LC (=)	L (8)	A	2	2020	ITSZ LEZ 201
Masticophis mentovarius (Duméril, Bibron & Duméril, 1854)	1,3,4	LC (?)	L (6)	A	3	1897	USNM24681
Mastigodryas cliftoni (Hardy, 1964)	2	DD (?)	H (14)	NL	1	1998	Ponce-Campos and Huerta-Ortega (1998)
Mastigodryas melanolomus (Cope 1868)	2,3,4,5	LC (=)	L (6)	NL	3	1881	NHM-LACM: 81.10.1.103
Oxybelis microphthalmus Barbour & Amaral, 1926	1,3,4,5	NE	NE	NL	2	1881	NHM-LACM: 81.10.1.107
Pituophis deppei (Duméril, 1853)	2	LC (=)	H (16)	NL	1	1894	CAS Herp 3126
Pseudoficimia frontalis (Cope, 1864)	2	LC (=)	M (10)	NL	3	1958	UIMNH 83010
Rhinocheilus lecontei Baird & Girard, 1853	1	LC (=)	L (8)	NL	2	1958	UIMNH 84165
Salvadora bairdii Jan & Sordelli, 1860	3	LC (=)	H (15)	Pr	1	2016	Woolrich-Piña et al. (2016)
Salvadora grahamiae Baird & Girard, 1853	2	LC (=)	M (10)	NL	2	1898	MNHN -RA 1898.251
Salvadora mexicana (Duméril, Bibron & Duméril, 1854)	2,3,4	LC (=)	H (15)	Pr	1	1958	UIMNH 84204
Senticolis triaspis (Cope, 1866)	2,3,4	LC (=)	L (6)	NL	4	1956	UIMNH83492
Sonora mutabilis Stickel, 1943	2,3,4	LC (?)	H (14)	NL	1	ND	AMNH 74951
Sympholis lippiens Cope, 1862	3	DD (?)	H (14)	NL	1	1894	CAS Herp 3127
Tantilla bocourti (Günther, 1895)	3,5	LC (?)	L (9)	NL	1	1964	McDiarmid et al. (1976)
Tantilla calamarina Cope, 1866	3,4,5	LC (=)	M (12)	Pr	1	1881	NHM LACM 81.10.1.12
Tantilla ceboruca Canseco-Márquez, Smith, Ponce-Campos, Flores-Villela & Campbell, 2007	3	NE	H (16)	NL	1	2003	Canseco-Márquez et al. (2007)
Tantilla yaquia Smith, 1942	2,3,4	LC (=)	M (10)	NL	2	ND	AMNH7 4949
Trimorphodon paucimaculatus Taylor, 1936	1,5	NE	H (15)	NL	1	1954	UPS PSM Herp-07530
Trimorphodon tau Cope, 1870	2,3,4	LC (=)	M (13)	NL	1	1956	TCWC Herpetology 12609
Family Dipsadidae							
Coniophanes lateritius Cope, 1862	3,4	DD (?)	M (13)	NL	1	1963	NHM-LACM: Herps9496
Diadophis punctatus (Linnaeus, 1766)	2,3	LC (=)	L (4)	NL	2	1959	NHM-LACM: Herps103860
Enulius oligostichus Smith, Arndt & Sherbrook, 1967	3,4	DD (?)	H (15)	Pr	1	1962	NHM-LACM: Herps36232
Geophis annuliferus Boulenger, 1894	3,4,5	LC (=)	M (13)	Pr	1	1925	CAS Herp 58680
Geophis dugesii Bocourt, 1883	3	LC (?)	M (13)	NL	1	2015	Luja-Molina and Grünwald (2015)
Hypsiglena affinis Boulenger, 1894	2,3,4	NE	H (14)	Pr	1	1962	UIMNH 85582
Hypsiglena torquata (Günther, 1860)	1,2,5	LC (=)	L (8)	Pr	1	1957	UIMNH 83059
Imantodes gemmistratus (Cope, 1861)	1,2,3,4,5	LC (=)	L (6)	Pr	3	1881	NHM-LACM: 81.10.1.101
Leptodeira maculata (Hallowell, 1861)	1,4	LC (=)	L (7)	Pr	1	1894	CAS Herp 3128
Leptodeira polysticta (Günther, 1895)	3,4	NE	L (8)	NL	3	1937	FMNH 95206
Leptodeira punctata (Peters, 1866)	1	LC (?)	H (17)	NL	1	1954	KU KUH 37598
Leptodeira splendida Günther, 1895	2,3,4	LC (?)	H (14)	NL	1	1964	CAS Herp 96885
Manolepis putnami (Jan, 1863)	3,4	LC (=)	M (13)	NL	1	1973	USNM 197999
Rhadinaea hesperia Bailey, 1940	3,4,5	LC (=)	M (10)	Pr	1	1960	NHM-LACM: Herps 103653
Rhadinaea taeniata (Peters, 1863)	3	DD (?)	M (13)	NL	1	2015	Luja-Molina and Grünwald (2015)
Sibon nebulatus (Linnaeus, 1758)	1,3	LC (=)	L (5)	NL	3	1959	NHM-LACM: Herps103863
Tropidodipsas philippii (Jan, 1863)	2,4	LC (=)	H (14)	Pr	1	1959	UIMNH 84567

	ECR	IUCN	EVS	Mex	Global	1st	Source
Family Elapidae							
Hydrophis platurus (Linnaeus, 1766)	1	LC (=)	NE	NL	6	1925	CAS Herp 58992
Micruroides euryxanthus (Kennicott, 1860)	2,3	LC (=)	H (15)	A	2	2013	MZFC 28306
Micrurus browni Schmidt & Smith, 1943	2,3,4	LC (=)	L (8)	Pr	3	1956	UIMNH 40369
Micrurus distans (Kennicott, 1860)	2,3	LC (=)	H (14)	Pr	1	1956	UMMZ 114443
Family Leptotyphlopidae							
Rena humilis Baird &Girard, 1853	1,3,4	LC (=)	L (8)	NL	2	1956	CAS SUR 19243
Family Loxocemidae							
Loxocemus bicolor Cope, 1861	1,2,4	LC (?)	M (10)	Pr	3	1957	UIMNH 82538
Family Natricidae							
Storeria storerioides (Cope, 1865)	2,3	LC (=)	M (11)	NL	1	1963	UAZ 26389
Thamnophis cyrtopsis (Kennicott, 1860)	2,3	LC (=)	L (7)	A	4	1980	CAS Herp 169687
Thamnophis eques (Reuss, 1834)	2	LC (=)	L (8)	A	2	1897	USNM 46429
Thamnophis errans Smith, 1942	2	LC (?)	H (16)	NL	1	1979	CAS Herp 169662
Thamnophis melanogaster (Peters, 1864)	2,3	EN (↓)	H (15)	A	1	1934	FMNH 126726
Thamnophis nigronuchalis Thompson, 1957	2	DD (?)	M (12)	Pr	1	1979	CAS Herp 169660
Thamnophis pulchrilatus (Cope, 1885)	2	LC (?)	H (15)	NL	1	1897	USNM 46420
Thamnophis rossmani Conant, 2000	3	DD (?)	H (18)	NL	0	1934	UIMNH 18836
Thamnophis validus (Kennicott, 1860)	1,3,4	LC (=)	M (12)	NL	1	1934	FMNH 115618
Family Viperidae							
Agkistrodon bilineatus (Günther, 1863)	1,2,3,4	NT (↓)	M (11)	Pr	3	1881	NHM LACM 81.10.1.95
Crotalus armstrongi Campbell, 1979	3	NE	H (18)	NL	1	1950	KU KUH 29501
Crotalus basiliscus (Cope, 1864)	1,2,3,4	LC (=)	H (16)	Pr	1	1956	UIMNH 84705
Crotalus campbelli Bryson, Linkem, Dorcas, Lathrop, Jones, Alvarado-Díaz, Grünwald & Murphy, 2014	3	NE	H (17)	NL	1	2015	Luja-Molina and Grünwald (2015)
Crotalus lepidus (Kennicott, 1861)	2,3	LC (=)	M (12)	Pr	2	1897	USNM 46333
Crotalus molossus Baird & Girard, 1853	2,3	LC (=)	L (8)	Pr	2	1977	MSB.UNM: Herp:32085
Crotalus pricei Van Denburgh, 1895	2,3	LC (?)	H (14)	Pr	2	1952	Klauber (1952)
Crotalus willardi Meek, 1905	2	LC (=)	M (13)	Pr	2	2021	Woolrich-Piña et al. (2021)
ORDER TESTUDINES							
Family Cheloniidae							
Chelonia mydas (Linnaeus, 1758)	1,5	EN (↓)	NE	P	5	1883	CAS Herp 4860
Eretmochelys imbricata Linnaeus, 1766	1,5	CR (↓)	NE	P	5	ND	AMNH 78717
Lepidochelys olivacea (Eschscholtz, 1829)	1,5	VU (↓)	NE	P	5	1956	UPS PSM Herp-07725
Family Dermochelyidae							
Dermochelys coriacea (Vandelli, 1761)	1,5	VU (↓)	NE	P	5	2007	Sarti-Martínez et al. (2007)
Family Emydidae							
Terrapene nelsoni Stejneger, 1925	2	DD	H (18)	Pr	1	1897	USNM 46252
Trachemys ornata (Gray, 1831)	1,3,4	VU (↓)	H (19)	NL	1	1961	UIMNH 12922
Family Geoemydidae							
Rhinoclemmys pulcherrima (Gray, 1855)	2,3,4	NE	L (8)	A	3	1897	USNM 46305
Family Kinosternidae							
Kinosternon cora Loc-Barragán, Reyes-Velásco, Woolrich-Piña, Grünwald, Venegas de Anaya, Rangel-Méndoza & López-Luna, 2020	1	NE	NE	NL	1	2020	Loc-Barragán et al. (2020)
Kinosternon hirtipes (Wagler, 1830)	2	LC (↓)	M (10)	Pr	2	1955	UIMNH 85855
Kinosternon integrum LeConte, 1854	1,2,3,4,5	LC (=)	M (11)	Pr	1	1881	NHM LACM 81.10.1.74
Kinosternon vogti López-Luna, Cupull-Magaña, Escobedo-Galván, González-Hernández, Centenero-Alcalá, Rangel-Mendoza, Ramírez-Ramírez & Cazares-Hernández 2018	4	NL	NE	P	1	2021	Rosales-Martínez et al. (2021)

Table 2. Summary of native species present in Nayarit by class, family, order and suborder. Status summary indicates the number of species found in each IUCN conservation status in the order DD, LC, NT, VU, EN, CR (see Table 1 for abbreviations). Mean EVS is the mean Environmental Vulnerability Score, scores ≥ 14 are considered to have high vulnerability (Wilson et al. 2013a, 2013b) and conservation status in Mexico according to SEMARNAT (2010) in the order NL, Pr, A, P (see Table 1 for abbreviations).

Scientific Name	Genera	Species	IUCN	EVS ($\bar{X}$)	SEMARNAT
CLASS AMPHIBIA			DD, LC, NT, VU, EN, CR		NL, Pr, A, P
ORDER ANURA	18	35	0,28,1,1,0,0	9.91	30,5,0,0
Bufonidae	2	5	0,4,0,0,0,0	10.2	5,0,0,0
Craugastoridae	1	5	0,5,0,0,0,0	11.6	5,0,0,0
Eleutherodactylidae	1	4	0,2,0,1,0,0	15.25	2,2,0,0
Hylidae	7	9	0,8,0,0,0,0	8.5	8,1,0,0
Leptodactylidae	1	1	0,1,0,0,0,0	6	1,0,0,0

Scientific Name	Genera	Species	IUCN	EVS (X̄)	SEMARNAT
Microhylidae	2	3	0,2,0,0,0,0	6.33	2,1,0,0
Phyllomedusidae	1	1	0,1,0,0,0,0	13	1,0,0,0
Ranidae	1	5	0,3,1,0,0,0	10.4	3,2,0,0
Scaphiopodidae	2	2	0,2,0,0,0,0	4.5	2,0,0,0
ORDER CAUDATA	2	2	0,2,0,0,0,0	13	0,1,1,0
Ambystomatidae	1	1	0,1,0,0,0,0	14	0,1,0,0
Plethodontidae	1	1	0,1,0,0,0,0	12	0,0,1,0
SUBTOTAL	20	37	0,30,1,1,0,0	10.1	30,6,1,0
CLASS REPTILIA					
ORDER CROCODYLIA	1	1	0,0,0,1,0,0	14	0,1,0,0
Crocodylidae	1	1	0,0,0,1,0,0	14	0,1,0,0
ORDER SQUAMATA	56	113	10,85,1,0,1,0	11.78	69,33,11,0
SUBORDER LACERTILIA	18	44	3,34,0,0,0,0	12.03	29,11,4,0
Anguidae	3	3	0,3,0,0,0,0	10	0,3,0,0
Corytophanidae	1	1	0,1,0,0,0,0	7	1,0,0,0
Dactyloidae	1	1	0,1,0,0,0,0	13	1,0,0,0
Eublepharidae	1	1	0,1,0,0,0,0	9	0,0,1,0
Helodermatidae	1	1	0,1,0,0,0,0	11	0,0,1,0
Iguanidae	2	2	0,2,0,0,0,0	13.5	0,1,1,0
Phrynosomatidae	4	22	1,18,0,0,0,0	12.57	19,2,1,0
Phyllodactylidae	1	3	0,1,0,0,0,0	11.5	3,0,0,0
Scincidae	2	6	2,3,0,0,0,0	12.6	4,2,0,0
Teiidae	2	4	0,3,0,0,0,0	13	1,3,0,0
SUBORDER SERPENTES	38	69	7,51,1,0,1,0	11.54	40,22,7,0
Boidae	1	1	0,0,0,0,0,0	10	1,0,0,0
Colubridae	17	28	2,22,0,0,0,0	11.04	21,4,3,0
Dipsadidae	11	17	3,12,0,0,0,0	11	9,8,0,0
Elapidae	3	4	0,4,0,0,0,0	12.33	1,2,1,0
Leptotyphlopidae	1	1	0,1,0,0,0,0	8	1,0,0,0
Loxocemidae	1	1	0,1,0,0,0,0	10	0,1,0,0
Natricidae	2	9	2,6,0,0,1,0	12.66	5,1,3,0
Viperidae	2	8	0,5,1,0,0,0	13.62	2,6,0,0
ORDER TESTUDINES	8	11	1,2,0,3,1,1	13.2	2,3,1,5
Cheloniidae	3	3	0,0,0,1,1,1	–	0,0,0,3
Dermochelyidae	1	1	0,0,0,1,0,0	–	0,0,0,1
Emydidae	2	2	1,0,0,1,0,0	18.5	1,1,0,0
Geoemydidae	1	1	0,0,0,0,0,0	8	0,0,1,0
Kinosternidae	1	4	0,2,0,0,0,0	10.5	1,2,0,1
SUBTOTAL	65	125	11,87,1,4,2,1	11.75	71,37,12,5
TOTAL	85	162	11,119,2,5,2,1	11.35	101,43,13,5

The species accumulation curves indicate that there have been three periods of rapid growth in the known species richness of the herpetofauna of Nayarit (Fig. 5). The first period was in the 1890s and represents the work of A.C. Buller, E. Nelson and E. Goldman, G. Eisen and F.H. Vaslit, J. Van Denburgh and L. Diguet, amongst others. The second period of rapid increases in the known species richness was in the 1950s and 1960s and was particularly dramatic for reptiles. This increase reflects the work of A.E. Leviton and H.E. Munsterman, A.S. Lokley, A.R. Phillips, C.H. Lowe, D. Langebartel, J. Schaffner and E. Widdows, J. Dixon, W. Duellman, F.A. Shannon and F.L. Humphrey, J. Maris, M.L. Johnson, T.H. Lewis, R. Abbuhl, G. Gates and A. Maas, R.B. Loomis and R.G. Webb, amongst others. The final increase has been in the past decade or so and is almost entirely the result of an increase in the number of reptiles identified in Nayarit. The current period of increased knowledge of the herpetofauna of Nayarit is the result of work by herpetologists,

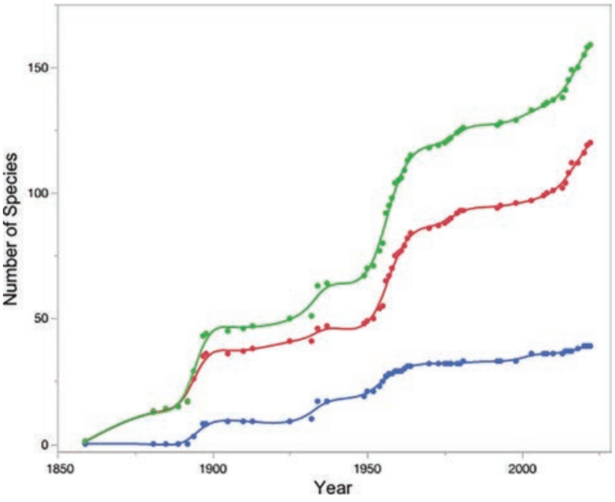

Figure 5. Species accumulation curves for all reptiles and amphibians (green), reptiles (red) and amphibians (blue) in Nayarit, Mexico.

Table 3. List of amphibian and reptile species that potentially occur in Nayarit. Region abbreviations: SMO (Sierra Madre Occidental); SMS (Sierra Madre del Sur); PL (Pacific Lowlands); TMVB (Trans-Mexican Volcanic Belt.

Taxon	Likely to occur in:
Class Amphibia	
Order Anura	
Family Bufonidae	
Anaxyrus compactilis (Wiegmann, 1833)	SMO
Anaxyrus debilis (Girard, 1854)	SMO
Anaxyrus mexicanus (Brocchi, 1879)	SMO
Anaxyrus punctatus (Baird & Girard, 1852)	PL and SMO
Family Eleutherodactylidae	
Eleutherodactylus saxatilis (Webb, 1962)	SMO
Eleutherodactylus wixarika Reyes-Velasco, Ahumada-Carrillo, Burkhardt & Devitt, 2015	SMO
Ranidae	
Rana neovolcanica (Hillis & Frost, 1985)	TMVB
Orden Gymnophiona	
Dermophiidae	
Dermophis oaxacae (Mertens, 1930)	PL and SMS
Order Caudata	
Family Ambystomatidae	
Ambystoma velasci Dugès, 1888	SMO
Class Reptilia	
Suborder Lacertilia	
Anguidae	
Barisia ciliaris (Smith, 1942)	SMO
Eublepharidae	
Coleonyx fasciatus (Boulenger, 1885)	SMS
Phrynosomatidae	
Callisaurus draconoides Blainville, 1835	PL
Phrynosoma asio Cope, 1864	SMS
Teiidae	
Aspidoscelis gularis (Baird & Girard, 1852)	TMVB
Scincidae	
Plestiodon bilineatus (Tanner, 1958)	SMO
Xantusiidae	
Xantusia sanchezi Bezy & Flores-Villela, 1999	SMO
Suborder Serpientes	
Colubridae	
Conopsis biserialis (Taylor & Smith, 1942)	TMVB
Lampropeltis webbi Bryson, Dixon & Lazcano, 2005	SMO
Masticophis taeniatus (Hallowell, 1852)	SMO
Pituophis catenifer (Blainville, 1835)	
Adelophis foxi Rossman & Blaney, 1968	SMO
Dipsadidae	
Conophis vittatus Peters, 1860	SMS
Pseudoleptodeira latifasciata (Günther, 1894)	SMS
Natricidae	
Thamnophis marcianus (Baird & Girard, 1853)	SMO
Viperidae	
Crotalus lannomi Tanner, 1966	SMS
Crotalus stejnegeri Dunn, 1919	SMO
Crotalus polystictus (Cope, 1865)	SMO
Geoemydidae	
Rhinoclemmys rubida (Cope, 1870)	SMS

based in Mexico: C. Grünwald, G. Woolrich-Piña, I. Ahumada-Carrillo, J. Reyes-Velasco, J. Loc-Barragán and M. López-Luna, amongst others. The species accumulation curves, particularly for reptiles and total herpetofauna, do not level off, suggesting that the knowledge of the herpetofauna, especially the reptiles, is incomplete and more species are likely to be described and discovered as modern molecular tools and renewed fieldwork continues.

Recent taxonomical changes

In the years since the writing and publication of Woolrich-Piña et al. (2016), there have been several taxonomic changes or additions with regard to the herpetofauna of Nayarit. In this section, we provide some information on these recent changes and additions.

Devitt et al. (2023) described a new species of *Eleutherodactylus* from the mountainous region of Sinaloa and Nayarit, this species representing populations of this region previously regarded as *Eleutherodactylus nitidus*. Campbell et al. (2018) described a new hylid frog from the southern Sierra Madre Occidental from Durango and southern Sinaloa, along the southern edge of the Mexican Plateau from Michoacán to Morelos. This new frog species replaces *Sarcohyla bistincta* in these States, including Nayarit. Pérez-Ramos and Luja-Molina (2022) studied populations of *R. forreri* from the Pacific Lowlands and distinguished two new species, one of them, *Rana cora*, inhabiting the States of Chihuahua, Sonora, Sinaloa, Nayarit and Colima. Flores-Villela et al. (2022) described a new species of *Sceloporus* from the mountainous region of Jalisco and Nayarit, this species representing populations of this region previously regarded as *Sceloporus torquatus*. Ramírez-Reyes et al. (2021a) presented genomic, phyllogenomic and morphological evidence showing that *Phyllodactylus* populations from María Cleofas Island, Nayarit, represent a new species related to *P. saxatilis*. Ramírez-Reyes et al. (2021b) showed that the arrangement of the subspecies *P. tuberculosus saxatilis* is polyphyletic. To avoid this polyphyletic arrangement and the use of infraspecific categories, they elevated this subspecies to the rank of species, whose distribution includes Nayarit. MacCranie et al. (2020) conducted a revision of the genus *Marisora* from Mexico and Central America and found support for the description of four new species, amongst them *M. aquilonaris* which includes populations previously regarded as *M. brachypoda* in Nayarit, south to Guerrero, Morelos and Puebla in the east. Hansen and Salmon (2017) presented a taxonomic update and a distribution analysis of the *Lampropeltis mexicana* group, providing morphological evidence that indicates the presence of *L. greeri* in Nayarit. Jadin et al. (2020) revised the taxonomy of *Oxybelis aeneus* recognising five additional taxa, including *O. microphthalmus* which is found in Nayarit. Grünwald et al. (2021) presented a molecular phylogeny of the Mexican snail-eating snakes, finding evidence that *Tropidodipsas annulifera* is more closely related to the genus *Geophis* than to other snail-eating snake genera, for which they proposed the new combination *Geophis annuliferus* Boulenger, 1894. Reyes-Velasco et al. (2020) demonstrated in the phylogenetic relationships within the genus *Micrurus*, that individuals distributed on the western coast of Mexico, from Nayarit to Guerrero, including *M. proximans*, represent *M. browni*. Loc-Barragán et al. (2020) showed that populations of *K. chimalhuaca* in Nayarit represent a new species (*K. cora*).

General distribution

Twenty-four of the 37 species of amphibians found in Nayarit are endemic to Mexico (Table 1). This compares to 21 country endemics reported by Woolrich-Piña et al. (2016). Eleven of these 24 endemics are distributed mainly in the Pacific Lowlands. Five more are typical of western-central Mexico, mainly in the States of Sinaloa, Nayarit, Jalisco, Colima and Michoacán. Another five are distributed in several ecoregions, including the Pacific Lowlands, Trans-Mexican Volcanic Belt, Mesa Central and Sierra Madre del Sur. One species (*Dryophytes eximius* [Baird, 1854]) is widely distributed in both Sierra Madre Occidental and Sierra Madre del Sur and the Trans-Mexican Volcanic Belt. Another one (*Isthmura bellii* [Grey, 1850]) is found in central Mexico with isolated populations in Tamaulipas and Nayarit. Finally, the last one (*Ambystoma rosaceum* Taylor, 1941) ranges mainly in the Sierra Madre Occidental. Six more of the 37 amphibian species found in Nayarit are distributed in the United States and Mexico. Four more are species distributed in Mexico and Central America or even South America. The last three amphibian species found in Nayarit are distributed from extreme south-eastern United States to Central America or South America (Table 1).

The only crocodile that inhabits Nayarit, *Crocodylus acutus* (Cuvier, 1807), is widely distributed with populations in southern Florida, USA, the Caribbean, the Gulf of Mexico, the Mexican Pacific, Central and South America.

Thirty-one of the 44 species of lizards that inhabit Nayarit are endemic to Mexico, one of them (*Phyllodactylus cleofasensis*) to Nayarit (Table 1). This is an increase of seven country endemic lizards from the 24 reported in Woolrich-Piña et al. (2016). Sixteen of the other 30 are distributed mainly in the Pacific Lowlands, but can also inhabit in the Sierra Madre Occidental, Sierra Madre del Sur, Mesa Central, Trans-Mexican Volcanic Belt and Balsas Depression. Five more are typical of western-central Mexico. Another six are widely distributed in the Sierra Madre Occidental, Mesa del Centro, Trans-Mexican Volcanic Belt and some of them even in the Sierra Madre del Sur, Balsas Depression and Sierra Madre Oriental. One more, *Sceloporus spinosus* Weigmann, 1828, occurs in the Mesa del Centro, Trans-Mexican Volcanic Belt, Sierra Madre Oriental and Sierra Madre del Sur. Another one, *Plestiodon parviauriculatus* (Taylor, 1933), is found in the western slopes of the Sierra Madre Occidental. The last of the Mexican endemic lizards (*Plestiodon* aff. *brevirostris* [Günther, 1860]) is mainly found in the Trans-Mexican Volcanic Belt. Eight more of the 44 species of lizards found in Nayarit are distributed in the United States and Mexico. Four more are species of lizards distributed in Mexico and Central America or even South America. The last species of lizard (*Gerrhonotus liocephalus* Weigmann, 1828), found in Nayarit, is distributed from extreme south-eastern United States to Central America.

One of the 69 species of snakes that occur in Nayarit is endemic to Nayarit (*Thamnophis rossmani*) and 39 are endemic to Mexico (Table 1). This matches the number of country endemics reported by Woolrich-Piña et al. (2016). Of the 29 species of snakes not endemic to Mexico that inhabit Nayarit, 15 are found in the United States and Mexico. Nine are distributed from Mexico to Central or South America. Four are found from central or southern United States to Central or South America. The last one is a sea snake distributed across the Pacific and Indo-Pacific Oceans.

Five of the 11 species of turtles found in Nayarit are endemic to Mexico (Table 1). This is an increase of one species over the four reported in Woolrich-Piña et al. (2016). One, *Kinosternon hirtipes* (Wagler, 1830) is found in the United States and Mexico. Another one (*Rhinoclemmys pulcherrima* (Gray, 1855) is found in Mexico and Central America. The other four have a circumtropical or circumglobal distribution (Table 1).

Ecoregions

The ecoregion containing the most species of amphibians and reptiles in Nayarit is the Trans-Mexican Volcanic Belt with 96 species inhabiting it, followed by the Sierra Madre Occidental with 95 species and the Sierra Madre del Sur with 77 species. The Pacific Lowlands with 62 species and the Nayarit Islands with 31 species are the least species-rich ecoregions by species of amphibians and reptiles in Nayarit. The mountain habitats in Trans-Mexican Volcanic Belt, Sierra Madre Occidental and Sierra Madre del Sur, host more species than the lowlands habitats in Pacific Lowlands and Nayarit Islands, such that Nayarit is dominated by species with temperate affinities of the first three ecoregions (Table 4). In general, the pattern of species richness amongst the ecoregions we found is similar to that in Woolrich-Piña et al. (2016); however, in the scheme we used, the Coastal Plain of Woolrich-Piña et al. (2016) is divided into the Pacific Lowlands and the Sierra Madre del Sur.

In general, the amphibians of the five ecoregions of Nayarit have none or a low percentage of species protected by the IUCN and SEMARNAT, except for the Trans-Mexican Volcanic Belt that has one species (*Eleutherodactylus teretistes* [VU]) in the IUCN protection categories and the Sierra Madre Occidental, which has one species (*Isthmura bellii* [A]) in the SEMARNAT protection categories and, although the percentages of amphibian species considered at high risk by the EVS are very different to those of IUCN and SEMARNAT, these are still relatively low, the highest of them in the Nayarit Islands with one (*Eleutherodactylus pallidus* [H-17]) of four species with a high risk EVS (Table 5).

On the other hand, the reptiles of the five ecoregions of Nayarit have greater protection than amphibians; however, these remain relatively low, not only for the IUCN and SEMARNAT, but also for the EVS assessments. These three lists have similar percentages for the Nayarit Islands: IUCN = 21.7%; SEMARNAT = 22.2%; and EVS = 23.8%. For the remaining four ecoregions, the EVS percentages are higher than those of the IUCN and SEMARNAT, in some cases more than double or triple (for example: Sierra Madre Occidental and Trans-Mexican Volcanic Belt) (Table 5).

Conservation status

A total of eight (= 5.8% [8/138]) species of amphibians and reptiles in Nayarit are IUCN-listed (i.e. Vulnerable, Endangered or Critically Endangered), 18 (= 11.1% [18/162]) are placed in a protected category (excluding NL and Pr, this last category being equivalent to the LC category of IUCN) by SEMARNAT and 49 (= 33.3% [49/147]) are categorised as high risk by the EVS (Table 2). Woolrich-Piña et al. (2016) found 6.7% were in threatened categories of the IUCN Red List, 11.4% in a protected category by SEMARNAT and 33.8% with high EVS (did not include marine species). For amphibians, 3.1% (1/32) are IUCN-listed, 2.7% (1/37) are protected by SEMARNAT and 23.5% (= 8/34) are at high risk according to the EVS (Table 2; Fig. 6). For reptiles, 6.6% (7/106) are listed by the IUCN, 13.6% (17/125) are protected by SEMARNAT and 36.3% (41/113) are at high risk according to the EVS (Table 2; Fig. 6). This summary suggests that the herpetofauna of Nayarit has relatively few species of conservation concern at a global and national scale (IUCN and SEMARNAT lists), but there might be greater conservation concerns using the EVS which is based on information specific to Mexico and Central America and so might be more likely to reflect the conservation status and needs of the Nayarit herpetofauna. The SEMARNAT list is also based on information specific to Mexico and, although this institution released a new update in 2019, it does not appear that conservation statuses have been re-evaluated since 2010 because all Nayarit statuses for amphibians and reptiles have remained the same. Therefore, although it is a local evaluation, it might not reflect the current conservation status of the species and so does not take into account the numerous recent taxonomic changes and the description of new species or more recent changes in conservation status or threats. There are several taxa that, based on their IUCN listing, SEMARNAT category or their EVS, are of conservation concern. Families with species of particular conservation concern include Eleutherodactylidae, Ranidae, Ambystomatidae, Crocodylidae, Eublepharidae, Helodermatidae, Iguanidae, Phyllodactylidae, Colubridae, Dipsadidae, Elapidae, Natricidae, Viperidae, Cheloniidae and Dermochelyidae (Table 2). The status of a species in Nayarit may differ (i.e. be worse or better) from the IUCN, SEMARNAT and EVS assessments. Thus, assessments at the State level are needed to fully understand the conservation or management needs for the Nayarit herpetofauna.

Table 4. Number of amphibian and reptile species in each physiographic province of Nayarit.

	Pacific Lowlands	Sierra Madre Occidental	Trans-Mexican Volcanic Belt	Sierra Madre del Sur	Nayarit Islands
Amphibians	19	25	25	23	4
Reptiles	43	70	71	54	27
TOTAL	62	95	96	77	31

Table 5. Number of amphibian and reptile species in each physiographic province of Nayarit listed and protected in the IUCN Red List or SEMARNAT list or with high EVS. Numbers in parenthesis represent the total number of species evaluated in each list. In some cases, the total numbers for each list differ because the IUCN Red List and EVS assessments have not yet been reported for all species, the only total equal to that of the species inhabiting each ecoregion is that of the SEMARNAT list.

	IUCN	SEMARNAT	EVS
Amphibians			
Pacific Lowlands	0 (16)	0 (19)	2 (18)
Sierra Madre Occidental	0 (24)	1 (25)	4 (24)
Trans-Mexican Volcanic Belt	1 (24)	0 (25)	5 (24)
Sierra Madre del Sur	0 (21)	0 (23)	3 (23)
Nayarit Islands	0 (4)	0 (4)	1 (4)
Reptiles			
Pacific Lowlands	6 (38)	8 (43)	9 (35)
Sierra Madre Occidental	2 (62)	8 (70)	25 (68)
Trans-Mexican Volcanic Belt	3 (59)	9 (71)	24 (69)
Sierra Madre del Sur	1 (45)	9 (54)	16 (53)
Nayarit Islands	5 (23)	6 (27)	5 (21)

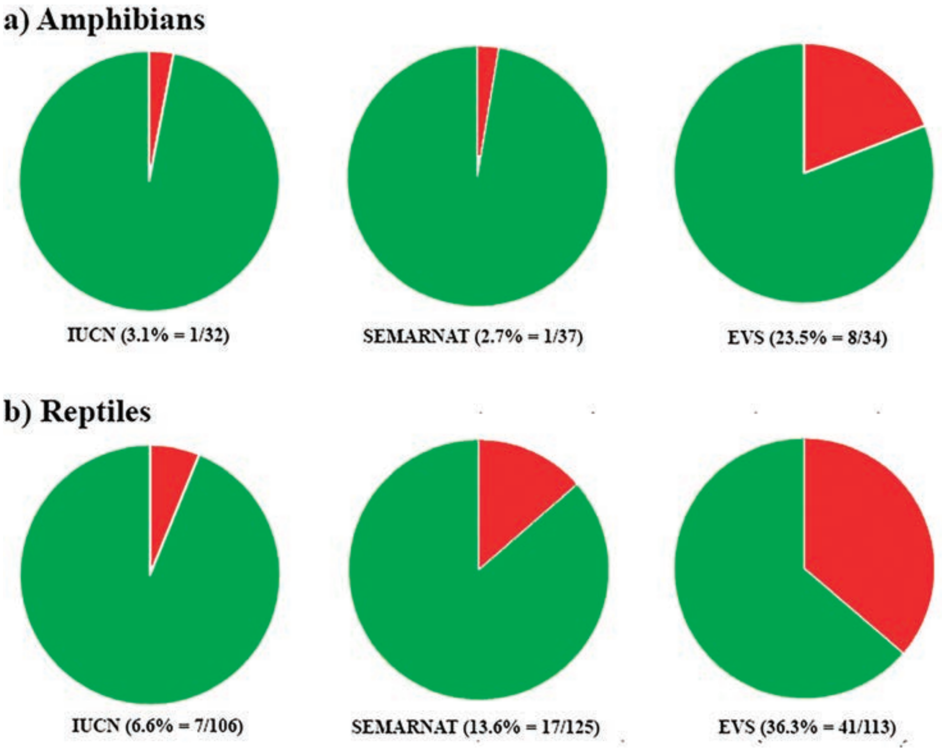

Figure 6. Proportion of **A.** Amphibians and **B.** Reptiles listed in protected categories on the IUCN Red List, SEMARNAT and high EVS for Nayarit. Green is the proportion in Data Deficient and Least Concern (IUCN); Not Listed and Subject to Special Protection (we regarded the category of Subject to Special Protection in SEMARNAT equivalent to Least Concern in IUCN) (SEMARNAT); or low or medium EVS. Red is percentage in protected categories or high EVS. N is the number of species assessed.

Comparison with neighbouring States

Overall, Nayarit shares the most herpetofaunal species with Jalisco, with 88.9% overlap (Table 6). Nayarit shares the most amphibian species with Jalisco (89.2%). Seven families, including Craugastoridae, Eleutherodactylidae, Hylidae, Leptodactylidae, Phyllomedusidae, Ambystomatidae and Plethodontidae, show complete overlap between Nayarit and Jalisco. Only four species of amphibians that inhabit Nayarit do not occur in Jalisco, two of them (*Anaxyrus kelloggi* and *Gastrophryne mazatlanensis*) reach their southernmost distribution in Nayarit, another (*Scaphiopus couchii*) reaches the southernmost distribution on the Mexican Pacific Coast extending its range eastwards to Hidalgo and Veracruz, the fourth (*Rana cora*) is a recently-described species that almost certainly occurs in Jalisco, but has not been reported there yet. The similarity between the amphibian composition of Nayarit and Sinaloa is also high (86.5%), showing complete overlap in almost the same families completely shared between Nayarit and Jalisco, with some slight differences. Sinaloa shows complete overlap with Nayarit in Bufonidae, Craugastoridae, Hylidae, Leptodactylidae, Microhylidae, Phyllomedusidae and Ambystomatidae. Only five species of amphibians that inhabit Nayarit do not occur in Sinaloa: *Eleutherodactylus modestus* is at its northernmost distribution in Nayarit; *Rana megapoda*, *R. psilonota* and *Isthmura bellii* are at their north-westernmost distribution in Nayarit; and *Spea multiplicata* extends to western Mexico in the Sierra Madre Occidental and Trans-Mexican Volcanic Belt of Nayarit and Pacific Lowlands of Jalisco. The neighbouring States that share the lowest percentage of amphibian species with Nayarit are Durango (48.6%) and Zacatecas (37.8%). The high similarity in amphibian composition between Nayarit and Jalisco and Sinaloa, but not with Durango and Zacatecas, is due to the dominance of amphibian species related to the region of the Pacific Lowlands, which are shared between these three States with wide Pacific coasts and the absence of these coasts in Durango and Zacatecas. Durango is mostly a mountainous State dominated by the Sierra Madre Occidental and the Chihuahuan Desert (Lemos-Espinal et al. 2019), whereas Zacatecas is also a mountainous State dominated by the Sierra Madre Occidental, Sierra Madre Oriental and the Chihuahuan Desert.

The number of reptile species shared between Nayarit and Jalisco is large, with 88.8% overlap. Sixteen of the 24 families of reptiles present in Nayarit show a complete overlap with Jalisco and 111 of the species that inhabit Nayarit are shared with Jalisco. Ten of the 14 species of reptiles that inhabit Nayarit, but have not been recorded in Jalisco, show their southernmost distribution in Nayarit (*Holbrookia elegans*, *Urosaurus ornatus*, *Plestiodon parviauriculatus*, *Gyalopion quadrangulare*, *Rhinocheilus lecontei*, *Tantilla yaquia*, *Leptodeira polysticta*, *Thamnophis nigronuchalis*, *Crotalus willardi* and *Kinosternon cora*), two more are endemic to Nayarit (*Phyllodactylus cleofasensis* and *Thamnophis rossmani*)

Table 6. Summary of the numbers of species shared between Nayarit and neighbouring Mexican States (not including introduced species). The percentage of Nayarit species shared by a neighbouring State are given in parentheses. Total refers to the total number of species found in Nayarit and four neighbouring States (i.e. regional species pool) and the number in parentheses in this column is the percentage of the regional species pool found in Nayarit. -- indicates either Nayarit or the neighbouring State has no species in the taxonomic group or none of that specific taxon is shared between the States, thus no value for shared species is provided.

	Nayarit	Jalisco	Sinaloa	Durango	Zacatecas	Total
AMPHIBIA	37	33 (89.2)	32 (86.5)	18 (48.6)	14 (37.8)	75 (49.3)
ORDER ANURA	35	31 (88.6)	31 (88.6)	17 (48.6)	12 (34.3)	70 (50.0)
Bufonidae	5	4 (80.0)	5 (100)	4 (80.0)	2 (40.0)	14 (35.7)
Craugastoridae	5	5 (100)	5 (100)	3 (60.0)	2 (40.0)	7 (71.4)
Eleutherodactylidae	4	4 (100)	3 (75.0)	1 (25.0)	–	14 (28.6)
Hylidae	9	9 (100)	9 (100)	4 (44.4)	3 (33.3)	13 (69.2)
Leptodactylidae	1	1 (100)	1 (100)	–	–	2 (50.0)
Microhylidae	3	2 (66.7)	3 (100)	–	1 (33.3)	4 (75.0)
Phyllomedusidae	1	1 (100)	1(100)	1 (100)	–	1 (100)
Ranidae	5	4 (80.0)	3 (60.0)	2 (40.0)	2 (40.0)	13 (38.5)
Scaphiopodidae	2	1 (50.0)	1 (50.0)	2 (100)	2 (100)	2 (100)
ORDER CAUDATA	2	2 (100)	1 (50.0)	1 (50.0)	2 (100)	5 (20.0)
Ambystomatidae	1	1 (100)	1 (100)	1 (100)	1 (100)	4 (25.0)
Plethodontidae	1	1 (100)	–	–	1 (100)	1 (100)
ORDER GYMNOPHIONA	–	–	–	–	–	1 (0)
Caecilidae	–	–	–	–	–	1 (0)
REPTILIA	125	111 (88.8)	86 (68.8)	61 (48.8)	57 (45.6)	261 (47.9)
CROCODYLIA	1	1 (100)	1 (100)	–	–	1 (100)
Crocodylidae	1	1 (100)	1 (100)	–	–	1 (100)
SQUAMATA	113	100 (88.5)	75 (66.4)	59 (52.2)	55 (48.7)	239 (47.3)
LACERTILIA	44	39 (88.6)	26 (59.1)	24 (54.5)	18 (40.9)	104 (42.3)
Anguidae	3	3 (100)	2 (66.7)	1 (33.3)	3 (100)	5 (60.0)
Corytophanidae	1	1 (100)	–	–	–	1 (100)
Crotaphytidae	–	–	–	–	–	2 (0)
Dactyloidae	1	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)
Eublepharidae	1	1 (100)	–	–	–	4 (25.0)
Helodermatidae	1	1 (100)	1 (100)	1 (100)	1 (100)	3 (33.3)
Iguanidae	2	2 (100)	2 (100)	1 (50.0)	1 (50.0)	5 (40.0)
Phrynosomatidae	22	20 (90.9)	12 (54.5)	16 (72.7)	9 (40.9)	52 (42.3)
Phyllodactylidae	3	1 (33.3)	2 (66.7)	1 (33.3)	–	6 (50.0)
Scincidae	6	5 (83.3)	4 (66.7)	2 (33.3)	2 (33.3)	13 (46.2)
Teiidae	4	4 (100)	2 (50.0)	1 (25.0)	1 (25.0)	12 (33.3)
Xantusidae	–	–	–	–	–	3 (0)
SERPENTES	69	61 (88.4)	49 (71.0)	35 (50.7)	37 (53.6)	135 (51.1)
Boidae	1	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)
Colubridae	28	24 (85.7)	26 (92.9)	17 (60.7)	18 (64.3)	57 (49.1)
Dipsadidae	17	16 (94.1)	12 (70.6)	4 (23.5)	5 (29.4)	33 (51.5)
Elapidae	4	4 (100)	3 (75.0)	–	1 (25.0)	7 (57.1)
Leptotyphlopidae	1	1 (100)	–	–	1 (100)	5 (20.0)
Loxocemidae	1	1 (100)	–	–		1 (100)
Natricidae	9	7 (77.8)	3 (33.3)	8 (88.9)	6 (66.7)	16 (56.3)
Viperidae	8	7 (87.5)	4 (50.0)	5 (62.5)	5 (62.5)	15 (53.3)
TESTUDINES	11	10 (90.9)	10 (90.9)	2 (18.2)	2 (18.2)	21 (52.4)
Cheloniidae	3	3 (100)	3 (100)	–	–	4 (75.0)
Dermochelyidae	1	1 (100)	1 (100)	–	–	1 (100)
Emydidae	2	2 (100)	2 (100)	–	–	5 (40.0)
Geoemydidae	1	1 (100)	1 (100)	–	–	2 (50.0)
Kinosternidae	4	3 (75.0)	3 (75.0)	2 (50.0)	2 (50.0)	7 (57.1)
Testudinidae	–	–	–	–	–	2 (0)
TOTAL	162	144 (88.9)	118 (72.8)	79 (48.8)	71 (43.8)	336 (48.5)

and the other two have a discrete distribution that includes Nayarit, but not Jalisco (*Plestiodon* aff. *brevirostris* and *Salvadora grahamiae*). Nayarit shares a smaller, but still high percentage of reptile species with Sinaloa, with 68.8% overlap. Nine of the 24 families of reptiles present in Nayarit show a complete overlap with Sinaloa and 86 of the species that inhabit Nayarit are shared with Sinaloa. Twenty-three of the 39 species of reptiles that inhabit Nayarit, but have not been recorded in Sinaloa, show their northernmost distribution in Nayarit or Jalisco (*Barisia imbricata*, *Basiliscus vittatus*, *Coleonyx elegans*, *Sceloporus asper*, *S. dugesii*, *S. heterolepis*, *S. horridus*, *S. huichol*, *S. melanorhinus*, *S. scalaris*, *S. unicanthalis*, *Plestiodon* aff. *brevirostris*, *Aspidoscelis lineattissimus*, *Holcosus sinister*, *Salvadora mexicana*, *Tantilla ceboruca*, *Hypsiglena affinis*, *Manolepis putnami*, *Rhadinaea taeniata*, *Sibon nebulatus*, *Micrurus proximans*, *Loxocemus bicolor* and *Kinosternon vogti*). Six more species show a discrete distribution that does not include Sinaloa (*Rena humilis*, *Thamnophis eques*, *T. errans*, *T. melanogaster*, *T. pulchrilatus* and *Salvadora grahamiae*). Another eight species are found in the Sierra Madre Occidental and/or Trans-Mexican Volcanic Belt (*Phrynosoma orbiculare*, *Sceloporus grammicus*, *Plestiodon lynxe*, *Thamnophis nigronuchalis*, *Crotalus armstrongi*, *C. campbelli*, *C. pricei* and *C. willardi*). The last two species not shared with Sinaloa are endemic to Nayarit (*Phyllodactylus cleofasensis* and *Thamnophis rossmani*). Nayarit shares 61 species of reptiles with Durango, representing a 48.8% overlap. This relatively low overlap compared to Jalisco and Sinaloa can be explained by the fact that Durango is dominated by species from the Sierra Madre Occidental and the Chihuahuan Desert, while the other three States (Nayarit, Jalisco and Sinaloa) are dominated by species from the Pacific Coast. Nayarit has a coastline of 307 km, Jalisco 351 km and Sinaloa 622 km. The lowest number of shared species between Nayarit and a neighbouring State is represented by Zacatecas with only 57 species shared with Nayarit, representing 45.6% overlap. Just as Durango, Zacatecas is dominated by species from the Sierra Madre Occidental and the Chihuahuan Desert, which may explain this low overlap with Nayarit.

The relationship between the neighbouring States of Nayarit shows that Nayarit is a transition State between species with Nearctic and Neotropical affinities; most of the species present in Nayarit, but absent in Sinaloa, show their northernmost distribution in Nayarit, contrary to most of the species present in Nayarit, but absent in Jalisco show their southernmost distribution in Nayarit. In addition, Nayarit is also a State that contains species from the Pacific Coast as well as the mountains of the Sierra Madre Occidental, Trans-Mexican Volcanic Belt and Sierra Madre del Sur.

Conclusions

One of the main take-home messages of our update of the list of the herpetofaunal species of Nayarit is that our understanding of the full complement of species is not complete, especially the reptiles. This is evidenced by the rapid accumulation of species in Nayarit in the past decade (see Fig. 5). In addition, our updated list has increased the number of country endemic species found in Nayarit. It seems likely, as herpetofaunal surveys and systematic studies continue, both in Nayarit and in other Mexican States, that the richness of amphibians and reptiles will increase.

Acknowledgements

We thank the logistic support provided by all the people of each locality visited in Nayarit. We are grateful to Alejandra Núñez Merchand from the National Commission for the Understanding and Use of Biodiversity (CONABIO) for kindly creating and providing the municipality, topographic, physiographic, climate and vegetation maps used in this publication and for generating the State border lengths of Nayarit's neighbouring States, to Isabel Cruz, also from CONABIO, for providing the satellite images of Nayarit and to Jesús Sigala-Rodríguez for allowing us access to an unpublished list of amphibians and reptiles of Zacatecas. Support for this study was provided by the Turtle Conservation Fund-TCF (096) and Mohamed bin Zayed Species Conservation Fund MZSC (220529355), through Project Ecological Observations of Cora Mud Turtle (*Kinosternon cora*) in Nayarit and Sinaloa, Mexico to JLB; TecNM projects 5293.19-P and 14548.22-PD, Subsecretaría de Educación Superior through the Dirección General de Educación Superior Universitaria e Intercultural and Dirección de Superación Académica granted the funds for the group Ecología, Distribución y Conservación de Fauna Silvestre IDCA 27963, Clave ITESZACA-CA-4, 2020–2021; and internal grants from the ITS Zacapoaxtla PI-LB to GAWP; and through the generous support provided by the Dirección General de Asuntos del Personal Académico – Programa de Apoyos para la Superación del Personal Académico de la UNAM (DGAPA-PASPA) through the scholarship assigned to JLE for his sabbatical year at the University of Colorado, Boulder and by the Dirección General de Asuntos del Personal Académico – Programa de Apoyo a Proyectos de Investigación e Innovación Tecnológicas (DGAPA-PAPIIT) through the project IN202021. We thank one anonymous reviewer and the subject editor (B. Rojas) for helpful comments that improved the manuscript.

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ÖGH Herpetozoa
Austrian Herpetological Society

Herpetozoa 37: 43–55 (2024)
DOI 10.3897/herpetozoa.37.e107986

Three brown frog species in Denmark have different abilities to colonise new ponds

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Academic editor: Günter Gollmann ♦ **Received** 14 June 2023 ♦ **Accepted** 7 February 2024 ♦ **Published** 20 February 2024

Abstract

For 29 consecutive years, the populations of three species of brown frogs, *Rana arvalis*, *R. dalmatina* and *R. temporaria*, were monitored in an open area in South Zealand, Denmark, with no direct influence of agriculture. Population sizes were recorded by counting egg clumps and showed large variations from year to year. The total population of *R. arvalis* differed by a factor of 100 between the years with the lowest and the highest numbers. A total of 19 initially unoccupied suitable waterbodies could potentially be colonised by the frogs. *Rana dalmatina* colonised all of the ponds, mostly in the very first year of existence. *Rana arvalis* colonised 17 ponds after an average of 10.5 years. *Rana temporaria* colonised eight ponds after an average of 13.4 years. Colonisation by *R. dalmatina* was independent of changes in total population size, whereas colonisation by *R. arvalis* predominantly occurred in years with considerable population increases. The results are discussed in relation to the movement patterns and philopatry of juvenile frogs of the three species. Juvenile *R. dalmatina* disperse far from the breeding site, but most individuals return to their natal site. This allows the species to be an efficient coloniser of new waterbodies and, at the same time, to have stable occurrence at the original site. The two other species show a more erratic type of dispersal and especially *R. temporaria* often shifts breeding site from year to year.

Key Words

dispersal, movements, philopatry, *Rana arvalis*, *Rana dalmatina*, *Rana temporaria*, time series

Introduction

This paper reports the results from monitoring of brown-frog populations covering a 29-year study period. The purpose is: 1) to present a long data series on the development of amphibian populations in an area where populations were not threatened by the influence of agriculture and 2) to report differences between species in colonisation events.

The study was conducted in a military training area in South Zealand, Denmark. Three species of brown frogs breed in this area: moor frog, *Rana arvalis* Nilsson, 1842, agile frog, *Rana dalmatina* Fitzinger in Bonaparte, 1838 and common frog, *Rana temporaria* Linnaeus, 1758.

Brown frog population size was monitored by counting the number of egg clumps in spring. Each mature female will usually produce a single egg clump per year in *R. dalmatina* (Hachtel and Grossenbacher 2014) and presumably also in other *Rana* species (Glandt 2014). Thus, the total egg clump count approximately indicates the number of mature females and, thereby, provides a population size index.

Several other studies have reported long-term monitoring results for European species of brown frogs by egg clump counts (Meyer et al. 1998; Hartel 2008; Ahlén 2013; Băncilă et al. 2016; Canova and Balestrieri 2018; Combes et al. 2018; Meek 2018; Schmidt et al. 2021). However, in all these studies, the study species was distributed in a way that left little opportunity to study colonisation events.

In the present study, populations were monitored in all waterbodies in an area covering nearly 2 km². During the

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study, 19 new suitable waterbodies were created or arose by natural flooding, which allowed monitoring of how fast each of the three species colonised waterbodies that had become available for breeding.

It is well known that amphibians are declining (e.g. Fog (1988)) and that the most efficient way to counteract such decline is the creation and improvement of ponds (Fog 1997a). Many projects in European countries have been conducted to improve the living conditions for threatened amphibian species by restoring old ponds and/or creating new ones. The largest efforts have probably been made in Switzerland, The Netherlands and Denmark (Fog 1997a; Stumpel and van der Voet 1998; Fog et al. 2019; Moor et al. 2022). In Denmark, for instance, thousands of ponds have been created since 1988. A survey of follow-up data (Fog 1997a) provided the results for 1,308 ponds. These were investigated again 5 years after the improvement. By then, 43% of the new ponds and 42% of the restored ponds had been colonised by natural immigration of the target species.

The colonisation process depends on several aspects of the species' biology, such as pond fidelity and the number of individuals that move far away from their natal pond. The importance of such traits is extensively discussed in literature on amphibian ecology, for example, by Smith and Green (2005) and Sinsch (2014). The present paper may add new aspects to that discussion.

Methods

Study area

I conducted the study in the military training area of Kulsbjerge in South Zealand, Denmark, situated at 55°N, 12°E (Fig. 1). The training grounds, comprising a total area of 4.96 km², were established in 1970. Before this, the area was used for intensive agriculture.

The study area covers about 1.8 km² in the eastern half of the military training area. It is hilly with elevations ranging from 50 to 100 m above sea level. The terrestrial habitats are mostly grassy vegetation with a height of less than 1 m. Many bushes are scattered around, mainly *Crataegus* and *Salix*. To the north, the study area is bordered by deciduous wood; to the south, mostly by detached houses with gardens. To the east and west, the military area extends beyond the study area.

Until 1970, only few possible breeding localities existed for amphibians in this area: a few small marl pits and a few larger swamps formed by peat diggings. The many depressions in the landscape were kept dry by drainage pipes. In the period up to 1992, when the study started, additional waterbodies had arisen, mostly as flooding in natural depressions after heavy military vehicles had destroyed the drainage pipes in the ground. The largest natural flooding covered 8,000 m². Water depth and vegetation vary amongst the waterbodies. Many of them have extensive reed belts of *Typha latifolia* along the banks. A few of the waterbodies have fish, but the fish species were not determined, except that pikes (*Esox lucius*) were observed in two waterbodies and a dead tench (*Tinca tinca*) was seen in another. Over the years, predation pressure from fish has varied. After some winters with long ice cover and after desiccation in dry summers, fish populations were reduced, after which breeding frogs immigrated into the respective waterbodies.

Out of the waterbodies that existed in the area in 1988, two were lakes with fish. Eleven were permanent waterbodies with no fish or moderate fish populations. These waterbodies were not changed further and were not defined as targets of colonisation. At the start of the study, 10 of them were inhabited by *R. dalmatina* already, seven by *R. arvalis* and five by *R. temporaria*. The latter species colonised some waterbodies that had existed unchanged all the time, but were not inhabited until some time had passed after the study period had started. Such cases were not included in data on colonisation, because it was not possible to indicate the number of years since the first existence of those waterbodies.

In 1988, the military administration agreed that a number of ponds were excavated or improved to benefit amphibians. Improvements comprised cutting down shading trees, removing bottom sludge by dredging and improving bottom profiles to create more gently-sloping banks. In these ways, a total of eight new or improved ponds were made in the years 1989 and 1992. Three of these were colonised by *R. dalmatina* already before the improvement. Thus, the remaining five ponds could potentially be colonised in the following years by this species and all eight ponds could potentially be colonised by the two other brown frog species. There were no fish in these ponds, except that a pike was observed in one of them, but this did not prevent subsequent colonisation there in the shallow parts by *R. arvalis* and *R. temporaria*.

During the 1992–2020 study period, many more floodings formed in natural depressions. Here, the term "flooding" indicates a depression that was previously dry, but which gradually became inundated with rainwater. These floodings were not in contact with streams or lakes and did not have fish. Eleven of them gradually turned into permanent waterbodies. For three of these 11, the first year when they could possibly be colonised was ambiguous, for instance, when they came into existence in one year, then were dry for several years and later came into existence again. For the remaining eight waterbodies, the first year of existence was well defined. One flooding was already colonised by *R. dalmatina* when the study started.

To sum up: altogether, 19 waterbodies where *R. arvalis* and *R. temporaria* were initially not present, could potentially be colonised by them. All of these 19 waterbodies are considered to be suitable for breeding of all three brown frog species. In four of these ponds, *R. dalmatina* was already present before the start of the study period, so only 15 vacant waterbodies were available for colonisation by *R. dalmatina* during the study period.

Fourteen other floodings formed during the study period, but these existed only in some years and were,

Figure 1. The location of the study area in south Zealand is marked with a red dot.

therefore, not considered suitable for permanent colonisation. Altogether, the number of waterbodies that existed in all years, or in most years, or in only some years, were 2 + 11 + 19 + 14 = 46. Eggs were also found in very temporary floodings that dried out a few weeks later and did not turn into permanent ponds in subsequent years. I did not classify such cases as colonisations, because no new breeding population could be established there. Such places were not included in the counts of waterbodies.

Detecting colonisation

I inspected all excavated and improved ponds annually to register when eggs of a species appeared for the first time. I inspected natural floodings even if they had just arisen and there was only little water. The first time that an egg clump of a species appeared was noted as "first appearance". If this was followed by continued presence of the species in some of the following years, this was classified as "colonisation". The first parent generation might have died after the first year, but if new egg clumps were found no later than 4 years after, this could possibly represent the breeding of the offspring from the previous eggs and so this was also classified as a colonisation. If the species had colonised the site continuously during a few years and then disappeared again, this was also considered as a colonisation.

Recordings

After a few observations in 1988 and 1990, I initiated systematic recordings in 1992. Each year, I surveyed all relevant waterbodies in the study area for *Rana* eggs, except that a few ponds were omitted in 1992 and a few others in 1993. The number of survey dates per year was 3–5, depending on the total number of egg clumps and, hence, how many days were necessary to investigate all waterbodies. Each locality was investigated first for *R. dalmatina* and then once again for the two other species, which breed slightly later in the spring. Six to 22 days passed between the first and the last visit. The number of visits depended on how concentrated the spawning season was in that particular year.

Very often, repeated visits of the same waterbodies were made to check if the first counts gave the full number of egg clumps or if additional egg clumps had been spawned later in the season. Data from such repeated visits allowed me to compare the results from a first and a second visit in the same spring of the same waterbody and also gave interesting information on the length of the spawning period in different years.

The search for *R. dalmatina* egg clumps was conducted by wading through all accessible parts of the waterbodies to a water depth of approx. 1.2 m and recording all egg clumps that could be seen from there. In most waterbodies, the search route followed a meandering pattern with a distance of 2–3 m between parallel tracks. During the recordings, some egg clumps remain fixed to straws at a depth of up to 1 m below the water surface. To detect such clumps, full sunshine is crucial, as half or more of the clumps below the water surface will be overlooked in overcast weather.

Egg clumps of *R. arvalis* and *R. temporaria* were usually, but not always, deposited in shallow water near the banks. They are typically placed so densely together in shallow water near banks the that they form “egg carpets”. Some clumps were detected at the bottom at depths of more than 50 cm or in floating vegetation far from the bank while searching for *R. dalmatina* clumps. Counting clumps in egg carpets required full sunshine to clearly identify borders between different clumps. If the eggs formed dense carpets, I lifted upper egg clumps to observe any additional clumps below them. In a few cases, when egg carpets were large and extensive, I measured the area of the egg carpet with a folding ruler and calculated the number of clumps from previous observations of number of clumps per m². For *R. arvalis*, this was most often ca. 100 clumps per m².

The results may be considered as an index of the population size. As I made all observations in the same waterbodies in the same manner and in the same weather conditions (full sunshine) every year, differences between years are a reliable indication of differences in this “index value”.

The main focus of the present study is colonisation. Here, the crucial data are presence or absence of the species rather than the exact population size. Newly arisen waterbodies had clear water, sparse vegetation and were often shallow. In such waterbodies, it can be assumed that 100% of the egg clumps had been detected.

Species determination

Species determination from egg clumps is not straightforward and requires experience. Description of specific characteristics of egg clumps, for example, in Nöllert and Nöllert (1992), do not generally hold true for all sizes of spawning females or in all geographic regions. Egg clumps of *R. dalmatina* are usually very characteristic, being fastened around submerged vertical straws. However, in case they are not, they may be determined from the structure of the egg gel and by the characteristic shape of the embryos shortly before hatching. At the beginning of the project, I placed a few eggs from egg clumps at Kulsbjerge in aquaria and reared them until the tadpoles were large enough to tell the species, in order to define what characterises egg clumps of the species in this local area. I concluded that distinguishing the egg clumps of *R. temporaria* from those of *R. arvalis* was usually possible, though not always. No single character will ensure a 100% certain determination. The most useful character is that the egg gel is firmer in *R. arvalis* than in *R. temporaria*. Furthermore, the colour hue is often, but not always, a useful characteristic.

In the Kulsbjerge area, *R. arvalis* and *R. temporaria* usually deposit their eggs mixed between each other in the same egg carpets. It is difficult to determine which clumps belong to each of the two species. Determining this requires the handling of every single egg clump to feel the firmness of the gel. In cases of doubt, I used additional options, for example, inspection of the egg clumps just when the newly-hatched larvae lie on top of the egg mass. In this stage, *R. temporaria* and *R. arvalis* differ by the length of the external gills (Fog et al. 2001; Pankratius and Assmann 2008). A further possibility is to catch tadpoles in the water later in the season and determine their species from the teeth rows in the mouth field, as described, for example, in Grillitsch et al. (1983), Nöllert and Nöllert (1992) and Fog et al. (2001). In some cases, I observed newly-metamorphosed froglets at the water’s edge. Fog (2008) has described which characteristics are useful in identifying *R. dalmatina*, *R. temporaria* and *R. arvalis* froglets. The species of froglets found always matched the species of eggs found in the spring.

Catching of adult frogs or hearing them calling was possible in only a few cases.

Determinations of *R. dalmatina* are here treated as being always correct. Most, but not all, determinations of *R. arvalis* and *R. temporaria* are considered to be certain. I noted some egg clumps as “likely *R. temporaria*”. Data on such egg clumps were used only in the presentation of the overall population trends, not for recordings of colonisation. Furthermore, a few egg clumps remained “undetermined”; this included some egg clumps that had dried out before I found them.

Differences in how rapidly species colonise

For each waterbody and for each of the three species, I recorded the length of the period (the number of years) until the species appeared there for the first time and until it had more permanently colonised the waterbody. A Kruskal-Wallis test was used to test for differences between the three species in the duration until appearance or until colonisation and, subsequently, a Wilcoxon-Mann-Whitney test was used as a post-hoc test for pairwise comparisons between two species. The calculations were made manually following the procedures given by Siegel and Castellan (1988).

It was of interest to examine if colonisations occurred mainly in years with large population increases. I chose an arbitrary criterion for what was a year with a large increase, namely that the increase in population size from the previous year was at least 50% of the average population size over the study period for that species. This reflects the absolute number of new recruits in the area, which is more relevant than the relative number of new individuals compared to the previous years. This criterion selected a suitable number of years, namely 5, 6 and 5 years, respectively, for *R. dalmatina*, *R. arvalis* and *R. temporaria*. Next, the probability that a particular colonisation event randomly occurred in one of these years, was calculated as follows: for each species and each year, the number of previously unoccupied waterbodies that could potentially be colonised was counted and the total number of colonisation opportunities that fell within the indicated 5 or 6 years was calculated as a proportion of all opportunities. For instance, in *R. arvalis*, a fraction of 0.198 of all colonisation opportunities fell within the 6 years referred to. Taking this as the probability in a binomial distribution, I used the binomial test for the H_0 hypothesis that the sum of the number of colonisation events in these selected years was no higher than expected, using the total number of colonisation events as the number of trials in the distribution.

Results

Population sizes

During the 29-year observation period, in all waterbodies combined, a total of approx. 60,000 egg clumps were counted, amongst which 35.6% were *R. dalmatina*, 55.5% were *R. arvalis* and 5.9% were certain *R. temporaria*, 1.2% were likely *R. temporaria* and 1.7% were undetermined (Suppl. Materials 1, 2). Populations were relatively small during the 1992–2003 period and considerably larger during the 2004–2020 period (Fig. 2). For *R. dalmatina*, the minimum number of egg clumps was 279, recorded in 2003. The maximum number counted was 2,179, recorded in 2020. The minimum number of reliably determined *R. arvalis* was 23, recorded in 1994, to which must be added most of the 39 undetermined egg clumps. The maximum number of *R. arvalis* egg clumps was 4,310, recorded in 2013. *Rana temporaria* had a maximum of 691 in 2020. There were no certain recordings of *R. temporaria* from 1995 to 2002, but, in most of these years, there were likely recordings or (in 1996) undetermined eggs that could also have been *R. temporaria*. Only one year, 1997, had no recordings of possible *R. temporaria*.

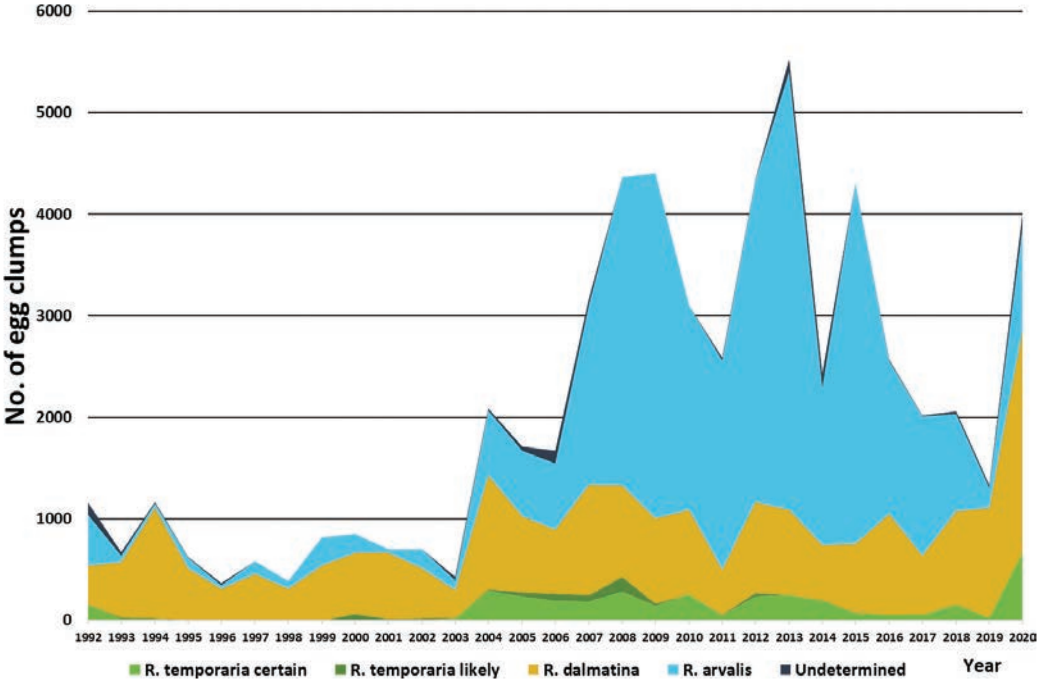

Figure 2. Total annual egg clump numbers from 1992 to 2020. From the bottom to the top, the sections of the graph show the figures for certain *R. temporaria*, likely *R. temporaria*, *R. dalmatina*, *R. arvalis* and egg clumps not determined by species. A few ponds were omitted in 1992 and a few others, in 1993. Data for these ponds were extrapolated from the general trends for each species in those years, in order to give a correct impression of the relative change from year to year during the whole study period. Without the inclusion of the extrapolated values, the total number of egg clumps would have been 18% lower and 7% lower than actually shown in 1992 and 1993, respectively.

The simultaneous increase in all three species from 2004 onwards is remarkable. The increase was not restricted to a few waterbodies, but was observed in many waterbodies all over the study area. There was an additional increase of *R. arvalis* from 2007 onwards, leading to a population size about 100 times higher than at the minimum in 1994. This increase, too, was distributed over many waterbodies.

The geographic pattern of colonisation

Rana dalmatina was recorded in all waterbodies in the study area at least once and colonisation of new sites took place rapidly (Fig. 3). Only a few waterbodies were situated far from a possible source locality.

In *R. arvalis*, no single waterbody was occupied every single year during the first study years. However, those seven waterbodies that were most permanently occupied, all situated in the west-central area, could be considered as likely source ponds (Fig. 4). Out of the 19 “target localities” that were available for permanent colonisation, 17 were eventually colonised. One was never visited by the species and one had eggs only in a couple of years, after which the species disappeared again.

In *R. temporaria*, no waterbodies were obvious source ponds. In some ponds, the species was absent during the mid-1990s for no longer than 3 or 4 years and the few individuals present may have shifted between some of these ponds. These are situated in the mid-central area (Fig. 5). Colonisers of other waterbodies may or may not have originated from these localities. Out of the 19 “target localities” that were available for permanent colonisation, three had no recordings of *R. temporaria*. All other “target localities” were either colonised or had sparse visits of the species.

The temporal aspects of colonisation

The average length of the period from the time when the first eggs could potentially appear until the time when

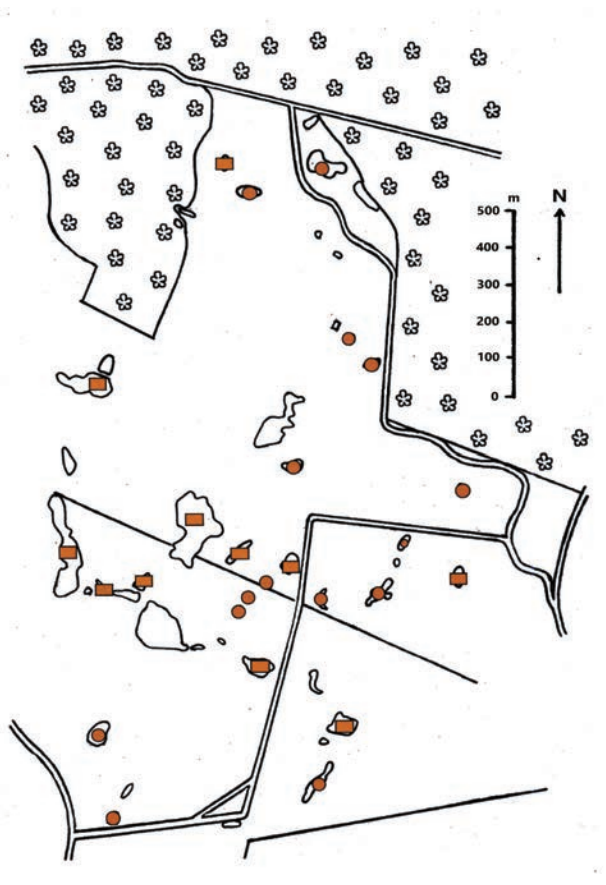

Figure 3. Map of the study area illustrating colonisations of *R. dalmatina*. Surrounding woods are shown. Any waterbodies existing at some time are shown with their outlines. Coloured rectangles show likely source localities. Circles indicate the 15 “target localities” that were available for permanent colonisation. Larger circles indicate permanent colonisation within 0–5 years. There is one smaller circle in the east-central area where colonisation occurred only after 8 years.

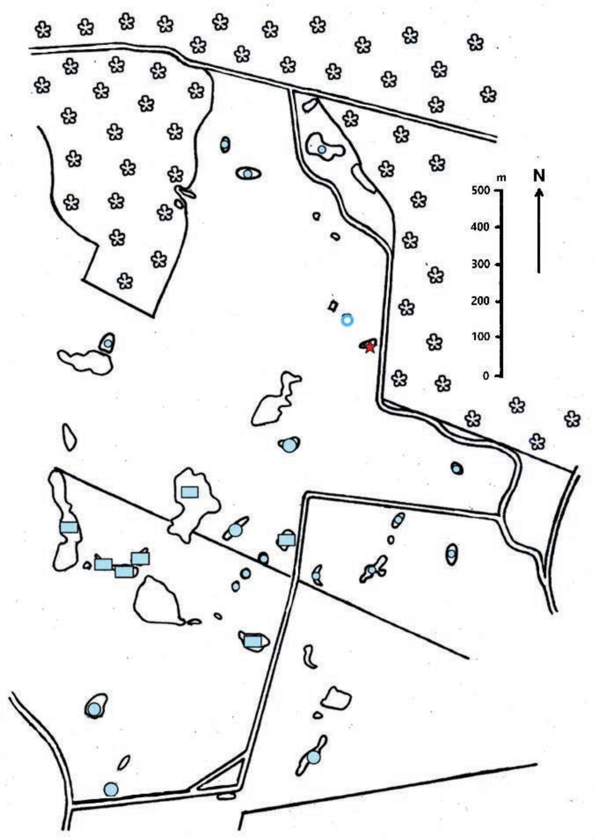

Figure 4. Map of the study area illustrating colonisations of *R. arvalis*. Coloured rectangles show likely source localities. Filled circles show colonised “target ponds”. Larger circles indicate permanent colonisation within 0–5 years, smaller circles permanent colonisation after more than 5 years. An open circle (north of the red asterisk) indicates that eggs had been found there, but colonisation was not permanent. A red star shows a “target pond” where the species never appeared. The species also colonised some other (older) ponds, which were not target ponds (not shown).

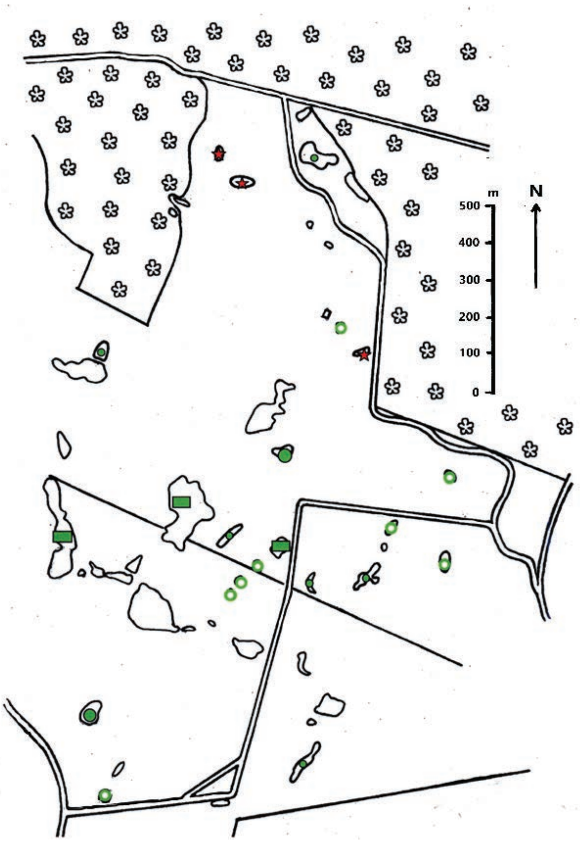

Figure 5. Map of the study area illustrating colonisations of *R. temporaria*. Coloured rectangles show likely source localities. Filled circles show colonised "target ponds". Larger circles indicate permanent colonisation within 0–5 years, smaller circles permanent colonisation after more than 5 years. Open circles indicate that eggs had been found there or a single adult had been observed, but colonisation was not permanent. The three red stars show "target ponds" where the species never appeared. The species also colonised some other (older) ponds, which were not target ponds (not shown).

the first egg clumps actually appeared differed between species. *Rana dalmatina* usually appeared in the very year that occurrence became possible. *Rana arvalis* and *R. temporaria,* on the other hand, usually arrived much later. In many cases, more than 10 years elapsed before the first eggs appeared (Table 1). In those new waterbodies where the species appeared, the average duration until egg clumps appeared for the first time was 0.5 years (SD 1.2) for *R. dalmatina*, 7.9 years (SD 5.8) for *R. arvalis* and 10.6 years (SD 6.9) for *R. temporaria*.

Table 1. Time to first appearance of eggs after a water body had become available for colonisation. Each cell gives the number of water bodies with first appearance after the number of years indicated in the headline atina.

Species	Number of years							No eggs found
	0	1–2	3–5	6–10	11–15	16–20	> 20	
R. dalmatina	11	3	1	0	0	0	0	0
R. arvalis	1	3	3	5	3	3	0	1
R. temporaria	0	1	4	2	4	2	2	4

However, the first egg clumps did not necessarily indicate the beginning of a more permanent colonisation. The species would often be absent for more than three consecutive years after the first colonisation event and more permanent colonisation would only occur later. *Rana dalmatina* colonised all waterbodies and no colonisation was delayed more than 8 years, whereas, in the other two species, about half of the colonisations were delayed more than 10 years. Two waterbodies were not permanently colonised by *R. arvalis*. Eleven waterbodies were not permanently colonised by *R. temporaria* (Table 2). For those waterbodies that were colonised, the average duration until a colonisation lasting for at least several years was 0.9 years (SD 2.2) for *R. dalmatina*, 10.5 years (SD 6.6) for *R. arvalis* and 13.4 years (SD 8.8) for *R. temporaria*.

Table 2. Time taken to colonise water bodies by the three frog species. Each cell gives the number of water bodies that were colonised after the number of years indicated.

Species	Number of years							Not colonised
	0	1–2	3–5	6–10	11–15	16–20	> 20	
R. dalmatina	10	3	1	1	0	0	0	0
R. arvalis	1	2	2	3	4	4	1	2
R. temporaria	0	1	1	2	1	2	1	11

The difference between *R. dalmatina* and the two other species in length of period until first appearance was highly significant. The same was true for the difference in length of the period until colonisation (Wilcoxon-Mann-Whitney test: all comparisons: $p < 0.0001$). In contrast, the difference between *R. arvalis* and *R. temporaria* was not significant ($p = 0.14$ for first appearance, $p = 0.48$ for colonisation).

This is true when analysing the whole study period. However, the opportunities for colonisation by *R. arvalis* and *R. temporaria* were relatively poor in the first half of the study period, whereas *R. dalmatina* had relatively better opportunities to colonise the northern waterbodies rapidly, because it was already present from the beginning in both the southern and the northern half of the study area (Fig. 3). *Rana arvalis* and *R. temporaria* had small populations until 2004 (Fig. 2). The situation for these two species was much better in the second half of the study period. Therefore, a second analysis was made to see if the differences between the species in the rate of colonisation persisted in the second half of the study period. This analysis was restricted to those waterbodies that were available for colonisation from 2004 onwards (number of available vacant waterbodies that were eventually colonised: *R. dalmatina*: 7; *R. arvalis*: 9; *R. temporaria*: 6). This new analysis showed the same pattern as the analysis for the whole period. *Rana dalmatina* still appeared earlier than *R. arvalis* and earlier than *R. temporaria* ($p < 0.0005$ resp. $p = 0.02$) and *R. dalmatina* also still colonised earlier than *R. arvalis* and *R. temporaria* ($p < 0.05$ in both comparisons).

In years with large population increases, some of the many new individuals may colonise new waterbodies. To examine if colonisations occur mainly as a result of population growth, I identified years in which a marked population rise occurred, applying the criterion described in the Methods section. Years with a large population rise or boost were for *R. dalmatina*: 1994, 2004, 2007, 2012 and 2020; for *R. arvalis*: 2007, 2008, 2012, 2013, 2015 and 2020; and for *R. temporaria*: 2004, 2008, 2012, 2018 and 2020. For *R. arvalis*, there was a significant trend that events of first appearance occurred in the boost years more often than expected (eight events out of 18; the occurrence of eight events or more has a probability of $p = 0.0153$). Likewise, concerning colonisation events, the figures are eight events out of 17, $p = 0.0103$. A similar pattern could not be demonstrated for the other species. Considering that three species were tested and one had a significant trend, we may apply a Bonferroni correction with a factor 3, giving the criterion that $p < 0.05/3 = 0.0167$, which is met for *R. arvalis*. In *R. temporaria*, there was a slight trend for first appearances to occur relatively frequently in boost years ($p < 0.25$) and also for colonisations of non-target localities to occur in boost years (data not shown). In contrast, there was no such trend in *R. dalmatina.* It appeared in many waterbodies in the very first year that they existed, most often in 1993, when its total population was relatively small. Therefore, what governed colonisations by *R. dalmatina* was availability of colonisable waterbodies, not the population size.

Temporary waterbodies

Some egg clumps were found in temporary floodings that did not hold water long enough to allow for successful breeding and that did not develop into more permanent ponds over the years. The total number per year of egg clumps deposited in such places was, on average, 12.2 for *R. dalmatina*, 14.9 for *R. arvalis* and 0.6 for *R. temporaria*. In addition, some egg clumps were found in water-filled wheel tracks made by heavy military vehicles. The average annual number egg clumps deposited in such places was 4.1 for *R. dalmatina*, 1.1 for *R. arvalis* and 0 for *R. temporaria*.

Local extinctions and pond shifts

Once *R. dalmatina* had colonised a waterbody, it nearly always remained there, except for years when the floodings had no water. Out of 15 suitable waterbodies existing from the start of the study, 10 contained *R. dalmatina* eggs every single year from 1992 to 2020. There were just two cases when the species disappeared from a pond due to gradual overgrowth with shading bushes. In one other pond, a target pond, the population size went from a record-high 494 egg clumps in 1994 to just 2 egg clumps in 2020. The main cause of this was probably that pikes had appeared there at some time after the pond was excavated.

For *R. arvalis* and *R. temporaria*, the pattern was different. There were just five waterbodies with stable occurrence of *R. arvalis* from 2004 onwards. Out of 15 permanently suitable waterbodies, existing from the start of the study, none contained *R. arvalis* or *R. temporaria* eggs every single year from 1992 onwards.

In some cases, *R. arvalis* or *R. temporaria* showed a very erratic course of colonisation. They suddenly appeared in a waterbody and spawned a large number of egg clumps there, but then were absent for a long period afterwards, even if the spawning had led to breeding success. *Rana arvalis* often colonised a pond preliminarily, then it was absent, often for 4 years or more and then colonised the pond again. In some cases, such local extinctions were synchronous between several ponds. In *R. temporaria*, there were many shifts between two or three neighbouring waterbodies, situated 100 – 200 m from each other, with breeding in one waterbody one or two years and in another in one or two other years. Many local extinctions were followed by subsequent recolonisations, but rarely in a synchronous pattern.

Repeated censuses of the same waterbodies

In those cases when all egg clumps in a waterbody were counted twice, the results were mixed. For *R. dalmatina,* the change in numbers counted from the first to the second visit was as follows, with indications of the proportion of the egg clumps represented in each category: in 20%, a large increase (an increase by 50% or more), in 34% a moderate increase (on average 23%), in 5% practically identical counts (± 1 egg clump) and in nearly 40% a decline in the numbers (on average an 18% decline). The large increases were in cases when the first visit was obviously too early in the season.

In 1996, a late spring with no eggs yet on 09/04, *R. dalmatina* on average spawned later than the other brown frogs. *Rana dalmatina* egg clumps that were clearly newly spawned were found at a second visit on 05/05 after a first visit on 23/04, indicating that egg clumps were spawned in the end of April or the first days of May. In contrast, in some other years, the spawning season had ended in many waterbodies much earlier, in one particular year already before 24/03.

The data on repeated visits concerning *R. arvalis* are dominated by results from a particular year when there was much ongoing spawning during the first half of April after the first visit. Apart from that year, the change in numbers counted from the first to the second visit was as follows: waterbodies representing nearly 50% of the egg clumps showed moderate increases (23% on average) at the second count. For 9% of the egg clumps there were identical results (± 1 egg clump) and for 42% of the egg clumps, there was a decline in numbers (on average a 22% decline). There were no indications that large increases were preferably in cases when the first visit was relatively early in the season.

There are no useful data for *R. temporaria* on this aspect.

Another procedure was to record any additional, newly-spawned egg clumps observed at visits made late in the season. For *R. dalmatina*, this added on average 6% to the previous counts. This percentage was lowest (3%) when only 1–7 days had passed since the previous visit and largest (12%) when more than 20 days had passed since the previous visit. For *R. arvalis,* this procedure added on average 4% to the previous counts, with no trend regarding the number of days between the visits.

Discussion

Population trends

Considering that few long time series of amphibian populations have been published, it is relevant to present the population trends in the present study (Fig. 2). There was a large increase in all three species in 2004 and again a large increase especially for *R. arvalis* in 2007–2008. These increases were observed in many waterbodies simultaneously and, thus, could not be explained by events in a few specific waterbodies. Rather, they could be caused by some weather conditions, such as the unusually mild winter 2006–2007. Analyses of what weather conditions might explain the ups and downs during the study period will be possible and are planned.

There were no certain recordings of *R. temporaria* from 1995 to 2002, but in most of these years, there were likely recordings. To classify these likely recordings as “undetermined” would have given the false impression that *R. temporaria* was most likely absent during a long period, when it was actually most likely present. To determine an egg clump that looks like *temporaria* as certainly *temporaria* is not easy. It took some years´ experience before I was able to conclude that feeling the firmness of the egg gel is the most reliable character. The problem is largest when there are very few such egg clumps. When there are more such egg clumps, it is easier to find additional evidence, for example to find egg clumps where the larvae have just hatched and can be determined from their gill branches.

When “certain *R. temporaria*” appeared from 2003 onwards, they might possibly have immigrated from sites outside of the study area. However, they had more likely been present in low numbers in the area all along. In spite of the apparent lack of any adults in 1997, a few juveniles may have survived to breed as three-year olds in 1998 or 1999.

Different rates of colonisation

In my view, all the pre-defined target ponds were equally suitable for all species, being newly formed and not yet densely grown in with swamp vegetation or willow bushes. Except for one pond where a pike was observed, they were all free from fish, having no connection to other waterbodies. If there were a difference in suitability for the different species, then most of them should be most suitable for *R. arvalis* and *R. temporaria,* because most of them were floodings with shallow zones of flooded grass vegetation, which is optimal for them.

In the study area, *Rana dalmatina* was a much more efficient coloniser of newly-available waterbodies than the other two species. It colonised most waterbodies at once when they arose or became suitable, in years when the total population had declined, as well as in years with an increase. Colonisation events in *R. arvalis* and *R. temporaria* occurred after much longer periods and mostly in years with a considerable general population increase. In these years, strong cohorts of juveniles must have survived until sexual maturity, so it may be assumed that the colonising individuals were first-time breeders.

In general, in animal populations, there will often be some individuals with high site fidelity and other individuals with low site fidelity, and this has been documented also in a few amphibian species (Denoël et al. 2018). If all individuals had high site fidelity, the species would never spread. Could the proportion showing site fidelity differ between species, thereby explaining the higher colonisation ability in *R. dalmatina*? The available evidence is that *R. dalmatina* individuals do not show less site fidelity to their natal pond than the two other species. A German study of newly-metamorphosed *R. dalmatina* that were individually marked (Kneitz 1997) showed that, two years later, 80–90% of the recaptured individuals had returned to their natal pond. Amongst the adults that were equipped with a transponder, nearly 100% returned to their breeding site. Hachtel et al. (2005) found that the percentage of marked adults that returned to the same breeding pond after one year was higher in *R. dalmatina* (51%) than in *R. temporaria* (23–26%).

In the present study, *R. dalmatina* populations were more stable from year to year than were the populations of *R. arvalis* and *R. temporaria*. In 10 out of 15 suitable waterbodies, *R. dalmatina* spawned in every single year. The tendency for frog populations to shift breeding site entirely from one year to the next was most marked for *R. temporaria*, even though this species spawned more rarely in temporary waterbodies than the two other species.

Data from other studies may confirm the trend for relatively stable occurrence in *R. dalmatina*. On the Swedish island of Öland, Ahlén (2013) monitored *R. dalmatina* breeding sites continuously over 20 years. Out of 150 ponds with the species, it spawned every single year in 53% of them. In comparison, in a moist forest area in Bavaria, Grözinger et al. (2012) monitored *R. temporaria* continuously during 7 years. Out of 43 ponds with the species, only 10 (23%) contained spawn every single year.

Movement patterns

Several types of evidence indicate that differences in movement patterns partially explained the different colonisation rates of the three species. Although the majority of individuals stay relatively close to their site of origin, evidence from a number of amphibian species showed that a few individuals migrate away over very large distances, in many species more than 10 km (Smith and Green 2005). Sinsch (2014) noted: “Theory predicts that juveniles should move farther, faster and more frequently than adults”. Therefore, it is relevant to discuss what is known about the movement patterns of juveniles of the three species of brown frogs treated here. Kneitz (1997) found that marked *R. dalmatina* juveniles moved an average of 26 m/day or 1 km in 8 weeks. He added that juveniles are of great importance in the expansion and colonisation of new habitats.

Unpublished observations made by the author in the study area and elsewhere in Denmark show that newly-metamorphosed froglets of the three species do not move equally quickly away from their natal pond. *Rana temporaria* moves faster away from the water than *R. arvalis*, but *R. dalmatina* moves even faster. At a pond in Kulsbjerge, studied at ten dates in June and July 2009, many newly-metamorphosed *R. dalmatina* moved 15–30 m into the surrounding dry land even when they still had remains of their tail and very few older froglets remained within 10 m from the water’s edge, even when the weather was dry. Newly-metamorphosed *R. arvalis* and *R. temporaria* stayed for a relatively long time in the water or within 2 m from the water’s edge. Moreover, even when their tail had been resorbed, it was only after rainy weather that they moved further away from the water’s edge than 15 m.

Unpublished data about the habitats of young brown frogs in their first year of life (n = 106 for *R. temporaria*, 41 for *R. arvalis* and 56 for *R. dalmatina*) were gathered by Per Holm Andersen in Denmark in the 1940s (cf. Fog (1988)). About half of the newly-metamorphosed *R. dalmatina* were found far from a pond (i.e. no presence of a pond was mentioned), which was true of very few *R. temporaria* and of no *R. arvalis*. For the juveniles of a few months of age, the proportion still found near breeding sites was 83% for *R. arvalis*, 61% for *R. temporaria* and 13% for *R. dalmatina*. This pattern was even more pronounced for juveniles after their first hibernation, whereas some adult *R. dalmatina* were found close to ponds. Consequently, it is likely that juvenile *R. dalmatina*, emigrating quickly from the pond, disperse rapidly and widely in the landscape. However, they may also have a high degree of philopatry, i.e. although they move far away, most of them return to their natal ponds, which is confirmed by the relatively high stability of *R. dalmatina* breeding populations.

A paradoxical relation may exist between site fidelity and long-range movements. Those moving far away may have a strong need to return exactly to their point of origin. Juveniles of the two other species do not leave the area around the breeding pond as quickly. When they reach adulthood, after having moved around, they may most often choose a breeding site in, or in the vicinity of, their natal pond. They may do so not just because of philopatry, but also as a consequence of not having moved far initially.

Altogether, the pattern that emerges is that most *R. dalmatina* individuals return as adults to their natal ponds after having moved far away as younger individuals. Even so, 10–20% settle elsewhere and breed in other ponds (Kneitz 1997). Once they have chosen a new pond, they may show a high degree of site fidelity to the new site as indicated by the relatively high stability of *R. dalmatina* populations in new ponds. In the two other species, the whole process is probably more random. The frogs do not move as far away, that is, the need for a strongly-developed philopatry is not as strong as in *R. dalmatina*.

If these are the three species’ movement patterns, this would explain the observations made here. In *R. dalmatina*, a relatively more constant proportion of the juveniles emigrate to other sites and, therefore, a high likelihood of colonisation exists even in years with a low surplus of emigrating juveniles. The emigration process is less strict in *R. temporaria* and *R. arvalis*; only in years with a large surplus of juveniles will many of them settle in new ponds due to random movements.

The data do not allow conclusions on how far colonising frogs have migrated because we do not know their precise origin. Furthermore, some may have originated from waterbodies outside the study area. Two of the waterbodies in the north of the study area were colonised by *R. arvalis* in 2008, when the distance from both of these to the nearest possible source locality was about 660 m. The most isolated target pond, located furthest to the southwest, was colonised by *R. dalmatina* after a 2–year period, by *R. arvalis* after a 3–year period and probably by *R. temporaria* temporarily after a 4–year period. It has a distance of 530 m to the two nearest possible source ponds within the study area and a distance of 510 m to the nearest possible source pond outside of the study area. A few other relatively isolated waterbodies have distances to possible source ponds in- and outside of the study area of slightly less than 500 m.

Other evidence on different colonisation rates

The results of the present study agree well with the impression that the author and other herpetologists have concerning brown frogs in Denmark — *R. arvalis* and *R. temporaria* colonise relatively slowly, whereas new ponds are very often colonised by *R. dalmatina* surprisingly rapidly, even over considerable distances. Other evidence supports this assertion. Out of 66 newly-created or restored ponds from different parts of Denmark, *R. dalmatina* had colonised 65% within 5 years, which was a higher percentage than for five other anuran species comprised by the same study (Fog 1997a).

Evidence from the Bonn area, Germany, agrees that *R. dalmatina* is a fast coloniser (Hachtel and Grossenbacher 2014), but it generally does not agree that *R. temporaria* is a slow coloniser, especially not where *R. dalmatina* does not occur. In a study in the Netherlands, *R. tem-*

poraria turned out to be the best coloniser out of eight amphibian species: this species already occurred in 72% of the newly-created ponds two years after construction (Laan and Verboom 1990). In another Dutch study, just above 50% of the two-year old ponds had been colonised by *R. temporaria* (Stumpel and van der Voet 1998). In a region of south Sweden, *R. arvalis* and *R. temporaria* both colonised 19 out of 21 new ponds within 2–6 years; *R. arvalis* was more dependent than *R. temporaria* on the proximity of a source population within 250 m (Almhagen 2007). Furthermore, *R. arvalis* generally moves shorter distances than the other species do (Glandt 2014).

The present study suggests that some species or some local populations of amphibians colonise much more slowly than others. This may be of concern when new ponds are made in efforts to save amphibian populations. It may possibly differ from one region to another precisely what species are poor colonisers.

The situation in the landscape in general

In other parts of South Zealand, in the agricultural landscape, *R. dalmatina* is also widespread, whereas *R. arvalis* and *R. temporaria* are in a much worse situation there than in the military training area. In recent decades, *R. arvalis* and *R. temporaria* have declined drastically in large parts of South Zealand (Fog, unpubl. data), whereas *R. dalmatina* has expanded (Fog 1997b). Recordings of 152 ponds in a 1 km wide zone along the coastline of South Zealand made by the author in 2016 yielded the following number of breeding sites: *R. dalmatina* 47, *R. arvalis* 11 and *R. temporaria* 3.

Rana temporaria is rather sparsely present in southeast Denmark. The“common” frog is uncommon in this part of Denmark. It is completely absent from all the south-eastern islands, including Lolland, Falster and Møn with a combined area of 2,935 km² (cf. Fig. 1). Falster and Møn are both situated just 8 km from Kulsbjerge. That *R. temporaria* is absent from all islands in southeast Denmark is contrary to what is stated in Gollmann et al. (2014: 318), but the statement there is false due to an erroneous translation of their source, Bringsøe and Graff (1995). *Rana arvalis* has declined considerably in recent years on these islands, whereas *R. dalmatina* is widespread there.

Furthermore, there is evidence in recent decades that *R. dalmatina* advances where the other two species retreat. This is most clear in the peninsula of Knudshoved Odde, a nature area in South Zealand with many ponds. Here, the situation in 1946 was that only *R. arvalis* and *R. temporaria* were present, whereas *R. dalmatina* occurred in a forest approx. 4 km from there (H. Volsøe, unpubl. data). From the 1980s and onwards, *R. dalmatina* migrated to the peninsula and colonised all ponds there, whereas *R. temporaria* and *R. arvalis* disappeared completely.

The trend that *R. dalmatina* advances in the coastal regions of south Zealand, whereas the two other species are retreating, may partially be explained by the large increase of herons there, which are now extremely numerous predators in some amphibian ponds. Sometimes they may completely exterminate a breeding colony of brown frogs. They are a threat to breeding *R. arvalis* and *R. temporaria*, which are active in the water surface at daytime. They are not a threat to breeding *R. dalmatina*, which are active only under the surface during night.

In recent decades, *R. arvalis* and *R. temporaria* have declined so much in this part of Denmark that their continued presence may be threatened. Therefore, the situation in the military area is remarkable. Both these species have survived a period of very low population size there and have then expanded greatly. Evidently, the military training ground which is an open landscape without agricultural utilisation is much more favourable to these species than the surrounding farmland. Kulsbjerge has become at kind of sanctuary for these amphibian species (as well as for *Hyla arborea*). This exemplifies the importance of large uncultivated areas in amphibian conservation.

Uncertainties and sources of error

The probability of detecting egg clumps was unknown. Schmidt et al. (2023) showed that the largest variation in detection probability (presence/absence) of amphibian species was among-observer heterogeneity. In the present study, all observations have been made by the same person and under the same weather conditions. Thus, differences between years are reliable indications of differences in the population size, so it is justified to consider the counts as an index variable. One study from southern Europe indicates that about 93% of all *R. dalmatina* egg clumps were detected by one observer as a percentage of those detected by another observer on the same day (Falaschi et al. 2022). However, the study by Falaschi et al. (2022) did not specify weather conditions, vegetation density or water clarity, all of which may be of importance. In general, a risk exists that some egg clumps are hidden from the observer’s view in waterbodies with dense swamp vegetation, turbid water and/or deep water (Hachtel and Grossenbacher 2014). Therefore, the detection probability is not necessarily the same in all waterbodies. However, in the present study, vegetation and turbidity changed little from year to year, so results for a given waterbody will be comparable between neighbouring years.

If counts were made too early in the season, eggs that had been spawned late would have been missed and the numbers counted would be too low. This introduced an error margin of unknown size. However, those cases with a large increase at the second visit in counts of *R. dalmatina* were cases where the first visit had clearly been made much too early in the season. Inspecting the dates confirmed that, in these cases, the first visit had been made relatively early, in the period 30/03–06/04, and had always been followed by a later visit. When the first visit to a waterbody was made relatively late in the season, then the last visit yielded only a moderate number of additional egg clumps – on average about 23% more in *R. dalmatina* and likewise, 23% more in *R. arvalis*. The hypothetical further increase after the last visit must have been smaller than this, which is also suggested by the recordings of only few newly-spawned egg clumps at the last visit. The change in population size from one year to the next, on average for all 28 comparisons of two neighbouring years, were as follows: The counts differed by a factor of 1.52 for *R. dalmatina*, 2.48 for *R. arvalis* and 2.78 for certain + likely *R. temporaria*. Relative to this, an adjustment by a factor less than 1.23 for late eggs gives an uncertainty less than the average change from year to year. Therefore, this uncertainty does not severely affect reported changes from year to year.

Acknowledgements

I thank the military administration at Vordingborg Kaserne (Vordingborg Barracks) for allowing the registrations to take place and for improving the ponds.

Henrik Bringsøe is thanked for help with the manuscript.

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Supplementary material 1

Raw data file

Authors: Kåre Fog

Data type: xlsx

Explanation note: Numerals indicate the number of egg clumps. If a cell is empty, it was not relevant to investigate the waterbody, for example, if it did not exist yet or did not exist in that year. “Dry” indicates that the waterbody was practically dry when investigated. This concerns cases when a waterbody holds water in most other years and so is believed to harbour an otherwise permanent population, which then has to move to another waterbody in that particular year. NI means Not Investigated. 0 means that the waterbody was investigated, but no eggs of that particular species were found. X means the species was present and spawned, but the eggs were not counted. Waterbody numbers refer to the map of the study area, with all 46 waterbodies shown. Names of waterbodies are the names used by the author.

Link: https://doi.org/10.3897/herpetozoa.37.e107986.suppl1

Supplementary material 2

Map with labelled localities

Authors: Kåre Fog

Data type: pdf

Explanation note: Map showing the waterbody numbers used in the raw data file (Suppl. material 1).

Link: https://doi.org/10.3897/herpetozoa.37.e107986.suppl2

OGH Herpetozoa
Austrian Herpetological Society

Herpetozoa 37: 57–63 (2024)
DOI 10.3897/herpetozoa.37.e116879

New data on the distribution of the *Vipera ammodytes* (Linnaeus, 1758) mitochondrial lineages place their contact zone in western Bulgaria

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Academic editor: Peter Mikulíček ♦ **Received** 5 December 2023 ♦ **Accepted** 9 February 2024 ♦ **Published** 23 February 2024

Abstract

Molecular studies have outlined several mitochondrial lineages of *Vipera ammodytes*, but the limits of their distribution ranges remain unclear due to limited sampling throughout the vast range of the species. One particularly understudied area is the Eastern Balkan Peninsula where at least three lineages occur, showing ranges that may be partly overlapping. We used two mitochondrial markers (cytb and ND2) to unveil mtDNA distribution patterns based on vipers from 31 localities across Bulgaria. Our results confirmed the presence of the north-eastern (NE) and the south-eastern (SE) mitochondrial clade in Bulgaria, the latter being represented by its southern (S) and eastern (E) subclades. Among the sampled localities, two were suspected to be potential contact zones between these mtDNA lineages based on old morphology-derived distributional records. The NE clade was absent from both potential contact zones. However, our data showed that in western Bulgaria, populations of this clade establish contact with populations of the E subclade in at least one area, and also come close to contact with populations of the S subclade. These results indicate the need for more detailed research in the potential areas of contact in western Bulgaria, integrating morphological data with extensive mitochondrial and nuclear DNA-sampling to better understand the phylogeographic patterns of intraspecific differentiation in *V. ammodytes*.

Key Words

Balkan Peninsula, hybridization, phylogeography, species, Viperidae

Introduction

Despite its relatively wide range, the Nose-horned Viper, *Vipera ammodytes* (Linnaeus, 1758), is one of the poorly studied European viper species. The species is distributed from north-eastern Italy and southern Austria, through most of the Balkan Peninsula and many Aegean islands, to north-western and northern Asia Minor and the Lesser Caucasus (Speybroeck et al. 2016). Seven different subspecies have been recognized in the past, based on various morphological traits (Boulenger 1913; Bruno 1968; Sochurek 1976; Biella 1983; Biella and Blättler 1989). Using multivariate morphological analyses, Tomović (2006) suggested only four valid subspecies: *V. a. ammodytes*, inhabiting northern Italy, southern Austria, Slovenia, Croatia, Bosnia and Herzegovina, Montenegro, northern Albania, most of Serbia, north-western North Macedonia, north-western Bulgaria, and western Romania; *V. a. montandoni*, inhabiting south-eastern Romania, most of Bulgaria, western Turkey, north-western, northern and north-eastern Greece, most of North Macedonia, the southernmost parts of Serbia, and south and central Albania; *V. a. meridionalis*, inhabiting central Greece, Peloponnese and the Cyclades; *V. a. transcaucasiana*, inhabiting the eastern parts of Turkey, Georgia and Armenia.

Recent studies based on mitochondrial DNA markers, however, showed that the current taxonomy needs revision because the species exhibits a high genetic diversity

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throughout its range, and especially in the Balkan Peninsula, which the morphology-based approach fails to reflect (Ursenbacher et al. 2008; Čubrić et al. 2019; Freitas et al. 2020). Seven major mtDNA clades were found within *V. ammodytes*: NW, NE, MO, PE, CY, SW, and SE, the latter being comprised of the E, S, T, and AM subclades (Ursenbacher et al. 2008). Very recently, a study based on a combination of genomic and mtDNA phylogenies confirmed the presence of all clades described by Ursenbacher et al. (2008) and reported the presence of an additional clade from north-eastern Peloponnese (NEP) (Thanou et al. 2023). Genomic-based analyses revealed two distinct clusters, corresponding to the north-western ("North Balkan Clade" or NBC) and south-eastern ("South Balkan Clade" or SBC) Balkan Peninsula, respectively. The first cluster includes the NW, NE and MO mtDNA clades (all of which belong to the *V. a. ammodytes* morphotype), while the second cluster consists of the rest of the clades (belonging to *V. a. montandoni*, *V. a. meridionalis* and *V. a. transcaucasiana* morphotypes) (Thanou et al. 2023). The results of Thanou et al. (2023) also indicated very limited or no gene flow between the two clusters. The authors proposed that the NBC and SBC clusters should be considered different species, namely *V. ammodytes* and *V. meridionalis*.

In Bulgaria, the Nose-horned Viper is widespread throughout the country, except in the high parts of the mountains or urbanized and intensively cultivated agricultural land (Stojanov et al. 2011). Both *V. a. ammodytes* and *V. a. montandoni* morphotypes have been reported from Bulgaria. They are considered to have a parapatric distribution, possibly coming into contact in the western parts of the country. Specifically, a narrow area of contact has been suspected to exist along the Iskar River, north-east from the northern end of the Iskar Gorge (Fig. 1) (Stojanov et al. 2011). Another putative contact zone might exist along the southern slopes of Stara Planina Mtn., the eastern slopes of Ihtimanska Sredna Gora Mtn. and the western slopes of Sashtinska Sredna Gora Mtn., within the "triangle" locked between the towns of Chelopech, Ihtiman and Panagyurishte (Fig. 1) (A. Stojanov personal communication to A. Dyugmedzhiev). A third contact zone might also exist in the valley along the upper reaches of the Struma River, situated on the south-western slopes of Vitosha Mtn. (the area between the villages of Bosnek and Chuipetlovo, Fig. 1) (Tzankov et al. 2014). However, molecular data are not available for these populations and the presence, location and extent of these contact zones have not been confirmed.

On the other hand, the north-eastern (NE) clade as well as the eastern (E) subclade of the SE clade (hereafter referred to as the SE/E subclade) inhabit the country (Ursenbacher et al. 2008; Thanou et al. 2023). The NE clade is confirmed to be present in north-western Bulgaria (Ursenbacher et al. 2008) and its distribution in the country might correspond to the distribution range of the *V. a. ammodytes* morphotype in Bulgaria, depicted by Stojanov et al. (2011). The SE/E subclade is confirmed to be present along the latitudinal gradient in the central and eastern parts of Bulgaria (Ursenbacher et al. 2008; Thanou et al. 2023). Its distribution in the country seems to correspond to a large degree to the distribution range of the *V. a. montandoni* morphotype in Bulgaria, depicted by Stojanov et al. (2011), with the exception of south-western Bulgaria, where the southern (S) subclade of the SE clade (hereafter referred to as the SE/S subclade) might be distributed (Thanou et al. 2023). The latter is found in immediate proximity to the south-western border of Bulgaria in northern Greece and western parts of North Macedonia (Ursenbacher et al. 2008; Thanou et al. 2023). However, its presence has not been confirmed yet with molecular studies, since neither Ursenbacher et al. (2008) nor Thanou et al. (2023) sampled populations from south-western Bulgaria.

The goals of the current study are to 1) evaluate the mitochondrial diversity of the Bulgarian *V. ammodytes* populations, 2) assess the geographical distribution of the mtDNA lineages across Bulgaria, and 3) reveal and define the spatial distribution of putative contact zones between these lineages. In order to achieve these goals, we conducted an extensive sampling, covering most of the territory of Bulgaria, and analyzed information derived from two mitochondrial markers. The widely used gene cytochrome b (cytb) was chosen to allow the comparison of Bulgarian populations with previously published data, while a second gene, the NADH dehydrogenase subunit 2 (ND2), was added to provide further resolution between Bulgarian populations.

Materials and methods

DNA sampling

Sampling was carried out between 2014 and 2023 in Bulgaria and included 62 individuals from 31 localities covering different parts of the distribution range of *V. ammodytes* in the country. Among these localities, we included two of the three potential contact zones of the different *V. ammodytes* morphotypes in western Bulgaria: along the Iskar River near Karlukovo (43.17°N, 24.06°E) and along the upper reaches of Struma River between the villages of Bosnek and Chuipetlovo (42.49°N, 23.21°E) (Fig. 1). In each locality, ventral scale samples were taken in the field from living individuals (n = 52), which were released after the procedure. Additionally, tissue samples from specimens found dead were collected (n = 10). The material was stored in absolute ethanol for long-term preservation.

Molecular procedures

Total DNA was extracted from tissue samples using DNeasy Blood & Tissue Kit (Qiagen), following the instructions of the manufacturer. Two mitochondrial markers were amplified through polymerase chain reaction (PCR) with the Thermo Scientific DreamTaq Green PCR Master Mix (Thermo Fisher Scientific) and according to

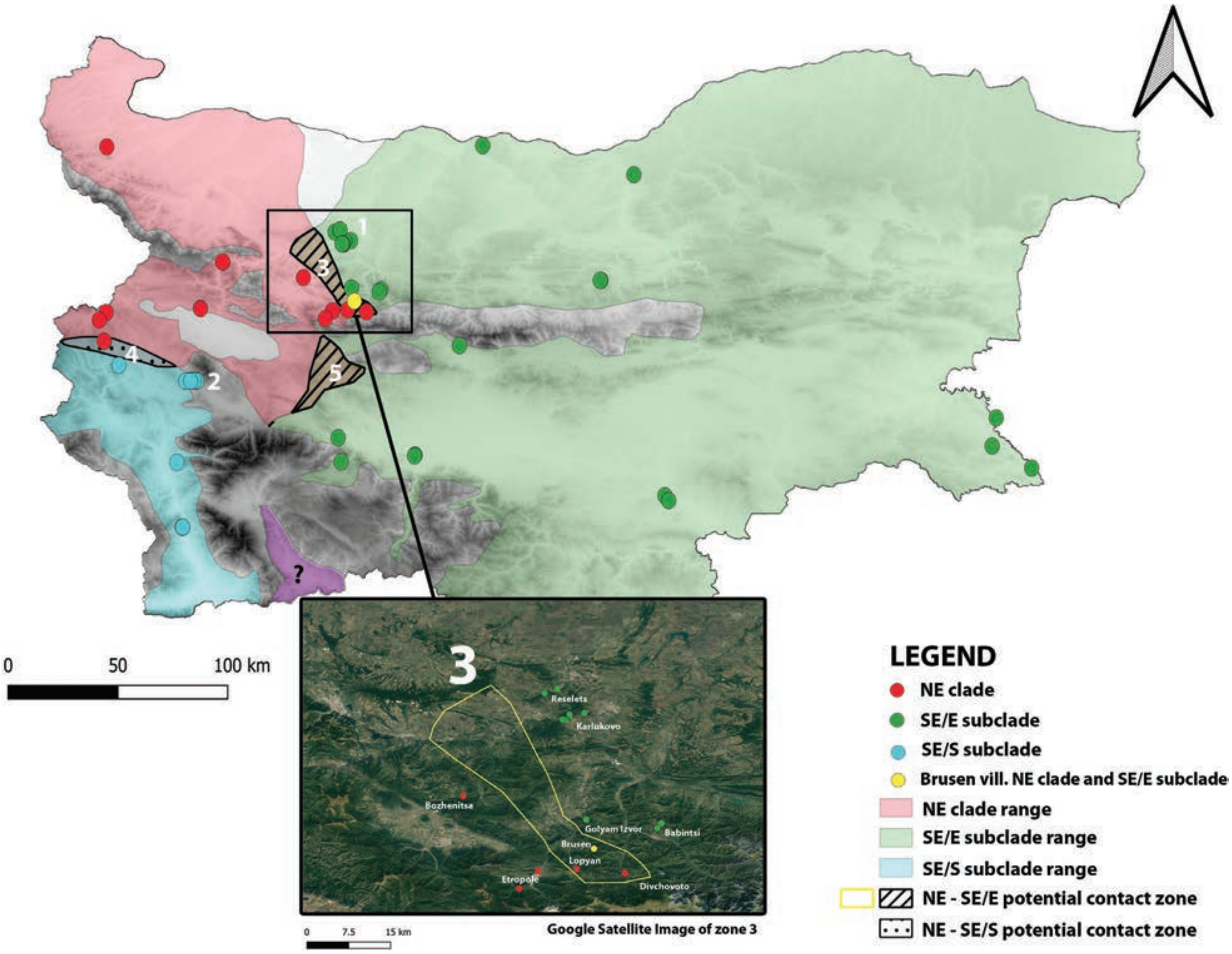

Figure 1. Distribution of the different mtDNA lineages of *Vipera ammodytes* in Bulgaria. Red, green and blue circles indicate the location of populations from which DNA samples were collected and their assignment to the respective clade; the yellow circle indicates the location of the population near Brusen. The red, green and blue areas indicate the potential distribution of the different clades in Bulgaria based on the distribution ranges depicted by Stojanov et al. (2011) and the authors' personal data; purple area with a question mark indicates the area around the valley of Mesta River, in which the clade-assignment of individuals needs to be clarified by DNA sampling. Numbers 1 and 2 indicate the suspected contact zones based on literature data (along the Iskar and Struma rivers, respectively) which were sampled during this study. The potential contact zones identified in the study are indicated with contours and numbered as follows: 3 – Iskar and Malki Iskar rivers (contact NE – SE/E), 4 – Krayste region (contact NE – SE/S), 5 - Stara Planina, Ihtimanska Sredna Gora and Sashtinska Sredna Gora Mtns (contact NE – SE/E, based on personal morphology-based data, which need confirmation by DNA sampling).

the instructions of the manufacturer. Specifically, cytb and ND2 were respectively amplified with the primer pairs L14724Vb / H15914Vb (Ursenbacher et al. 2006 and references therein) and L4437b / tRNA-trp (Kumazawa et al. 1996; Ashton and de Quieroz 2001) and the cycling protocols of Ursenbacher et al. (2006) and Garrigues et al. (2005). Sanger sequencing was performed by Macrogen Europe BV as an external service.

Phylogenetic analyses

The obtained chromatograms were visualized, trimmed, and assembled with CodonCode Aligner v. 8.0.2 (CodonCode, Dedham, MA, USA). The two fragments were tested for stop codons with DAMBE v. 7.3.11 (Xia 2018).

The phylogenetic analysis was based on the cytochrome b fragment, which is commonly used in molecular studies of vipers. DNA sequences of *V. ammodytes* from a previous European study (Ursenbacher et al. 2008) were downloaded from Genbank and added to the newly obtained data (see Suppl. material 1) to provide context. Sequences were aligned using Mega X (Kumar et al. 2018). The best substitution models for each coding position were estimated with PartitionFinder ver. 2.1.1 (Lanfear et al. 2017). Phylogeny was reconstructed through Bayesian inference (BI) accomplished in MrBayes v. 3.2.7 (Ronquist et al. 2012). Four simulations of Markov chains and 4 × 10^6 generations were run, sampling one of every 100 trees. The chain parameters were examined in Tracer ver. 1.7.1 (Rambaut et al. 2018). The first 25% of trees were discarded as burn-in.

Genetic diversity of Bulgarian clades

In order to further study Bulgarian populations, we concatenated cytb and ND2 fragments (ND2+cytb hereafter), thus obtaining a larger mitochondrial dataset. Haplotype networks were based on the concatenated mitochondrial alignment using the TCS method (Clement et al. 2002) implemented in PopART 1.7 (Leigh and Bryant 2015).

Results

Genetic diversity and distribution patterns of *V. ammodytes* in Bulgaria

New molecular data from Bulgaria included 62 DNA sequences from two genes; from one sample only ND2 was obtained. No stop-codons were detected in the protein coding genes. Localities, geographical coordinates and Genbank accession numbers are given in Suppl. material 1.

The cytb alignment, used to infer the general phylogenetic tree of *V. ammodytes,* consisted of 103 in-group sequences (61 own and 41 previously published data from the species, and one outgroup *Vipera berus* (Linnaeus, 1758)). The final matrix was 927 bp long. The ND2 fragment used for haplotype networks included new data only and was 864 bp long.

The arrangement of the Bulgarian samples in the Bayesian phylogenetic tree confirmed the presence of the north-eastern clade (NE) and the south-eastern clade (SE) in the country, the latter being represented by its southern (S) and eastern (E) subclades (Fig. 2). The samples collected in the central and northern parts of western Bulgaria (n = 18) belonged to the NE clade. The samples from the southernmost parts of central-western and south-western Bulgaria (n = 14) were arranged within the SE/S subclade. Finally, those collected along the latitudinal gradient from the easternmost parts of western Bulgaria, throughout central to eastern Bulgaria (n = 30) belonged to the SE/E subclade (Fig. 1 and Suppl. material 1). Two individuals from a single locality (Brusen, 42.89°N, 24.12°E) were arranged within separate mtDNA clusters (NE and SE/E, see below).

The (ND2+cytb) dataset used for haplotype network reconstruction was 1788 bp long. The haplotype network confirmed the presence of three distinct clusters in Bulgaria (Fig. 3). Based on our concatenated dataset, the estimated genetic distance was ca. 5% between NE and E, and ca. 2.8% between S and E clusters.

Analysis of individuals from contact zones

In order to investigate the potential contact zones, we sampled 10 individuals from the area along the Iskar River and seven from the area along the upper reaches of Struma River. Additional samples were also collected from two individuals captured near the village of Reselets, along the Iskar River (43.24°N, 24.02°E). All individuals captured from the first area and Reselets belonged to the SE/E subclade, and those from the second area belonged to the SE/S subclade. No individuals belonging to the NE clade were discovered in either of the two sites.

The closest sampled populations from the NE clade and the SE/S subclade occur in the Krayshte region, western Bulgaria, approximately 15 km from each other (Fig. 1). More interestingly, representatives of both the NE clade and the SE/E subclade were discovered within a narrow perimeter of less than 100 m, in the area between the valleys of Malki Iskar and Cherni Vit rivers, near Brusen, north-western Bulgaria (42.89°N, 24.12°E) (Fig. 1). Four neonate *V. ammodytes* (three females and one male) were captured there on 18.09.2022 and DNA was collected from one of the females. The site was visited again on 28.09.2023 when the remains of a dead subadult female were found 7 to 60 m away from where the four neonate vipers were found the previous year. Our analysis grouped the first female within the SE/E subclade and the second one within the NE clade (Fig. 3).

Discussion

Genetic diversity and distribution patterns of *V. ammodytes* in Bulgaria

The Bayesian phylogenetic tree constructed in our study was in general agreement with earlier mitochondrial phylogenies (Ursenbacher et al. 2008; Thanou et al. 2023). Our results confirm the presence of both the NE clade and the SE/E subclade in Bulgaria, as shown by Ursenbacher et al. (2008) and Thanou et al. (2023) and reveal that the distribution range of the former reaches further into the east than previously known. Furthermore, we confirm that the SE/S subclade is also distributed in Bulgaria, as depicted by Thanou et al. (2023), extending its distribution range further into the north-east. The NE clade inhabits the central and northern parts of western Bulgaria, the SE/S subclade is found in the southernmost parts of central-western Bulgaria and south-western Bulgaria, while the SE/E subclade occurs throughout the rest of the country (Fig. 1). During the course of this study, we were not able to collect any samples from nose-horned vipers along the Mesta River Valley in south-western Bulgaria (the separate purple triangle area with question mark on Fig. 1). Genetically admixed individuals between the SE/S and SE/E subclades are found nearby, to the south of this area in northern Greece, where their respective distributional ranges might overlap as a result of secondary contact following periods of population expansion (Thanou et al. 2023). So, further DNA sampling is needed in order to clarify the clade-assignment of individuals from this part of Bulgaria.

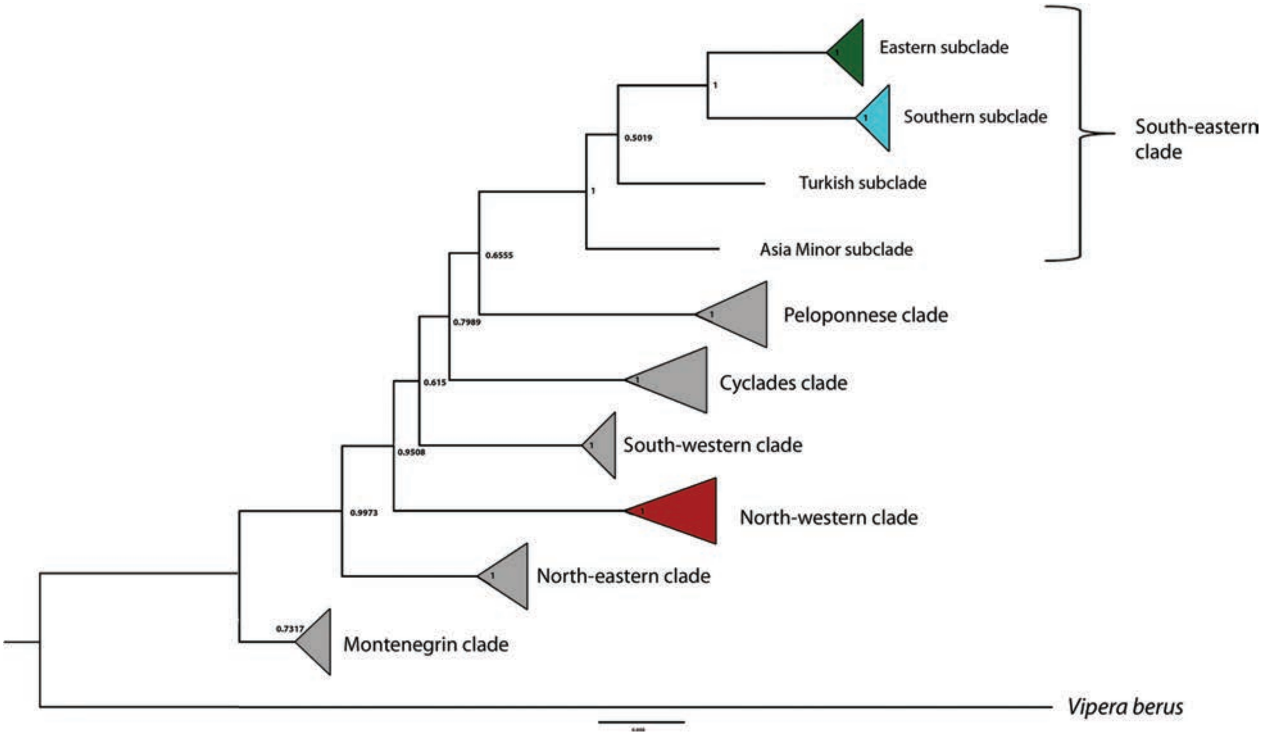

Figure 2. Bayesian phylogenetic tree of *Vipera ammodytes* inferred from the mitochondrial cytochrome b gene. Colored clades/subclades are found in Bulgaria, each color corresponding to the red, green and blue sampling localities shown in Fig. 1.

Distribution within the potential contact zones

According to Thanou et al. (2023), gene flow and hybridization between the NE clade (comprising the NBC genomic cluster) and both the SE/E and SE/S subclades (comprising the SBC genomic cluster) is absent or very limited. The authors considered the two clusters allopatric since the closest observed distances between them were at 50 and 70 km in Serbia and North Macedonia, respectively. Based on the geographical isolation and the deep divergence time, Thanou et al. (2023) suggested that speciation between the two clusters is complete and proposed that NBC and SBC could be considered different species, *Vipera ammodytes* and *V. meridionalis*, respectively. The authors, however, did not include samples from Bulgaria (with the exception of only one locality for the SE/E subclade from the south-eastern part of the country); the easternmost samples from the NE clade in their study being from western North Macedonia and central Serbia. Our results show that the NE clade and the SE/S subclade come very close to each other in the Krayshte region (Fig. 1), a pattern that is also suggested by the distributional ranges depicted in Ursenbacher et al. (2008) and Čubrić et al. (2019). During the course of the current study, the SE/S subclade was newly discovered in the southern parts of Krayshte, near the village of Sirishtnik (42.57°N, 22.78°E), and the NE clade was newly discovered with several sampled populations in the northern and central parts of Krayshte, the southernmost of them being located 15 km to the north-west from the SE/S subclade's population, near the village of Stanyovtsi (42.69°N, 22.70°E). Even more interestingly, there is evidence that the NE clade and the SE/E subclade, although parapatric, have reached contact at least in one area between the valley of the rivers Malki Iskar and Cherni Vit in western Bulgaria. This contact zone is situated between several closely sampled localities for both of the two mtDNA lineages within the area between the "triangle" locked between the Malki Iskar and Iskar Rivers (Fig. 1). For the NE clade, these localities are near the village of Bozhenitsa (43.01°N, 23.83°E), along the town of Etropole (42.84°N, 24.00°E and 42.80°N, 23.95°E), between the villages of Lopyan and Yamna (42.84°N, 24.08°E), and near the village Divchovoto (42.83°N, 24.19°E). For the SE/E subclade, they are near the villages Reselets, Karlukovo, Babintsi (42.93°N, 24.26°E and 42.94°N, 24.27°E), and Goliam Izvor (42.95°N, 24.10°E) (Fig. 1). Several small and medium-sized river valleys pass through this entire area, providing natural and easily accessible bio-corridors for dispersal of individuals (Fig. 1, small picture). Therefore, it could be expected that at least some mixed populations could be found there.

Although two of the localities sampled in this study (labelled as 1 and 2 in Fig. 1), were considered areas of contact between the *V. a. ammodytes* and *V. a. montandoni* morphotypes (Stojanov et al. 2011; Tzankov et al. 2014), we didn't find any genetic evidence in either of them that would support the occurrence of such a contact zone. Unfortunately, all potential and existing contact zones remain very poorly studied. Future studies should integrate morphological data with extensive

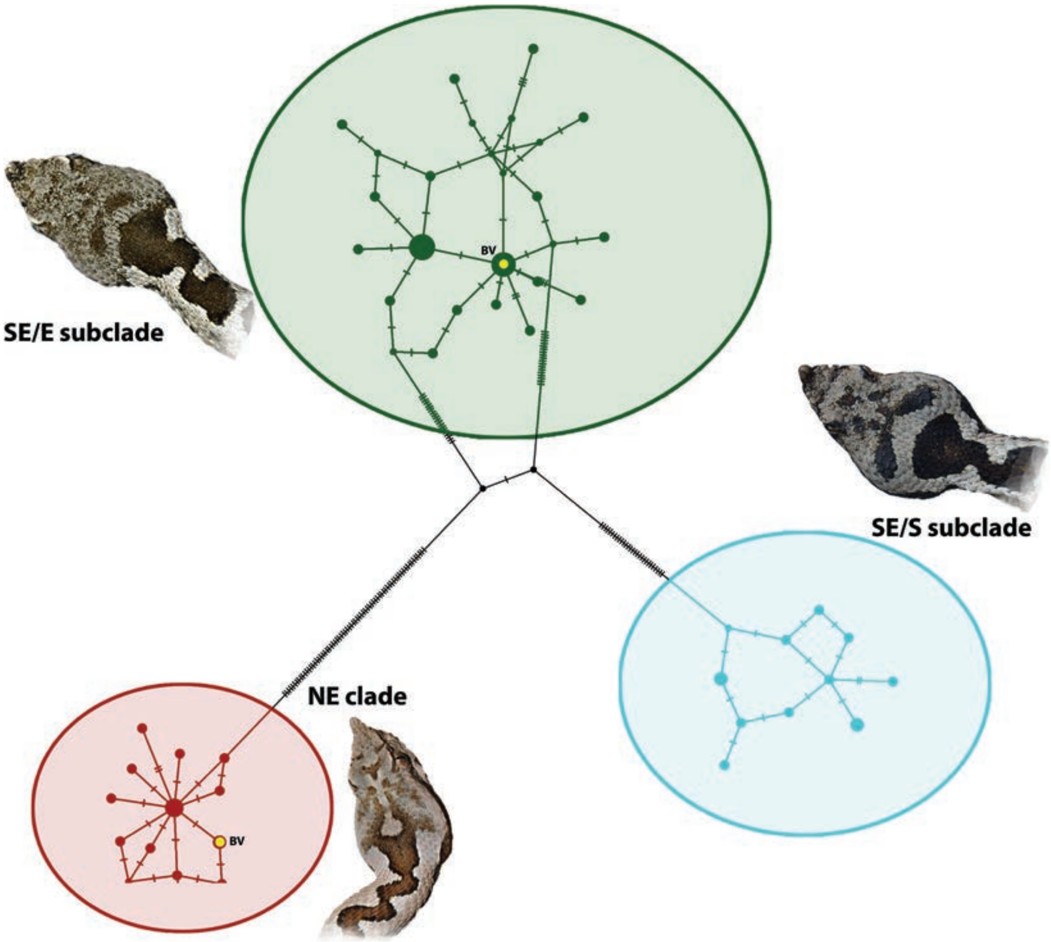

Figure 3. TCS haplotype network based on a 1788 bp. mitochondrial fragment (ND2+cytb) including all samples from the NE clade, the SE/E and the SE/S subclades, collected from Bulgaria. The size of the circle is proportional to the number of samples showing the respective haplotype. The yellow circles indicate the placement of the individuals from Brusen within the NE clade and the SE/E subclade, respectively.

DNA sampling in order to fully understand the ecological drivers of coexistence and segregation and estimate possible gene flow between evolutionary units of *V. ammodytes*. Molecular analyses should involve nuclear markers in order to better assign individuals to evolutionary units and to shed additional light on gene flow and hybridization. Such studies should also focus on the area locked between the southern slopes of Stara Planina Mtn., the eastern slopes of Ihtimanska Sredna Gora Mtn. and the western slopes of Sashtinska Sredna Gora Mtn. (labelled as 5 in Fig 1), which has also been considered a potential contact zone between the morphotypes.

Acknowledgments

This work was funded by the Bulgarian National Science Fund under Grant contract “KP-06-M51/2” from 11.11.2021. All fieldwork was carried out in accordance with the Ministry of Environment and Water of Bulgaria, Permits № 520/23.04.2013, № 656/08.12.2015, № 767/24.01.2019 and № 861/13.01.2021.

We would like to thank Borislav Naumov, Deyan Duhalov, Emiliya Vacheva, Georgi Krastev, Irina Lazarkevich, Kalina Valchinkova, Martin Tsvetkov, Miroslav Slavchev, Nikola Stanchev, Simeon Lukanov, Vladimir Todorov and Vladislav Vergilov for their help during some of the fieldwork for collecting the DNA-samples; Andrei Stoyanov, Nikolay Tzankov (who both sadly passed away in June 2016), Georgi Krastev, Martin Tsvetkov and Miroslav Slavchev for sharing their personal observation records on the species in the potential contact zones; Nikolay Todorov for designing a special box, which facilitated the safe photographing of the vipers; Sylvain Ursenbacher for his valuable advices concerning the analyses of the DNA-samples. We thank the anonymous reviewers and the subject editor whose comments and suggestions helped to improve the manuscript.

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Supplementary material 1

Suplement DNA data

Authors: Angel Dyugmedzhiev, Kostadin Andonov, Georgi Hristov, Simeon Borissov

Data type: xlsx

Explanation note: This is the suplementary DNA data from the rusults of our manuscript. If the manuscript is accepted for publication, this suplementary information will be uploaded into a International depository (Genbank).

Link: https://doi.org/10.3897/herpetozoa.37.e116879.suppl1

ÖGH Herpetozoa
Austrian Herpetological Society

Herpetozoa 37: 65–72 (2024)
DOI 10.3897/herpetozoa.37.e119492

First description of the female of *Cyrtodactylus dianxiensis* Liu & Rao, 2021, with extended diagnosis of this species (Squamata, Gekkonidae)

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https://zoobank.org/B09AB19D-EE93-4F49-9F27-AA6AED129B48

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Academic editor: Ben Wielstra ♦ **Received** 24 January 2024 ♦ **Accepted** 24 February 2024 ♦ **Published** 8 March 2024

Abstract

Cyrtodactylus dianxiensis Liu & Rao, 2021 was originally described based on only two adult male specimens from Tongbiguan Nature Reserve, Dehong Autonomous Prefecture, western Yunnan, China. So far, no information on the females of this species is available. During comprehensive herpetofaunal investigations in 2022, one female specimen of *C. dianxiensis* was collected from Tongbiguan Nature Reserve. The female specimen agrees well with the original description of *C. dianxiensis*, and also shows some slight differences in coloration. This study reported the female specimen of this species for the first time, and provided a description and photos of the female specimen; meanwhile, we extended the diagnosis of this species.

Key Words

bent-toed gecko, China, morphology, Yunnan

Introduction

Cyrtodactylus Gray, 1827 (bent-toed geckos) is the most speciose and ecologically diverse gekkonid genus, with more than 350 recognized species distributed from the Western Himalayas through southeast Asia to the Western Pacific (Wood et al. 2012; Uetz et al. 2024). Most species of *Cyrtodactylus* are karst-adapted or granite-adapted species (Grismer et al. 2020). With the in-depth investigation of karst areas, the underestimated biodiversity of *Cyrtodactylus* has been gradually revealed (Grismer et al. 2018; Liu et al. 2021, 2023).

So far, ten species of *Cyrtodactylus* have been recognized in China, and all are distributed in southwestern China, namely Yunnan Province and Tibet Autonomous Region. Four of them were recorded in Tibet: *C. tibetanus* (Boulenger, 1905), *C. cayuensis* Li, 2007, *C. zhaoermii* Shi & Zhao, 2010 and *C. kamengensis* Mirza, Bhosale, Thackeray, Phansalkar, Sawant, Gowande, Patel & Kamengensis, 2022. The other six were recorded in Yunnan: *C. dianxiensis* Liu & Rao, 2021, *C. gulinqingensis* Liu, Li, Hou, Orlov & Ananjeva, 2021, *C. hekouensis* Zhang, Liu, Bernstein, Wang & Yuan, 2021, *C. zhenkangensis* Liu & Rao, 2021, *C. menglianensis* Liu & Rao, 2022 and *C. caixitaoi* Liu, Rao, Hou, Wang & Ananjeva, 2023, among which, *C. dianxiensis* belong to the *C. khasiensis* species group, and the remaining belong to the *C. chauquangensis* species group.

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Cyrtodactylus dianxiensis is similar to *C. khasiensis* and previously the species have been confused with one another (Liu and Rao 2021; Wang et al. 2022). Liu and Rao (2021) describe the population of *C. khasiensis* distributed in Western Yunnan as a new species, *C. dianxiensis*. *Cyrtodactylus dianxiensis*, currently the only forest-dwelling *Cyrtodactylus* species recorded in Yunnan Province, China, was described based on one adult male holotype (KIZL2019044) from Tongbiguan Township, Yingjiang County, and one adult male paratype (KIZ2003170) from Longchuan County, both in Tongbiguan Nature Reserve, Dehong Autonomous Prefecture, western Yunnan, China (Liu and Rao 2021); no female information about this species was available. Identification verification of *C. khasiensis* voucher specimens deposited in museums could potentially yield additional specimens of *C. dianxiensis*. However, we have not yet been given the opportunity to examine the relevant specimens in other museums.

During a comprehensive herpetofaunal survey of Tongbiguan Nature Reserve in 2022, one female specimen of *Cyrtodactylus* was collected from Tongbiguan Nature Reserve, Xueli Village, Taiping Town, Yingjiang County, Dehong Prefecture, Yunnan Province, China (Fig. 1). Morphological and molecular phylogenetic analyses revealed that the specimen is *C. dianxiensis*. Here, we provide morphological data for the newly collected female specimen of *C. dianxiensis*, and extend the diagnosis of this species.

Materials and methods

Sampling

The specimen was sampled by hand at night. Sex was further determined based on whether the ventral side of the tail base is swollen. After euthanasia, liver tissues were taken and preserved in 95% alcohol. The specimen was then directly preserved in 75% ethanol without formalin fixation. The voucher specimen was deposited in Kunming Natural History Museum of Zoology, Kunming Institute of Zoology, Chinese Academy of Sciences (KIZ).

Morphological analyses

The adult female specimen preserved in 75% ethanol was measured with digital calipers to the nearest 0.1 mm. Morphological terminology followed Liu and Rao (2021). Measurements included the following: snout-vent length (SVL), from tip of snout to anterior margin of cloaca; trunk length (TRL), axilla to groin distance; body width (BW), maximum width of body; tail length (TL), from posterior margin of cloaca to tip of tail; tail width (TW), maximum width of tail; head length (HL), from tip of snout to posterior margin of jaw; head width (HW), maximum width of head; head

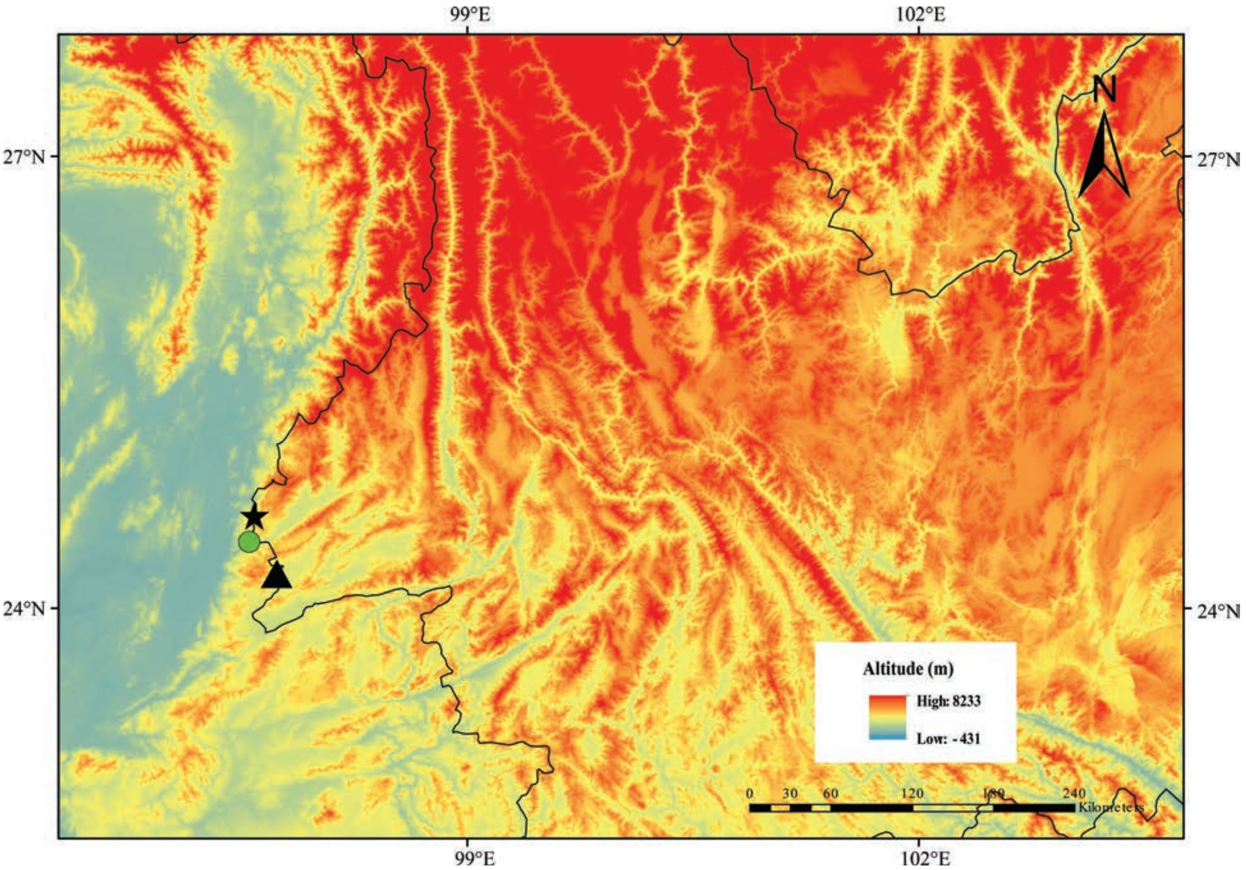

Figure 1. Sampling sites of *Cyrtodactylus dianxiensis*: solid black star, the locality of the holotype; solid black triangle, the locality of the paratype; solid green circle, the locality of the newly collected female specimen.

height (HH), from occiput to underside of jaws; forearm length (FL), from the base of the palm to the elbow; crus length (CL), from the base of heel to the knee; eye diameter (ED), greatest diameter of eye; nostril to eye distance (NE), from nostril to anterior corner of eye orbit; snout to eye distance (SE), from tip of snout to anterior corner of eye orbit; orbit to ear distance (EE), from posterior corner of eye orbit to anterior margin of ear opening; ear length (EL), greatest diameter of ear; internarial distance (IN), measured between inner borders of nostrils; interorbital distance (IO), measured across narrowest point of frontal bone. The counting of the following characteristics also followed Liu and Rao (2021): dorsal tubercle rows (DTR); ventral scale rows (MVSR); paravertebral tubercles (PVT); supralabials (SL); infralabials (IL); precloacal pores (PcP); precloacofemoral pores (PcFP); femoral pores on each thigh (FP); subdigital lamellae under the fourth finger (LF4) and under the fourth toe (LT4).

Table 1. Locality, voucher ID, and GenBank accession (ND2) for all samples used in this study. * denotes the holotype of *Cyrtodactylus dianxiensis*.

Species	Locality	Voucher	Accession
Cyrtodactylus aaronbaueri	Mizoram, India	MZMU 2015	MW596520
Cyrtodactylus agarwali	Meghalaya, India	MZMU 2158	MW596515
Cyrtodactylus arunachalensis	Arunachal Pradesh, India	BNHS 2777	MT341522
Cyrtodactylus aunglini	Mandalay, Myanmar	LSUHC 13948	MH764589
Cyrtodactylus ayeyarwadyensis	Ayeyarwady, Myanmar	CAS 212459	JX440526
Cyrtodactylus bapme	Meghalaya, India	BNHS 2756	MW367435
Cyrtodactylus bengkhuaiai	Mizoram, India	MZMU 1985	MW596516
Cyrtodactylus chrysopylos	Shan State, Myanmar	LSUHC 13937	MH764604
Cyrtodactylus dianxiensis	Yunnan, China	KIZ059201	MW971927
Cyrtodactylus dianxiensis	Yunnan, China	KIZL2019044*	MW971926
Cyrtodactylus dianxiensis	Yunnan, China	KIZ2022159	PP394340
Cyrtodactylus exercitus	Meghalaya, India	MZMU 2545	OK247679
Cyrtodactylus gansi	Chin State, Myanmar	CAS 222412	JX440537
Cyrtodactylus guwahatiensis	Assam, India	BNHS 2146	KM255194
Cyrtodactylus jaintiaensis	Meghalaya, India	BNHS 2248	KM255195
Cyrtodactylus karsticola	Meghalaya, India	MZMU 2156	MW596513
Cyrtodactylus kazirangaensis	Assam, India	BNHS 2147	KM255170
Cyrtodactylus khasiensis	Meghalaya, India	BNHS 2249	KM255188
Cyrtodactylus lungleiensis	Mizoram, India	MZMU 2428	MZ645742
Cyrtodactylus montanus	Tripura, India	BNHS 2231	KM255200
Cyrtodactylus mombergi	Kachin State, Myanmar	LSUHC 14734	MN059875
Cyrtodactylus nagalandensis	Nagaland, India	BNHS 2253	KM255199
Cyrtodactylus namtiram	Manipur, India	BNHS 2751	MW367433
Cyrtodactylus ngopensis	Mizoram, India	MZMU 2360	OM912605
Cyrtodactylus septentrionalis	Assam, India	BNHS 1989	MH971164
Cyrtodactylus siahaensis	Mizoram, India	MZMU 2445	OK247677
Cyrtodactylus tripuraensis	Tripura, India	BNHS 2238	KM255183
Cyrtodactylus urbanus	Assam, India	VR/ERS/ZSI/688	MN911174
Cyrtodactylus vairengtensis	Mizoram, India	MZMU 2903	OP874800
Cyrtodactylus slowinskii	Sagaing, Myanmar	CAS 210205	JX440559

DNA extraction, PCR amplification, and sequencing

Total genomic DNA was extracted from tissue samples preserved in 95% ethanol. The tissue sample was then digested using proteinase K, and subsequently purified using DNeasy Tissue Kit (QIAGEN). A fragment of NADH Dehydrogenase subunit 2 (ND2) was amplified and sequenced using the primers L4437b and H5934 (Macey et al. 1997). The experiment protocols are the same as Liu and Rao (2021). New sequence was assembled and edited using SEQMAN in Lasergene 7.1 (DNASTAR Inc., Madison, WI, USA).

Molecular analysis

Phylogenetic relationships within the *C. khasiensis* species group were inferred from ND2. The homologous sequences of the *C. khasiensis* species group, and the outgroups species *C. slowinskii*, were downloaded from GenBank (Clark et al. 2016) (Table 1). Sequences were aligned using MUSCLE 3.6 (Edgar 2004), then checked by eye for accuracy and trimmed to minimize missing characters in MEGA 6.0.6 (Tamura et al. 2013).

Phylogenetic reconstruction was conducted using Bayesian inference (BI) and maximum likelihood (ML) methods based on the ND2 gene. The best-fit substitution model was selected under the Bayesian Information Criterion by the program MODELFINDER (Kalyaanamoorthy et al. 2017) implemented in IQ-TREE 1.6.12 (Nguyen et al. 2015). Bayesian inference analyses were performed in MRBAYES 3.2.7 (Ronquist et al. 2012) based on the model GTR+F+I+G4. Two independent runs were initiated, each with four simultaneous Markov Chain Monte Carlo (MCMC) chains for one million generations and sampled every 100 generations with a burn-in of 25%. The convergence was examined with an average standard deviation of split frequencies less than 0.01 and effective sample size (ESS) values greater than 200 in TRACER 1.5 (Rambaut and Drummond 2009). Maximum likelihood analyses were conducted using IQ-TREE 1.6.12 (Nguyen et al. 2015), based on the model TIM+F+I+G4. Nodal support was estimated by 1,000 bootstrap replicates using the ultrafast bootstrap feature. Pairwise divergences were calculated using uncorrected *p*-distances implemented in MEGA 6.0.6 (Tamura et al. 2013).

Results

Bayesian inference and ML trees showed consistent topology. The newly collected female specimen clustered with the specimens (including the holotype) of *C. dianxiensis* with strong support by both BI and ML (BI/ML=1/100, Fig. 2). The genetic distance (uncorrected *p*-distance) between the newly collected female specimen and the specimens (including the holotype) of *C. dianxiensis* was only 0.9% (Table 2).

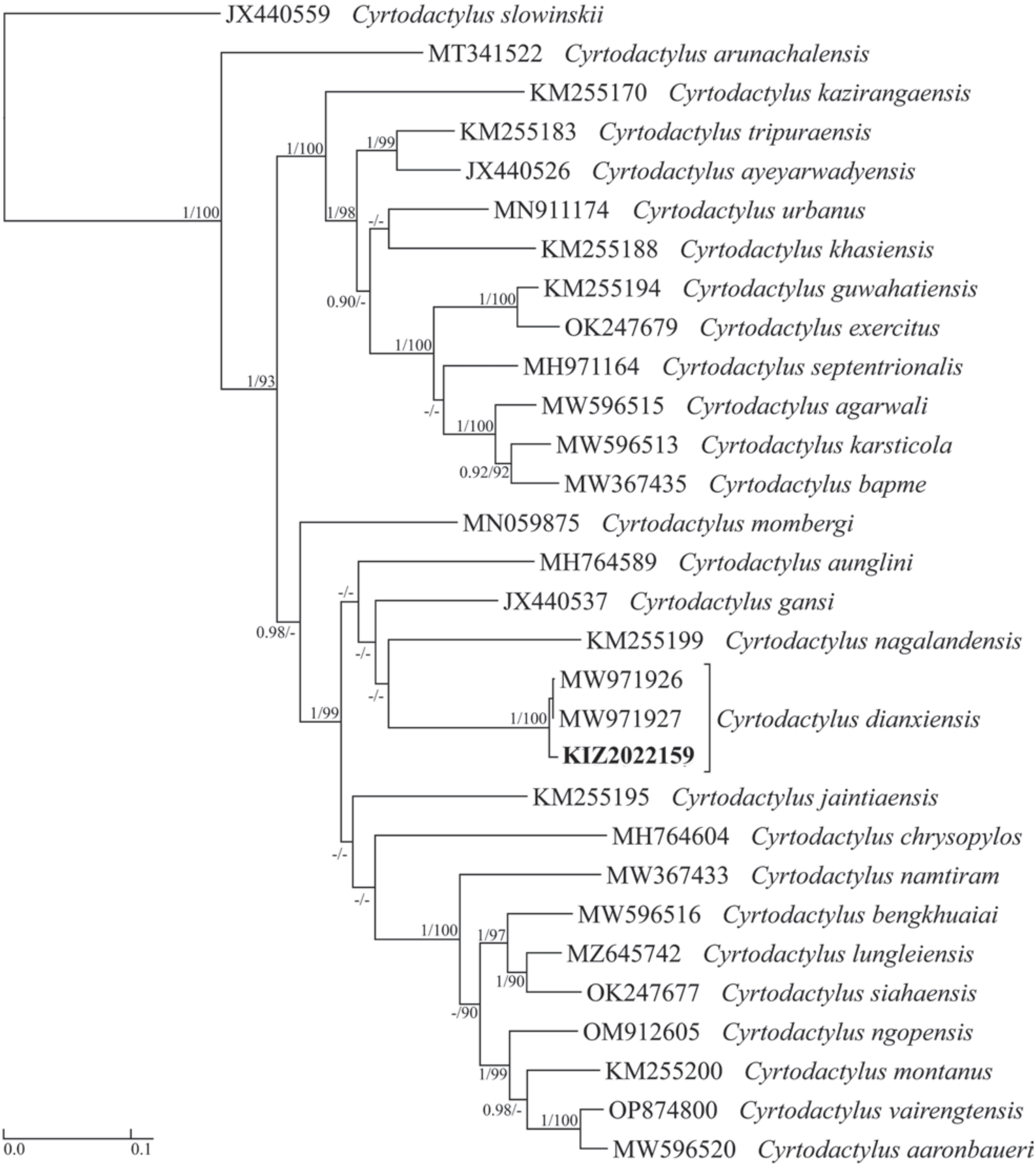

Figure 2. Bayesian phylogram of the *Cyrtodactylus khasiensis* species group inferred from ND2 sequences. Numbers before slashes indicate Bayesian posterior probabilities and numbers after slashes indicate the ML ultrafast bootstrap. Values below 0.90/90 are not shown.

Cyrtodactylus dianxiensis Liu & Rao, 2021

Figs 3–4, Table 3

Specimen examined. KIZ2022159, adult female, collected on 13 September 2022 by Shuo Liu from Tongbiguan Nature Reserve, Xueli Village, Taiping Town, Yingjiang County, Dehong Prefecture, Yunnan Province, China (24°26'35"N, 97°33'1"E; at an elevation of 380 m) (Fig. 1).

Description of the female specimen. SVL 75.0 mm; head relatively large (HL/SVL 0.26, HW/HL 0.68), depressed (HH/HL 0.45), distinct from neck; loreal and interorbital region concave, canthus rostralis slightly swollen; snout moderately long (SE/HL 0.40); eye large (ED/HL 0.24); pupil vertical with crenulated margins; ear opening oval, obliquely oriented (EL/HL 0.08); rostral with mid-rostral suture dorsally; two large supranasals, separated by one small internasal; dorsal head scales heterogeneous,

Table 2. Uncorrected pairwise divergence (%) between *Cyrtodactylus khasiensis* species group members based on ND2 sequences.

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27
1	*C. aaronbaueri*																											
2	*C. agarwali*	24.3																										
3	*C. arunachalensis*	23.4	20.6																									
4	*C. aunglini*	19.6	20.8	24.2																								
5	*C. ayeyarwadyensis*	21.9	14.8	20.2	18.2																							
6	*C. bapme*	22.9	7.2	20.8	20.7	14.2																						
7	*C. bengkhuaiai*	12.0	23.6	20.9	18.3	19.9	21.9																					
8	*C. chrysopylos*	21.7	21.4	23.1	18.5	18.9	21.9	19.2																				
9	*C. dianxiensis*	21.2	20.3	21.9	16.6	18.8	20.4	21.0	18.8																			
10	KIZ2022159	21.0	20.6	21.8	19.8	21.7	20.7	21.0	22.0	0.9																		
11	*C. exercitus*	22.5	11.8	18.4	21.7	16.2	13.6	22.5	21.9	21.3	21.4																	
12	*C. gansi*	19.2	21.0	22.0	15.8	16.9	20.9	18.2	17.6	15.5	18.4	21.1																
13	*C. guwahatiensis*	22.6	12.5	20.3	21.6	15.4	13.6	22.1	22.9	22.4	22.7	4.5	21.1															
14	*C. jaintiaensis*	19.2	23.1	22.9	19.1	21.8	22.5	17.3	22.0	19.1	19.3	21.4	19.0	22.4														
15	*C. karsticola*	22.9	6.4	19.1	19.2	12.6	6.7	23.8	21.1	20.0	20.1	13.4	21.1	13.1	22.0													
16	*C. kazirangaensis*	24.7	20.0	21.5	22.5	16.7	19.6	22.9	23.7	21.0	21.3	18.3	21.7	19.8	23.0	17.1												
17	*C. khasiensis*	22.7	17.2	21.0	21.5	15.6	16.9	19.5	22.1	21.9	22.6	17.0	20.0	16.9	21.5	15.2	18.5											
18	*C. lungleiensis*	12.1	21.6	19.9	18.3	19.2	20.7	7.2	20.4	20.3	19.7	20.2	18.5	21.1	18.0	22.0	21.8	18.8										
19	*C. mombergi*	20.0	20.4	20.4	17.4	17.3	19.9	20.4	19.2	17.2	20.3	20.4	15.9	21.8	19.7	19.6	21.0	20.3	18.4									
20	*C. montanus*	10.3	22.9	22.6	19.6	20.0	20.5	12.1	21.5	19.5	20.1	20.0	19.6	21.9	18.8	22.0	23.3	21.7	10.9	19.6								
21	*C. nagalandensis*	21.5	24.8	22.3	19.8	21.7	23.8	19.2	22.7	19.0	19.4	22.6	18.0	23.6	19.0	22.0	23.1	22.0	19.1	20.6	19.6							
22	*C. namtiram*	15.7	22.6	22.5	20.4	20.9	22.3	14.2	20.7	21.9	22.0	23.3	20.3	23.3	20.0	24.1	23.7	21.9	13.9	19.5	15.4	19.4						
23	*C. ngopensis*	9.9	23.6	22.0	18.3	20.7	21.1	11.2	20.3	20.2	19.9	21.0	18.4	21.6	17.8	22.7	24.2	22.2	11.5	19.6	10.5	19.7	14.8					
24	*C. septentrionalis*	21.2	11.1	20.6	20.8	14.6	11.6	20.5	21.7	20.3	21.0	10.9	19.8	11.3	21.4	10.7	18.7	15.7	19.1	20.6	19.6	23.6	21.5	20.1				
25	*C. siahaensis*	11.4	22.9	20.0	18.7	20.1	20.9	8.6	20.8	19.3	19.1	21.8	18.9	21.1	17.5	23.4	22.5	19.4	6.1	19.1	11.0	17.2	14.2	10.3	19.3			
26	*C. tripuraensis*	21.8	13.2	18.4	18.8	7.5	12.3	18.4	19.6	18.9	19.8	14.7	16.7	12.9	18.6	13.1	14.5	12.5	19.1	17.1	18.2	19.0	21.1	18.8	11.5	18.6		
27	*C. urbanus*	23.8	15.6	20.8	21.3	13.7	15.1	21.5	21.5	20.8	21.1	14.9	20.0	15.2	21.8	14.3	17.3	14.2	20.1	20.5	21.8	22.9	21.6	21.4	13.4	20.1	11.5	
28	*C. vairengtensis*	4.0	24.4	24.2	18.9	21.1	22.2	11.2	21.1	20.8	20.5	21.9	18.5	22.2	18.8	22.3	24.5	22.8	11.7	20.4	10.2	20.1	15.9	10.3	20.5	10.6	20.0	22.3

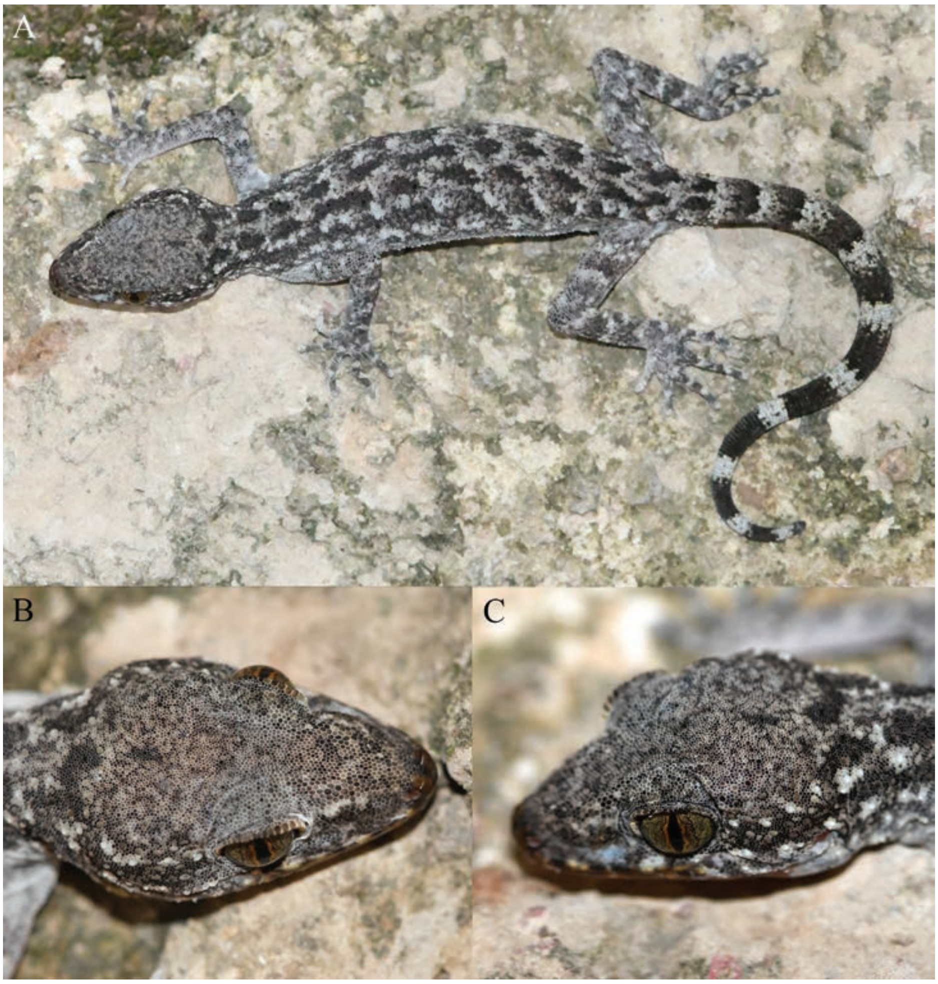

Figure 3. The female specimen (KIZ2022159) of *Cyrtodactylus dianxiensis* in life. **A.** Dorsal view; **B.** Close-up dorsal view of the head; **C.** Close-up dorsolateral view of the head.

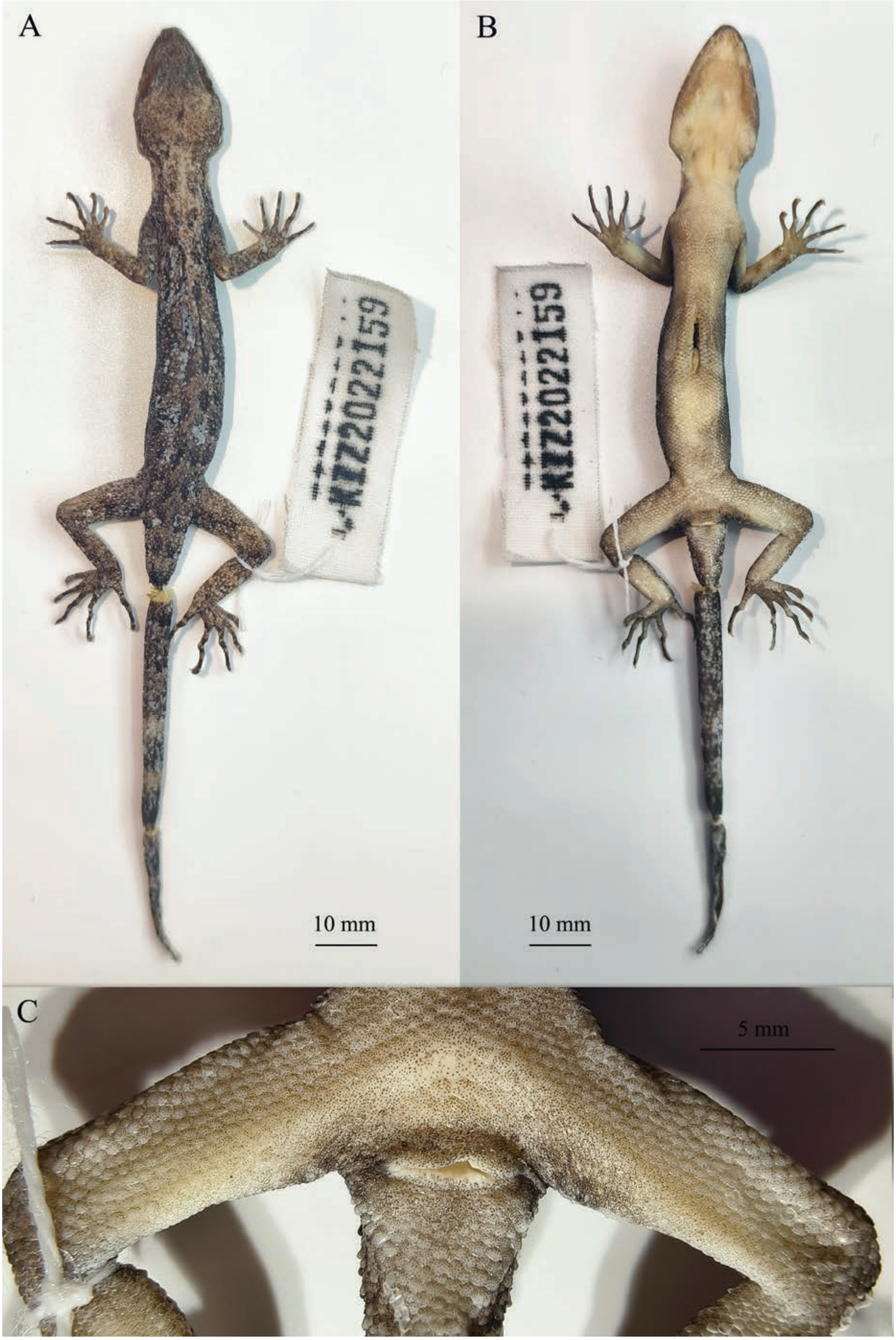

Figure 4. The female specimen (KIZ2022159) of *Cyrtodactylus dianxiensis* in preservative. **A.** Dorsal view; **B.** Ventral view; **C.** Close-up view of the precloacal and femoral regions.

Table 3. Measurements (mm) and meristic data for the female specimen of *Cyrtodactylus dianxiensis*. See Materials and methods for abbreviations.

	KIZ2022159		KIZ2022159
SVL	75.0	EE	5.9
TRL	33.8	EL	1.6
BW	11.6	IN	2.5
TL	73.7	IO	2.6
TW	6.8	FP	0
HL	19.3	PcP	0
HW	13.2	MVSR	35
HH	8.6	PVT	32
FL	11.5	DTR	17
CL	13.3	SL (R/L)	9(7)/10(7)
ED	4.6	IL (R/L)	9/8
NE	6.1	LF4	17/16
SE	7.8	LT4	20/20

rounded, granules; mental triangular with a pair of enlarged postmentals followed by some gradually decreasing chin-shields; scales on other region of the ventral head almost homogeneous, small, rounded, granules; supralabials seven to midorbital position on both sides, nine to angle of the jaw on right side and ten to angle of the jaw on the left side; infralabials nine on right side and seven on left side.

Body slender (TRL/SVL 0.45); dorsal scales heterogeneous, primarily small rounded granules, intermixed with irregularly arranged large circular tubercles, tubercles on nape and occipital region smaller than those on dorsum; ventrolateral folds present; ventral scales larger than dorsal; enlarged femoral scales and femoral pores absent; precloacal scales enlarged, precloacal pores absent; no precloacal groove; cloacal spurs indistinct.

Limbs slender (FL/SVL 0.15, CL/SVL 0.18); digits strongly inflected at each joint, all bearing robust, recurved claws; relative length of digits: I<II<V<III<IV (manus) and I<II<III<V<IV (pes); scales on dorsal forelimbs heterogeneous, granules, with small tubercles interspersed, scales on dorsal hind limbs heterogeneous, granular, with large conical tubercles interspersed; ventral scales of limbs almost homogeneous, granular, smaller than those on ventral body.

Original tail broken but exists, subequal to body length (TL/SVL 0.98); dorsal tail scales heterogeneous, with small tubercles interspersed; two rows of subcaudal scales slightly enlarged.

Coloration in life. Dorsal surface of head almost uniform brownish gray; upper lip brownish gray with many white spots, an indistinct black postorbital streak that extends backwards above ear opening on each side; a disconnected W-shaped black stripe on the occiput; dorsal surface of body brownish gray with many short black and white streaks roughly forming longitudinal rows; dorsal surface of limbs gray with indistinct white bands and spots; dorsal surface of tail grayish black with ten white bands; ventral surfaces of head, body, and limbs white, ventral surface of tail checkered with brownish gray and white; iris bronze with dark reticulations, edge of pupil orange red.

Extended diagnosis. Body size moderate (SVL 73.8–79.9 mm in adults); 9–12 supralabials; 8–11 infralabials; 17–19 longitudinal rows of rounded, conical dorsal tubercles; 31–32 paravertebral tubercles; 35–41 ventral scales between ventrolateral folds; no precloacal groove; femoral scales not enlarged, no femoral pores; 7–8 precloacals in males, no precloacal in female; 16–17 total subdigital lamellae beneath finger IV, 19–20 total subdigital lamellae beneath toe IV; subcaudal scales not transversely enlarged or two median rows slightly enlarged. Dorsum with light and dark blotches roughly forming longitudinal markings; a W-shaped black stripe present on occipital region; tail with 8–10 alternating dark and light bands; iris blueish gray or bronze with orange red edge.

Discussion

The female specimen agrees well with the original description (Liu and Rao 2021) of *C. dianxiensis* in measurements and meristic counts, however, there are some slight differences in coloration between them. The female specimen has a relatively lighter body color; the dorsal head of the holotype of *C. dianxiensis* is mixed with brown and grayish white, while it is almost uniform brownish gray in the female specimen; the iris of the holotype is blueish gray with no orange red edge, while the iris is bronze with orange red edge in the female specimen. Therefore, in this species, the coloration of dorsal head is not always mottled, but may also be uniform; in addition, the iris of this species has two color types, blueish gray and bronze with orange red edge.

The holotype of *C. dianxiensis* was collected at an altitude of 1170 m and the paratype was collected at an altitude of 1200 m (Liu and Rao 2021). However, the female specimen was collected at a much lower altitude. This record extends the lowest elevational distribution of this species to 380 m.

Cyrtodactylus dianxiensis was previously known based on only two adult males and one juvenile (Liu and Rao 2021). This study reported the female specimen of this species for the first time, and provided a detailed description and photos of the female of this species. However, we have only collected one female specimen of this species, and it is unknown whether some differences between male and female are permanent. In addition, the range and population status of this species will need to be determined by collecting more specimens in the future.

Acknowledgements

Thanks to the editors and reviewers for their work on the manuscript. We also would like to thank Yunnan key laboratory of biodiversity and ecological conservation of Gaoligong Mountain for its support. This study was supported by the Science-Technology Basic Condition Platform from the Ministry of Science and Technology of the People's Republic of China (No. 2005DKA21402) and Yunnan Applied Basic Research Projects (No. 202301AT070312).

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ÖGH Herpetozoa
Austrian Herpetological Society

Herpetozoa 37: 73–76 (2024)
DOI 10.3897/herpetozoa.37.e117207

The first record of the rarely observed rat snake, *Elaphe urartica* Jablonski et al., 2019 (Squamata, Colubridae) for Iraq

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Academic editor: Silke Schweiger ♦ **Received** 11 December 2023 ♦ **Accepted** 15 February 2024 ♦ **Published** 21 March 2024

Abstract

The Urartian rat snake, *Elaphe urartica* Jablonski, Kukushkin, Avcı, Bunyatova, Ilgaz, Tuniyev & Jandzik, 2019, is a recently described species within the genus *Elaphe*, found in eastern Anatolia, Caucasian region and the northern Middle East. It stands as one of the rarely observed snake species, with its distribution and other aspects of natural history largely unexplored. This is particularly true for the territories of Iraq and Iran. Therefore, we report on the presence of the species in Iraq, the first documented evidence of the species' distribution within the country. In conjunction with this record, we provide initial data regarding the species' habitat and ecology in the region. The distribution record presented here is further supplemented by a new species record from the border regions between Iraq and Iran, encouraging further herpetological exploration within the Zagros Mountains.

Key Words

Colubridae, distribution, *Elaphe quatuorlineata* group, Iran, Kurdistan, rat snake

The Urartian rat snake, *Elaphe urartica*, was described in 2019 following molecular and biogeographic analyses that revealed its differentiation from the closely related species *E. sauromates* (Pallas, 1814), both belonging to the *E. quatuorlineata* group (Jablonski et al. 2023). Presently, this species is known to inhabit various regions, including the Armenian Highlands, the southeast and east foothills of the Great Caucasus, the northern Zagros and Alborz mountains, along with the associated Kura-Aras lowlands. Its distribution spans eastern Turkey, Georgia, Armenia, Azerbaijan, Nagorno-Karabakh, Iran, and Dagestan (Russia) (Arakelyan et al. 2011; Jablonski et al. 2019). In literature, its easternmost range extends to Iran's Semnan and Golestan provinces, with the southernmost boundary noted in Kermanshah province (Safaei-Mahroo et al. 2015; Kamali 2020). Surprisingly, there is also a record of an isolated population from northeastern Khorasan provinces mentioned by Darvish and Rastegar-Pouyani (2012) (under the name *E. quatuorlineata*). Two museum specimens are known from Iran in publicly available databases: FMNH 130818 (locality unknown) and SMNS-Z-HE-2990 (Tehran). However, there is a lack of recent systematic examination of records within Iranian provinces regarding the presence of *E. urartica*, with existing records often relying on older sources and being frequently inaccurate (e.g., Latifi 1991). Although data from neighboring Turkey are missing, the probability of the species' presence in the West Azerbaijan and Kurdistan provinces of Iran is expected. The species' presence there is mentioned by Kamali (2020) (under the name *E. sauromates*) but again lacking comprehensive details. Similarly, Sindaco et al. (2013) mentioned the species' presence in the Iraq – Iran border but with uncertainty. Consequently, Jablonski et al. (2019, 2023) have indicated a questionable status for the species' range in these regions due to these conflicting data. Thus, the species' presence in this region remain elusive and recent, well supported data are, according to our best knowledge, unknown. Although the presence of the species in different parts of Zagros mountains is expected (Sindaco et al. 2013), there is no official report from the territory of Iraq (Jablonski et al. 2019). Thus, given the scarcity or fragmentation of data, every observation in these poorly explored areas is important.

We here report the first record of *E. urartica* in Iraq, discovered in the easternmost mountain part of Iraqi Kurdistan. The species was found and killed by a local man who alerted us about the presence of big snakes living in his yard. The observations came from Laradar village (35.7339°N, 46.2065°E, 1984 m a. s. l.) of the Penjween area in the Sulaymaniyah Governorate approximately 1 km from the Iranian border (Fig. 1A). From May to July 2023, locals informed us about four killed snakes. They were observed and photographed on 31 May (Fig. 2A) at 1:40 pm, 20 June (Fig. 2B) at 11:25 am, 6 (Fig. 2C) at 9:40 am and 25 July 2023. All individuals were adult specimens with the total length up to 1200 mm. The basic morphology and coloration clearly correspond with characters of *E. urartica*, particularly having a pattern formed by black, irregular but rounded blotches on the dorsal part of the body with a row of dark flanks on the yellowish background laterally. The dorsal part of the head of all specimens was typically black with the postocular stripe extending towards the mouth corner. The coloration and pattern among observed individuals slightly differentiated (see Fig. 2A–C).

To search for this species and see the habitat, we visited the location on 6 September 2023. We spent several hours searching in the environment where snakes had been previously sighted. Unfortunately, we did not come across any individuals. Interestingly, a local resident mentioned observing this species for the first time in this area what indicates the rarity of species. Local people are active in the wild mostly during spring months when they collect herbs and mushrooms. According to their information, they killed snakes because they believe that "*due to their size, they are venomous and pose a threat to humans*." Regrettably, encounters with snakes in Iraqi Kurdistan often result in the killing of nearly all individuals, a common occurrence. This trend significantly impacts the conservation of local reptiles, emphasizing the urgent need for enhanced education among local people about the snake fauna of Iraqi Kurdistan. The presence of the large and highly venomous *Macrovipera lebetinus* (Linnaeus, 1758) frequently prompts locals to proactively eliminate any sizable snakes. We made efforts to explain that this

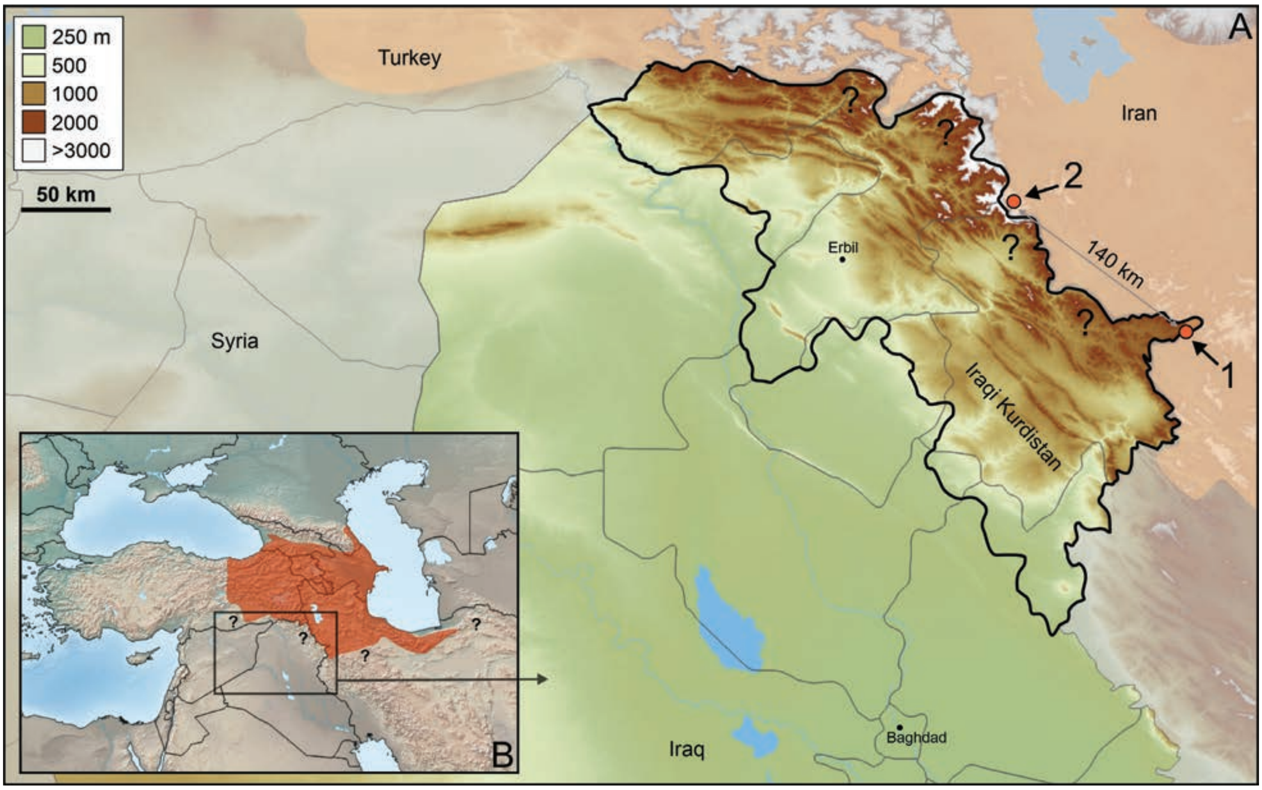

Figure 1. A. Depicts the physical map of northern Iraq (delineating Iraqi Kurdistan with a solid line) with the first record of *Elaphe urartica* in Iraqi Kurdistan (1) and the record from Iran (2), positioned three kilometers away from the Iraqi border. The question mark indicates potential areas where further field research is necessary to ascertain the species' presence. The orange shading represents the anticipated distribution range within the southern region of Turkey and western Iran; **B.** Depicts the distribution range of the species based on the combination of Sindaco et al. (2013), Kamali (2020), and Jablonski et al. (2023). Question marks denote regions where the presence of the species is uncertain and/or requires verification.

snake species is exceedingly rare and poses no threat. Fortunately, the locals assured us that they would take measures to protect *E. urartica* in the future. The habitat where these individuals were observed lies near the timberline zone at an elevation of nearly 2,000 meters. It primarily consists of a mixed oak forest dominated by *Quercus lebani*, *Q. infectoria*, *Q. aegilops*, *Pistacia eurycarpa*, and *Acer monspessulanum*. Additionally, agricultural lands are also present in the area (see Fig. 2E). The mountain peaks are covered with vegetation dominated by *Astragalus* spp. and *Daphne* sp. The temporary mountain streams are also found in the area, but they are extensively utilized by people for irrigation purposes and during summer months most of them disappear.

As *E. urartica* is currently considered a rarely observed species of the snake fauna of the Western Palearctic (Jablonski et al. 2019), the first record for Iraqi Kurdistan has importance for the protection of local populations. We would like to highlight several threats that this species could face there: 1) forest fires threatened the general habitat of the area. The sources of forest fires include mine explosions, army conflicts and bombardments, deliberate human firing, other anthropogenic factors, and sometimes fires come from the Iran border; minefields in the area made the forest fire impossible to control, 2) habitat loss and deforestation in the entire habitat of the area is under threat of extensive land use for agricultural purposes (manipulating surface and ground water sources for irrigation, cutting natural forest for expansion of agricultural lands, cutting trees for fuel and charcoal, and using pesticides), 3) killing of this species by local people due to a lack of awareness about snakes, thinking they all are venomous (see above). It is thus beholden on local conservation and education authorities to provide protection and obtain more information about this new, big but rare snake species of Iraqi Kurdistan.

To highlight the importance of distribution of *E. urartica* in Iran and Iraq and place the Iraqi record in context, we also report on two observations of the species from the

Figure 2. Individuals of *Elaphe urartica* from Iraq (**A–C**. Photography by Hemn Penjweeny) and Iran (**D.** Photography by Azad Shamsi) reported in this study; **E.** The species habitat (photography by Soran H. Ahmed) in Laradar village, Penjween area, Sulaymaniyah Governorate, Iraqi Kurdistan.

border area on the Iranian side. The species was observed on 14 May 2015 and 30 May 2023 at 11:07 am in Qandil Mountains (36.5323°N, 45.0577°E, 2,580 m a.s.l.). The observations were provided and photographed by local mountaineer Azad Shamsi. The first observations possess one individual sitting and basking in mountain vegetation (Fig. 2D), the second observation includes two snakes found during mating. Both observations come from the mountain valley, approximately 3 km (straight line) from the Iraqi border in the Kurdistan Province of Iran (neighboring the northeastern part of the Sulaymaniyah Governorate of the Iraqi Kurdistan and not so far from the border with the Erbil Governorate). This record is approximately 140 km distant (straight line) from the first species record in Iraq mentioned above. At the same locality in Iran, *Montivipera raddei* (Boettger, 1890) was also observed.

As is evident, the range of *E. urartica* in Iraq has a peripheral character (Fig. 1) but the species may extend to other parts of northwestern Zagros Mountains. Both records presented here significantly improve our knowledge about species distribution and provide well documented records for both countries. Moreover, they also confirm published suggestions that this species is present in these areas of Iran (Kamali 2020) and can be possibly found in the same areas of Iraq (Sindaco et al. 2013), i.e., Erbil and Sulaymaniyah Governorates. The presence in Dohuk Governorate of northern Iraq is also not excluded, and further research should answer the question about the distribution range of the species in both countries.

Acknowledgments

We would like to express our gratitude to Hemn Penjweeny and Azad Shamsi for their detailed information and photos of *E. urartica* and their kind permissions to be cited in this note. This work was supported by the Scientific Grant Agency of the Slovak Republic VEGA 1/0242/21.

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ÖGH Herpetozoa
Austrian Herpetological Society

Herpetozoa 37: 77–84 (2024)
DOI 10.3897/herpetozoa.37.e112716

Kleptoparasitism in *Micrurus mipartitus* (Squamata, Elapidae) competing for the same *Caecilia* sp. (Gymnophiona, Caeciliidae) in western Colombia

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Academic editor: Günter Gollmann ♦ **Received** 19 September 2023 ♦ **Accepted** 8 March 2024 ♦ **Published** 22 March 2024

Abstract

Kleptoparasitism, or food theft, is seldom reported in wild populations of snakes. Here, we describe as case where two Red-tailed Coral Snakes, *Micrurus mipartitus*, were observed competing for the same caecilian prey, either *Caecilia leucocephala* or *C. perdita*. This took place at night in a rainforest habitat in Valle del Cauca Department, western Colombia. Upon our arrival, the battle had already started as the two coral snakes kept bite-holds on the caecilian. They continued biting the prey at different places on the anterior parts and tugging in opposite directions. The snakes also made rotations along the longitudinal axis as they maintained their bite-holds. Surprisingly, one snake also bit the body of the other snake once. After 17 minutes of observation, the losing coral snake released its bite-hold on the caecilian. The winner then moved away from the losing snake which did not follow. It is well-known that *M. mipartitus* and other coral snakes eat caecilians, but this is the first observation of kleptoparasitism in elapid snakes in the wild. It is considered likely that they rely on chemoreception when detecting caecilians, notably in this case as two coral snakes detected the same prey item. In general, kleptoparasitism may occur more frequently amongst snakes than indicated by the very few published cases considering that numerous cases from captivity are known.

Key Words

caecilian, chemoreception, combat for food, coral snake, odour, resistance, scent, theft, Valle del Cauca, venom

Introduction

Kleptoparasitism is a feeding strategy defined as intraspecific or interspecific, deliberate stealing of already procured food (Broom and Ruxton 1998). It is known in a wide array of animals (Iyengar 2008) and is particularly conspicuous and well-described in birds (Brockmann and Barnard 1979) and in many invertebrates (Vollrath 1984). Iyengar's (2008) review includes some examples in lizards and turtles, but none amongst snakes. Kleptoparasitism is seldom reported in crocodiles, but is suspected to be widespread and hitherto overlooked (Platt et al. 2007).

The Red-tailed Coral Snake, *Micrurus mipartitus* (Duméril, Bibron & Duméril, 1854), is a widespread elapid species in Colombia (mainly the western half), but its overall distribution includes adjacent countries in South America and eastern Panama (Köhler 2008; Rios-Soto et al. 2018). This species is bicoloured with black body rings alternating with white, cream or yellow rings, but the parietal and caudal rings are red-orange and its total length is normally 60–75 cm, but rarely up to 120 cm (Campbell and Lamar 1989; Rios-Soto et al. 2018). The black rings may have a brown tinge as seen in one of the individuals in this paper. The pattern is variable and five subspecies are recognised, four of which occur in Colombia,

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namely the nominate subspecies, *anomalus*, *decussatus* and *popayanensis*, whereas *semipartitus* is extralimital and is distributed in northern Venezuela (Roze 1996). The nominate subspecies is known from the Pacific area of Colombia (including the San Cipriano area and Valle del Cauca, in general), as well as eastern Panama.

In this paper, we describe a rare event as two *M. mipartitus mipartitus* were observed in a vigorous dispute for the same caecilian prey in western Colombia. This is a new observation of kleptoparasitism which has not been documented in coral snakes or any other elapids in the wild before.

Observations

On 10 March 2023, two adult *Micrurus mipartitus* (estimated total lengths 55–70 cm) competing for an adult caecilian (estimated total length 25–40 cm) were observed in the Reserva Natural San Cipriano, Valle del Cauca Department, western Colombia. Coordinates: 3°49'51"N, 76°53'16"W; altitude 106 m a.s.l. The habitat consisted of a dirt road in the rainforest. Upon our arrival at 20:52 h, we witnessed that the two coral snakes kept bite-holds on the caecilian. None of the three individuals was handled or otherwise touched during the combat and feeding event. The following observations have been based on photographic and video documentation taken by Henrik Bringsøe and Niels Poul Dreyer. The observations were made by the authors, Alejandro Grajales and a local guide.

In this report, the term "winner" is used for the coral snake which eventually kept and carried away the caecilian, whereas "loser" is used for the snake eventually losing the caecilian. The two coral snakes could be distinguished from their different patterns and colourations being conspicuous in photos and video sequences. After the winner had moved away with its caecilian prey, the loser was photographed and unique characters were observed (Fig. 5) and then comparisons between the two snakes in an earlier close-up picture were made (Fig. 3). The ground colour of the winner appeared brownish-black, whereas that of the loser was black and the winner appeared larger than the loser. Furthermore, marked differences in the pattern in the right side of the red-orange parietal ring of the two individuals were recorded as follows:

The loser: Just behind the right eye, a fine black streak in the border between two head scales reached into the red-orange area. Moreover, the demarcation between the anterior part of the red-orange parietal ring and the posterior part of the black head colouration in the labial region was highly irregular, but sharp. The demarcation between the posterior part of the red-orange ring and the adjacent black area was also irregular because two fine black streaks penetrated into the red-orange area; a black chevron-shaped marking appeared between these two streaks.

The winner: Behind the right eye, a black streak was absent. The demarcation between the anterior part of the red-orange parietal ring and the posterior part of the black head colouration appeared blurred and small poorly-defined brownish spots were present in the posterior part of the red-orange ring, but there were no conspicuous irregularities in the posterior demarcation of the red-orange ring.

From the beginning, we observed both snakes being active and energetic biting the caecilian and tugging at it in opposite directions. That implies that the event must have started before our arrival at 20:52 h. Generally, they made their bite-holds on the anterior parts of the caecilian, close to its head (Figs 1–3). From 20:54 h, rotations along the longitudinal axis were performed by the snakes as they maintained their bite-holds, but mostly made clockwise by the loser (Suppl. material 1, 2).

From 20:56 h, mucous, probably produced by the caecilian, was noticed on the caecilian around the places of the snakes' bite-holds (Fig. 3).

At 20:58–21:01 h, the loser kept a bite-hold on the caecilian's body, while the winner constantly and eagerly moved along the loser's and the caecilian's bodies. The loser, while trying to secure bite-holds on the caecilian, often reacted with rapid jerks when its head was touched by that of the winner (Suppl. material 3).

From 21:06 h, the winner got a bite-hold on the caecilian's head and at 21:07 h, the loser moved its bite-hold to the central part of the caecilian's body.

At 21:08–21:09 h, the loser and the caecilian appeared entwined, while the winner continued biting the caecilian.

At 21:09 h, the loser bit the body of the winning snake, while the winner maintained its bite-hold on the caecilian's head (Fig. 4). The duration of one snake's bite-hold on the other snake was approx. 30 seconds. A bit later at 21:09 h, the loser released its bite-hold on the caecilian and subsequently the winner carried the caecilian into the vegetation and moved away from the loser which did not try to follow.

At 21:26 h, the loser was photographed alone which made it possible to register morphological characteristics (Fig. 5) in comparison with the winner.

In neither of the described events did the caecilian attempt to bite the two snakes in defence.

Discussion

Our observation involving two individuals of *M. mipartitus* competing for the caecilian is the first published case of kleptoparasitism in the family Elapidae in the wild. Since we did not touch any of the three animals involved, precise identification of the caecilian is difficult, considering that the most reliable way to identify caecilians is by groove counts, but it is a member of the genus *Caecilia* which is represented by five species in the Pacific lowlands of Colombia (Fernández-Roldán and Lynch 2023). Of these, only *C. leucocephala* and *C. perdita* have light-coloured heads in contrast to the dark body, but *C. perdita* (< 50 cm total length) attains larger sizes than *C. leucocephala* (< 30 cm total length) which implies that it is uncertain whether this caecilian belongs to either *C. leucocephala* or *C. perdita* (Fernández-Roldán, pers. comm. 2023).

Figure 1. Overview of the two *Micrurus mipartitus* competing for a caecilian, *Caecilia* sp., on a dirt road in the rainforest at night. Both coral snakes kept bite-holds on the anterior parts of the caecilian's body. Here, the loser with black ground colour is most conspicuous because its head is fully visible. 10 March 2023 at 20:53 h. Photo HB.

Figure 2. During the bite-holds, the two *Micrurus mipartitus* were tugging in opposite directions. The loser to the left, the winner to the right. 10 March 2023 at 20:56 h. Photo HB.

Figure 3. Mucous, probably produced by the caecilian, appeared on the caecilian around the places of the snakes' bite-holds. The winner to the left, the loser to the right. 10 March 2023 at 20:56 h. Photo HB.

Figure 4. One coral snake (the loser) was biting the body of the winning snake which shortly later moved away from the losing snake with its caecilian prey. 10 March 2023 at 21:09 h. Photo NPD.

Figure 5. The losing *Micrurus mipartitus* photographed some minutes after the winner had moved away with its prey. The pattern of the right side of the red-orange parietal ring exhibited unique characteristics in comparison with the same area of the winner (see the text for details). 10 March 2023 at 21:26 h. Photo HB.

The diet of coral snakes of the genus *Micrurus* is known to consist largely of elongate prey, such as snakes, amphisbaenians, lizards, caecilians and, to a lesser extent, fishes, notably eels (Roze 1982; Banci et al. 2017; Fernández-Roldán and Gómez-Sánchez 2021). Generally speaking, that also applies to the diet of *M. mipartitus* which is known to prey on snakes, amphisbaenians, lizards, caecilians (including *Caecilia* spp.) and frogs (Rios-Soto et al. 2018; Fernández-Roldán et al. 2021). However, predation events by *Micrurus* spp. in the wild are seldom observed (Maffei et al. 2009; Banci et al. 2017).

Fernández-Roldán and Gómez-Sánchez (2021) briefly reviewed toxins produced by New World caecilians against predators. Additionally, new research indicates that Latin American caecilians, including the genus *Caecilia*, have evolved resistance to neurotoxins of *Micrurus* and other elapid snakes (Mancuso et al. 2023). That may explain why the caecilian in this case appeared unaffected by the numerous bite-holds of the two coral snakes during the 17 minutes we watched the event. Several cases support that coral snakes are specialised feeders on elongate or vermiform prey (Fernández-Roldán and Gómez-Sánchez 2021) and such predator-prey relationships usually work as evolutionary arms-races escalating adaptations and counter-adaptations against each other. Caecilians' toxins, resistance to neurotoxins and increased production of mucus making them slippery may serve as anti-predatory responses to attacks of coral snakes. However, other cases of coral snakes preying on caecilians resulted in the weakening or death of the prey within the first minutes to an hour (Viana and Mendes 2015; Fernández-Roldán and Gómez-Sánchez 2021), which may suggest that neurotoxic resistance is not widespread, contrary to the reports of Mancuso et al. (2023). In the important study by Jorge da Silva and Aird (2001), it was concluded that prey are more susceptible to venoms of *Micrurus* species that feed upon them than to venoms of those that eat other animals. Thus, further research on neurotoxic resistance will be required, perhaps even including the sampling of several populations within widespread species.

Another factor impacting the effect of coral snake venom on its prey is the amount of venom injected implying that caecilians injected with very small quantities of venom may potentially suffer less. In a recent study of venom production in nine Mexican species of *Micrurus*, tremendously diverse yields were recorded ranging from 0.3 mg to 59 mg per extraction (Neri-Castro et al. 2024). To some extent, this variability was explained by snake lengths, but it was suggested that other important factors also influenced the amount of obtained venom. The extractions were conducted by inducing the snakes to bite plastic spoons covered with Parafilm and pressure to the venom glands was not applied. It is an open question whether smaller yield fluctuations might have been achieved if the snakes' venom glands had been exposed to pressure. The determinants of the quantity of venom either injected voluntarily or expressed manually remain relatively poorly studied (Wüster, pers. comm. 2024). In a literature review of venom yields of Brazilian *Micrurus* spp., the fluctuations were generally smaller (Carvalho et al. 2014). To our knowledge, venom production of *Micrurus mipartitus* has not been studied.

Repeatedly, each of the two coral snakes performed rotations about their own longitudinal body axis as they had secured bite-holds on the caecilian. This element of feeding behaviour is seldom observed in snakes, but previously reported in another species of coral snake preying upon a caecilian (Fernández-Roldán and Gómez-Sánchez 2021). Such rotations about the longitudinal body axis may also be called "death rolls"; however, that spinning manoeuvre is usually used for rapid rotations to tear off limbs or meat and is typically seen in crocodiles (Fish et al. 2007; Drumheller et al. 2019). In this case, on the contrary, the rotations were slow and were apparently a sequence of movements to untwist or unknot the caecilian.

It is noteworthy that one of the coral snakes bit the body of the other snake for approx. 30 seconds at the end of the battle. During the event, we used our lamps which may have blinded the snakes temporarily. Often the snakes were entwined with the caecilian which, in turn, produced mucous which was transferred to the snakes. Thus, the caecilian's odour particles probably covered much of the snakes' surfaces. Since it has been suggested that coral snakes often rely on chemosensory prey detection rather than vision when foraging (Marques et al. 2017), we believe it was an accident that one coral snake bit the body of the other, confusing it with the caecilian, though it cannot be ruled out that one coral snake might have attempted to prey on the other coral snake considering that cannibalism in *Micrurus* spp. has already been reported in literature. Apparently, the bitten snake was not affected by the bite.

Kleptoparasitism in snakes has not been paid much attention. Several cases in captivity are known (Naulleau 1967; Kelleway 1982; Burghardt and Denny 1983; Grimpe 1984; Yeager and Burghardt 1991; Firmage and Shine 1996; Doody et al. 2021), but few are reported in the wild. These events in the wild may involve multiple individuals of the same species (Platt et al. 2018), competitions associated along with ritualised combats (*Agkistrodon conanti*, Farrell 2022, pers. comm. 2023) and interspecific interactions, either including other congeners (Platt et al. 2020) or lizards (McConchie and Wilkinson 2004). Greene (in Iyengar 2008) suggested that the likelihood that a feeding snake will be challenged for a food item by another animal was small considering the widely-spaced intervals of feeding in many snakes. It is expected that kleptoparasitism in snakes would be particularly common in places with high population densities, as in the observations made by Platt et al. (2020) and Farrell (2022). Due to its crepuscular and nocturnal habits (Rios-Soto et al. 2018), *M. mipartitus* is rarely seen, albeit easy to detect because of its vivid, characteristic colouration. However, based on our field experience, we consider its populations densities may potentially be low. We consider that both coral snakes may have been attracted by the caecilian odour, as some species of snakes have been reported to trace back scents from hundreds of metres or even more than two kilometres (e.g., Brown and MacLean 1983; Andrén 1986). A coral snake pulling a caecilian out of the ground, after allegedly having detected it by chemoreception, has even been reported (Fernández-Roldán and Gómez-Sánchez 2021).

Kleptoparasitism amongst snakes in captivity has been known for decades and probably even centuries and basic warnings of not feeding two snakes one prey item have been communicated in a wide variety of books on herpetoculture including beginners' guides that even warn keepers that larger snakes may swallow the smaller (e.g., Klingelhöffer 1959; Roberts 1975; Frank 1979; Trutnau 1994). Albeit rarely reported, it is probable that kleptoparasitism may occur more frequently amongst wild snakes than previously thought.

Acknowledgements

Juan David Fernández-Roldán (Universidad Nacional de Colombia, Bogotá) provided valuable information as he tried to identify the caecilian prey in this study. Thanks to Terence M. Farrell (Stetson University, DeLand, Florida) for inspiring discussions about kleptoparasitism and to Wolfgang Wüster (Bangor University, UK) for useful hints on snake venom and resistance to venom. Alejandro Grajales (Armenia, Colombia) and Leonilda Viera (Nuquí, Colombia) arranged our excellent journey in western Colombia in March 2023. Mark Hanger (Copenhagen, Denmark) made valuable contributions and reviewed the English language.

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Supplementary material 1

Video A1

Authors: Henrik Bringsøe, Niels Poul Dreyer

Data type: mp4

Explanation note: Two *Micrurus mipartitus* in a competition for the same caecilian prey. Both snakes were biting the anterior part of the caecilian and tugging in opposite directions. Initially, the distance between them was 1½–2 times the diameter of the caecilian. However, the winning snake moved its bite-hold closer to the loser so that the two snakes' snouts and/or chins eventually touched each other. As they touched, the loser completed a clockwise rotation around its longitudinal body axis. Reserva Natural San Cipriano, Valle del Cauca, Colombia.10 March 2023 at 20:57 h. Duration: 24 seconds. Recorded by NPD.

Link: https://doi.org/10.3897/herpetozoa.37.e112716.suppl1

Supplementary material 2

Video A2

Authors: Henrik Bringsøe, Niels Poul Dreyer

Data type: mp4

Explanation note: Two *Micrurus mipartitus* in competition for the same caecilian prey. Solely, the loser had a bite-hold on the caecilian and made 3½ clockwise rotations, while the winner eagerly followed them and kept close physical contact. Reserva Natural San Cipriano, Valle del Cauca, Colombia.10 March 2023 at 20:58 h. Duration: 22 seconds. Recorded by NPD.

Link: https://doi.org/10.3897/herpetozoa.37.e112716.suppl2

Supplementary material 3

Video A3

Authors: Henrik Bringsøe, Niels Poul Dreyer

Data type: mp4

Explanation note: Two *Micrurus mipartitus* in competition for the same caecilian prey. The loser maintained a bite-hold in the caecilian's neck region, while the winner moved actively around close to the loser and the caecilian. Notice the rapid jerks performed by the loser when its head was touched by that of the winner. Reserva Natural San Cipriano, Valle del Cauca, Colombia.10 March 2023 at 21:01 h. Duration: 97 seconds. Recorded by HB.

Link: https://doi.org/10.3897/herpetozoa.37.e112716.suppl3

ÖGH Herpetozoa
Austrian Herpetological Society

Herpetozoa 37: 85–93 (2024)
DOI 10.3897/herpetozoa.37.e117370

At home in Jiangsu: Environmental niche modeling and new records for five species of amphibian and reptile in Jiangsu, China

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Academic editor: Yurii Kornilev ♦ **Received** 15 December 2023 ♦ **Accepted** 4 March 2024 ♦ **Published** 25 March 2024

Abstract

Environmental niche models are useful tools for generating hypotheses for the distribution of species and informing conservation planning, especially at the edge of species' ranges and for those with limited data. Here we report on the recent documentation of four species of amphibian (*Hylarana latouchii*, *Odorrana tianmuii*, *Polypedates braueri*, and *Zhangixalus dennysi*) and one reptile (*Protobothrops mucrosquamatus*) with few or no previous geolocated records from Jiangsu, China. We combined our opportunistic field sampling data from Jiangsu, which is at the edge of each of these species' ranges, with publicly available occurrence records and climatic data to generate environmental niche models for these five species using Maxent. All models showed good model performance with AUC values ranging from 0.899 to 0.983. Additional potentially suitable areas within southern Jiangsu were predicted for the four amphibian species, although the significant anthropogenic habitat modifications in the province may limit their contemporary distributions. For all five species, the climatic variable that contributed most to the model was the precipitation of the driest month (Bio 14), indicating they are limited by moisture availability. Our study adds new information about the climatic preferences of these five species and highlights the value of complementing environmental niche modeling with field surveys for robust inferences and conservation planning, particularly at the edge of species' ranges.

Key Words

distribution, Maxent, Ranidae, Rhacophoridae, Viperidae

Introduction

Reptiles and amphibians are often difficult to detect, as many are small, nocturnal, and cryptic (Tanadini and Schmidt 2011; Durso and Seigel 2015; Hammond et al. 2021), which can make determining their distributions difficult. Yet, this baseline information is crucial for conservation, especially in light of contemporary anthropogenic pressures (Luedtke et al. 2023). One tool available to better understand the range of species

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is species distribution modeling, also known as environmental (or ecological) niche modeling, which combine species occurrence data with environmental data to generate predictions (Elith and Leathwick 2009). While models are imperfect approximations of reality (Merow et al. 2013), they can still be very useful for guiding field surveys for target species (Rhoden et al. 2017; Sarker et al. 2019), predicting responses to climate change (Duan et al. 2016), increasing ecological knowledge of species (Ananjeva et al. 2015; Hou et al. 2023), and informing conservation planning (Kidov and Litvinchuk 2021; Shin et al. 2021). Models that incorporate both presence and absence data are ideal (Lawson et al. 2014; Fiedler et al. 2018), but absence data is rarely available for reptiles, amphibians, and many other taxa. Other approaches only need presence data, though many of these rely on pseudo-absences (Barbet-Massin et al. 2012). One commonly used open-source software that utilizes presence-only data is Maxent (Phillips et al. 2017), which has been used to investigate a wide variety of amphibians and reptiles at past, present, and future scales (Kim et al. 2020; Shin et al. 2021; Andersen et al. 2022). Model accuracy improves with additional data if spatial bias in sampling is addressed, and as such the value of gathering new species occurrence records should not be overlooked (Kramer-Schadt et al. 2013; Zhu and Qiao 2016).

During field surveys in southern Jiangsu, People's Republic of China, we encountered two Ranid frogs (*Hylarana latouchii* and *Odorrana tianmuii*; Fig. 1), two Rhacophorid frogs (*Polypedates braueri* and *Zhangixalus dennysi*), and one viper (*Protobothrops mucrosquamatus*) that had few or no previously reported geolocated records from the province. Jiangsu is located in eastern China and has 20 native species of amphibian and 56 native reptiles (Zou and Chen 2002; Fei et al. 2012). The province straddles the Palearctic and Indomalayan biogeographic realms (Munguía et al. 2012), and each of these five species with limited records from Jiangsu are Indomalayan species found at the northeastern edges of their respective ranges. We used Maxent to model the current climatic niche of these species to better understand their ecological requirements and potential for further occurrence in Jiangsu.

Methods

Field sampling took place at night (~19:00–24:00 h) on 10, 14, and 25 September 2023 in the southern portion of Jiangsu, China, near the county-level cities of Yixing and Liyang (Fig. 2). These sites were selected due to presence of mountains contiguous with southern latitudes, thus exhibiting a higher probability of detecting Indomalayan

Figure 1. Study amphibian species: *Zhangixalus dennysi* (**a**), *Polypedates braueri* (**b**), *Hylarana latouchii* (**c**), and *Odorrana tianmuii* (**d**). Photos **a, c, d**: Kenneth Chin; **b**: Zhenqi Wang.

species. Surveys consisted of opportunistic sampling to maximize the number of reptile and amphibian species encountered and involved driving along roads slowly as well as walking on foot in and along streams and rivers (Niemiller 2005; Dodd Jr. 2016). Rocks, logs, and other possible cover objects were overturned and replaced. All reptile and amphibian species encountered were photo vouchered for iNaturalist and released at the point of capture within minutes and GPS coordinates were obtained using cell phones.

We modeled the climatic niches of four amphibians and one reptile we encountered during our surveys that were at the edge of their respective known ranges (Fei et al. 2012): *Hylarana latouchii* Boulenger, 1899 (taxonomy following Sun et al. 2021); *Odorrana tianmuii* Chen, Zhou & Zheng, 2010 (taxonomy following Li et al. 2017); *Polypedates braueri* Vogt, 1911 (taxonomy following Kuraishi et al. 2013); *Zhangixalus dennysi* Blanford, 1881 (taxonomy following Jiang et al. 2019); and *Protobothrops mucrosquamatus* Cantor, 1839 (taxonomy following Guo et al. 2019b). All field identifications were based on visual observations, made easier by the absence of sister or cryptic species for any of the species in the area (Fei et al. 2012). The exception to this was *H. latouchii*, which is visually similar to *Odorrana tormota* Wu, 1997; for this species, individuals were identified by lateral patterning, shape of dorsolateral fold, and tympanum shape (Chen et al. 2018). For the other four species (*P. mucrosquamatus*, *O. tianmuii*, *P. braueri*, and *Z. dennysi*) dorsal patterning was sufficient to distinguish them from similar locally occurring species (Fei et al. 2012; Guo et al. 2021). Our records for these five species are available as a Suppl. material 1 (DOI: 10.17632/gz87mn8nnw.2).

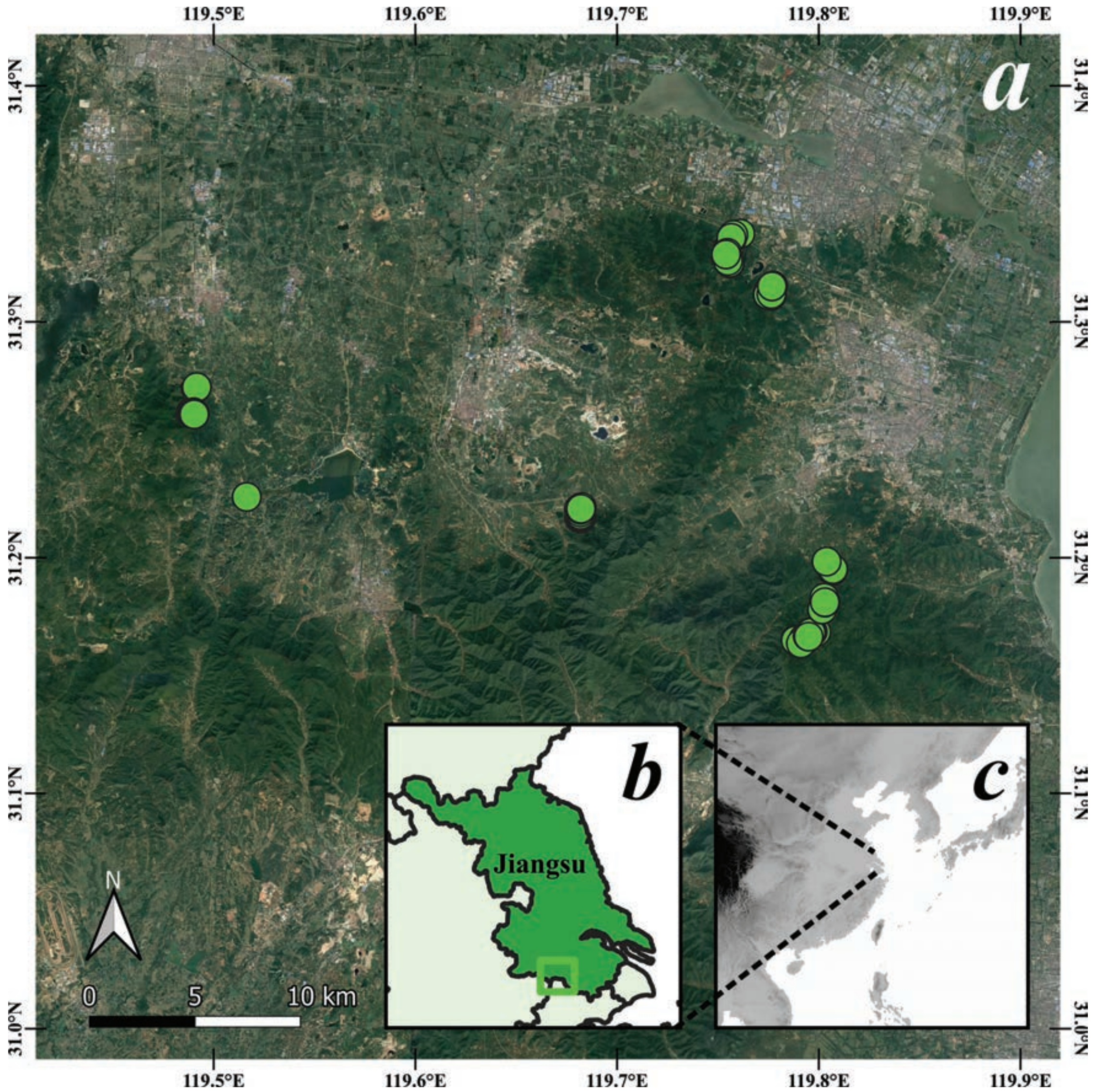

Figure 2. Sampling localities in Jiangsu, China. Map *a* shows the locations, represented by green dots, of all amphibians and reptiles observed during our field sampling (Satellite map: Google 2023 TerraMetrics). Inset *b* shows the focal area of the field surveys in southern Jiangsu outlined with a green rectangle. Inset *c* shows the broader location of Jiangsu (Elevation layer: Ryan et al. 2009).

In addition to our sampling data, we downloaded species occurrence data from GBIF.org, filtering for records with coordinates and no geospatial issues, for the five focal species (DOI: *P. mucrosquamatus* 10.15468/DL.AGK3WJ; *H. latouchii* 10.15468/DL.BXFK83; *P. braueri* 10.15468/DL.QBJWRW; *Z. dennysi* 10.15468/DL.TQTAP9; *O. tianmuii* 10.15468/DL.BRTRR3). For *P. braueri*, we searched GBIF for all *Polypedates* records within the range of the species as roughly outlined by Kuraishi et al. (2013), as *P. braueri* should be the only *Polypedates* species within this area but due to confusion regarding the species complex many records are incorrectly listed as *P. megacephalus* or *P. leucomystax*.

We also searched the scientific literature and other authoritative sources for records of these species from Jiangsu specifically. We found and incorporated nine geolocated locality records for *O. tianmuii* throughout its range from Li et al. (2017) and one record of *P. mucrosquamatus* from Jiangsu (Ye et al. 2024). For *H. latouchii*, the only existing geolocated record in Jiangsu we found was reported on iNaturalist (https://www.inaturalist.org/observations/153953701). For *Z. dennysi* there was only an undated museum record at Harvard's Museum of Comparative Zoology (MCZ:Herp:A-5624) from "Nanking" (no coordinates and not a precise locality; Museum of Comparative Zoology 2023). For *P. braueri* we found no geolocated records for Jiangsu.

For the environmental data for our modeling, we downloaded 19 bioclimatic layers, covering 1970–2000, from the WorldClim 2.1 database (Fick and Hijmans 2017) at a 30" resolution (~1 km^2). Layers were clipped to cover the full range of the study species using QGIS v. 3.32.2, and the built-in GRASS plug-in was used to calculate Pearson's correlation coefficients for all pairs of variables (Zhu and Qiao 2016, QGIS.org 2023). For highly correlated pairs ($|r| > 0.8$), we selected bioclimatic layers based on knowledge of the species and those shown to be relevant in modeling of related species (Table 1; Najibzadeh et al. 2017; Kidov and Litvinchuk 2021; Rai et al. 2021; Rupasinghe et al. 2021; Jiang et al. 2022).

To account for spatial bias in sampling, all occurrence records were spatially thinned at a distance of 10 km using *Wallace* v2.0.6 (Kramer-Schadt et al. 2013; Zhu and Qiao 2016; Kass et al. 2023). Additionally, for *P. mucrosquamatus, H. latouchii*, and *P. braueri*, the GBIF datasets contained significantly more records from the Island of Taiwan compared to the remainder of these species' ranges. To account for this disparity, which was caused by differing numbers of iNaturalist users and is not reflective of relative abundance, we standardized the dataset from the Island of Taiwan. We divided the land area of the Island of Taiwan (~36,200 km^2) by the total land area of each species' range, estimated using the measuring tool in QGIS, and thinned the records from the Island of Taiwan further until the proportion of records in the Island of Taiwan compared to the rest of the range was equal to the respective ratios of land area, rounding up to the nearest whole number (Kramer-Schadt et al. 2013). As a result, the following numbers of records for each species were used: *P. mucrosquamatus* (*n* = 104); *H. latouchii* (*n* = 66); *O. tianmuii* (*n* = 34); *P. braueri* (*n* = 125); *Z. dennysi* (*n* = 74).

Table 1. Bioclimatic variables from the WorldClim 2.1 database (Fick and Hijmans 2017) used in Maxent modeling for four amphibians (*H. latouchii*, *O. tianmuii*, *P. braueri*, *Z. dennysi*) and one reptile (*P. mucrosquamatus*).

Class	Bioclimatic Variable	
Amphibian models	Bio 1	Annual mean temperature
	Bio 2	Mean diurnal temperature range
	Bio 4	Temperature seasonality
	Bio 12	Annual precipitation
	Bio 14	Precipitation of driest month
	Bio 15	Precipitation seasonality
	Bio 18	Precipitation of warmest quarter
Reptile model	Bio 1	Annual mean temperature
	Bio 2	Mean diurnal temperature range
	Bio 3	Isothermality
	Bio 7	Temperature annual range
	Bio 12	Annual precipitation
	Bio 14	Precipitation of driest month
	Bio 15	Precipitation seasonality
	Bio 18	Precipitation of warmest quarter

Environmental niche modeling was done using Maxent 3.4.4 (Phillips et al. 2017). We used a random seed with the random test percentage set to 25, a regularization multiplier of 1, and a sampling of 10,000 replicates to run 20 bootstrap replicates using the default feature classes. We also applied a jackknife analysis to estimate the relative contributions of each variable. We used the maximum training sensitivity plus specificity threshold (MTSS) generated by Maxent for each species as the threshold for suitability (Liu et al. 2013).

Results

Our environmental niche models (Fig. 3) all had moderately or very high area under the receiver-operator curve (AUC) values (Swets 1988), which is a common measure of model fit (Merow et al. 2013). The AUC values were as follows: *P. mucrosquamatus*: 0.901; *H. latouchii*: 0.961; *O. tianmuii*: 0.983; *P. braueri*: 0.899; *Z. dennysi*: 0.924. The model for *P. mucrosquamatus* predicted no suitable area above the MTSS threshold in Jiangsu, though the models for all four amphibians showed some suitable habitat in the southern half of the province.

For all five species, the precipitation of the driest month was the variable with the greatest percent contribution to the model (Table 2), contributing between 42.1% (*P. mucrosquamatus*) and 83.3% (*Z. dennysi*). Other variables that contributed more than 10% to models included annual precipitation (*P. mucrosquamatus*), mean diurnal temperature range (*H. latouchii*), annual mean temperature (*O. tianmuii* and *P. braueri*), and temperature seasonality (*P. braueri*; see Table 2).

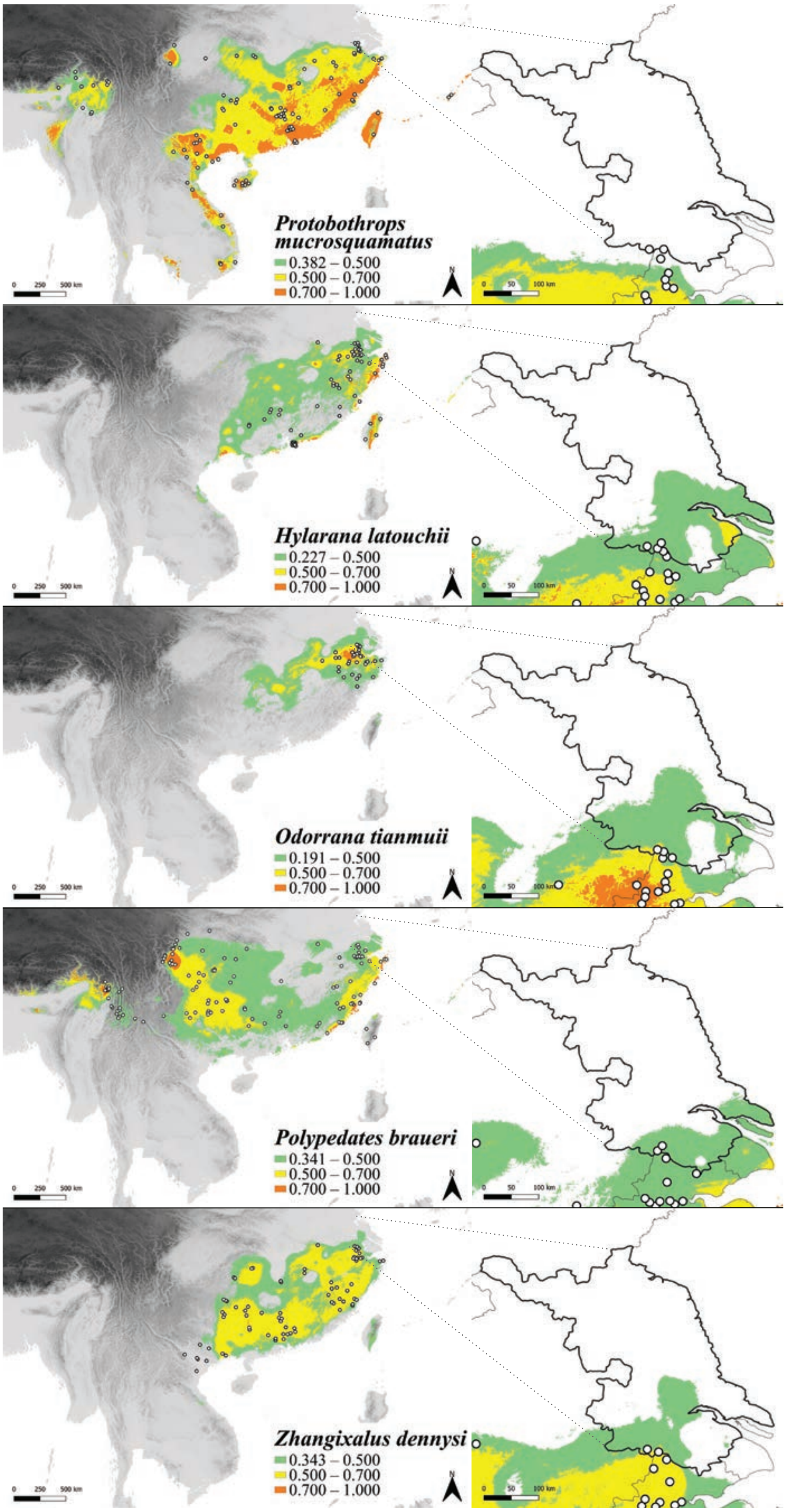

Figure 3. Full Maxent environmental niche models of the five focal species are shown on the left (elevation layer: Ryan et al. 2009) with a view of Jiangsu on the right. We used each models' maximum training sensitivity plus specificity threshold (MTSS) as the minimum threshold for suitability (green; MTSS–0.5), with higher values corresponding to medium (yellow; 0.5–0.7) and high (orange; 0.7–1) predicted climatic suitability. Locality records used in modeling are represented with white dots.

Table 2. Percent contribution of bioclimatic variables to environmental niche models for the five study species, with the maximum and minimum contributions across all 20 runs given in parentheses. Variables contributing more than 10% bolded and those not included indicated by a dash.

Variable	*P. mucrosquamatus*	*H. latouchii*	*O. tianmuii*	*P. braueri*	*Z. dennysi*
Annual mean temp. (Bio 1)	7.1 (1.4–12.4)	1.3 (0.0–4.1)	**10.6** (4.5–18.6)	**12.2** (7.7–21.8)	0.8 (0.0–2.2)
Mean diurnal temp. range (Bio 2)	9.1 (1.6–19.9)	**13.7** (1.8–24.8)	0.3 (0.0–2.3)	4.3 (0.2–16.1)	2.8 (0.5–6.1)
Isothermality (Bio 3)	2.2 (0.1–10.5)	–	–	–	–
Temp. seasonality (Bio 4)	–	0.9 (0.0–2.7)	9.9 (2.8–17.7)	**21.8** (12.5–31.7)	3.8 (1.1–11.8)
Temp. annual range (Bio 7)	2.6 (0.3–7.9)	–	–	–	–
Annual precip. (Bio 12)	**31.7** (16.8–43.8)	0.8 (0.0–3.1)	0.5 (0.0–1.6)	8.7 (2.1–16.9)	3.0 (0.0–11.3)
Precip. of driest month (Bio 14)	**42.1** (32.6–54.5)	**76.6** (68.6–87.1)	**71.8** (64.2–78.8)	**45.9** (30.4–60.0)	**83.3** (67.8–92.4)
Precip. seasonality (Bio 15)	1.5 (0.0–5.1)	0.6 (0.0–4.4)	2.5 (0.0–5.0)	5.4 (1.1–11.5)	0.2 (0–1.6)
Precip. of warmest quarter (Bio 18)	3.7 (0.1–21.2)	6.2 (3.0–12.3)	4.4 (2.6–6.7)	1.7 (0.5–6.7)	6.1 (1.2–13.3)

Discussion

All five environmental niche models had moderately high AUC values and fit our understanding of each species' contemporary range (Fig. 3). The model for *P. mucrosquamatus* predicted no suitable habitat in Jiangsu, indicating that the individual we found was likely on the very edge of the species' range. Conversely, the models for each of the four anurans predicted some suitable habitat in the southern half of the province, although for these species no specific records in Jiangsu exist north of the records we obtained. Anthropogenic impacts are a possible limiting factor in the distribution of the study species in Jiangsu, which is highly developed, and our models only included climatic conditions and did not account for human impacts (Du et al. 2014; Chuai et al. 2016). The extirpation of species such as the Chinese alligator (*Alligator sinensis*) from Jiangsu due to habitat loss and fragmentation has been documented (Zou and Chen 2002; Pan et al. 2019), and it is possible that these four anurans previously had more extensive ranges in the province. Conversely, the presence of the species could be linked to recent expansions in distribution caused by contemporary climate change (Duan et al. 2016; Biber et al. 2023). This scenario may be more likely for *P. mucrosquamatus* than the amphibians, as the field surveys were conducted during the period of the highest movement for Indomalayan snakes (Rahman et al. 2013) and our observation of this species was very close to both the edge of the province boundary and its predicted range.

In two instances, a model predicted sizable amounts of climatically suitable areas where the species likely does not occur. First, our model for *Z. dennysi* predicted a substantial amount of suitable area in the Island of Taiwan. The ocean is an obvious biogeographic barrier that may explain this absence, but if *Z. dennysi* were to be introduced to Taiwan anthropogenically, our results indicate that, at least in terms of climatic suitability, it could easily become established. Second, our model for *O. tianmuii* showed a substantial amount of suitable habitat west of the species' actual range, although this area is occupied by the closely related *O. schmackeri* (Li et al. 2017), indicating that these two species may have very similar climatic niches. Interestingly, the model for *P. braueri* strongly matched the distribution given by Kuraishi et al. (2013). While the degree of range overlap between *P. braueri* and *P. megacephalus* remains unclear, this result does lend support to recognizing the *Polypedates* in southernmost China as a distinct species (*P. megacephalus*) from those in the remainder of the country (*P. braueri*).

Surprisingly, the precipitation of the driest month (Bio 14) was the variable with the highest percent contribution in each of the five models, despite the evolutionary distance between these species. This indicates that moisture availability in the driest portion of the year is potentially a limiting factor for each of these organisms. Climate change is forecasted to have significant effects on the distribution of amphibians and reptiles (Duan et al. 2016; Biber et al. 2023), and given the importance of this bioclimatic variable for all five models, changes in rainfall patterns will likely be more influential on these species than temperature changes (Qian et al. 2007; Guo et al. 2019a).

While our field sampling efforts were opportunistic and not comprehensive, they still yielded observations of five species with few or no specific formal records from Jiangsu. This indicates a lack of previous sampling, and additional survey effort can more accurately delineate the range of these species within Jiangsu, especially considering that the models for all four anurans predicted some suitable habitat beyond our sampling sites. Furthermore, we anticipate that more sampling may yield records of previously undocumented Indomalayan reptiles and amphibians for the province. For example, our surveys did not detect *Kurixalus inexpectatus* (Rhacophoridae), a species described in 2022 in Zhejiang in mountains contiguous with our sampling sites (Messenger et al. 2022). We did not attempt to model the distribution of *K. inexpectatus* due to insufficient locality records (van Proosdij et al. 2016), but consider it likely to be found in Jiangsu in the future.

While species distribution models can be generated solely from the vast amounts of existing publicly available data, models are most effective when they both inform and are informed by field efforts. Environmental niche modeling can guide field surveys to be conducted in areas where target species, or even new species, are likely to be found (Rhoden et al. 2017; Sarker et al. 2019). Conversely, obtaining additional occurrence data, especially data that extend the known range of a species, improves the accuracy of models at present as well as past and future scales (Qin et al. 2017; Zhang et al. 2019; Yousaf et al. 2022). The positive feedback loop between modeling and field sampling should be utilized to improve conservation efforts and deepen ecological knowledge of species.

Authors contributions

Conceptualization: AB and DBK; Investigation: all authors; Methodology: XZ, KRM, and DBK; Supervision: AB; Writing - original draft: DBK; Writing - review and editing: SNO, DG, AB, DBK, KRM, XZ, HA, VKP; Visualization: DBK and KCYA.

Acknowledgements

This work was supported by the Foreign Youth Talent Program (QN2023014004L) from the Ministry of Science and Technology of the People's Republic of China to AB. We also thank two anonymous reviewers and the editor for their helpful comments on a previous version of this manuscript.

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Supplementary material 1

Records for *Protobothrops mucrosquamatus*, *Hylarana latouchii*, *Odorrana tianmuii*, *Polypedates braueri*, and *Zhangixalus dennysi*

Authors: Dallin B. Kohler, Xiaoli Zhang, Kevin R. Messenger, Kenneth Chin Yu An, Deyatima Ghosh, Siti N. Othman, Zhenqi Wang, Hina Amin, Vishal Kumar Prasad, Zhichao Wu, Amaël Borzée

Data type: xlsx

Link: https://doi.org/10.3897/herpetozoa.37.e117370.suppl1

OGH Herpetozoa
Austrian Herpetological Society

Herpetozoa 37: 95–105 (2024)
DOI 10.3897/herpetozoa.37.e116071

Hiding in the valley: a new national record of *Ablepharus eremchenkoi*, with rediscovery of *Ablepharus alaicus* in China: phylogeny, morphology and natural history notes

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https://zoobank.org/1B80F815-2554-477E-848E-0E46FADD626C

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Academic editor: Philipp Wagner ♦ **Received** 22 November 2023 ♦ **Accepted** 29 March 2024 ♦ **Published** 10 April 2024

Abstract

The genus *Ablepharus* Lichtenstein, 1823 contains the common snake-eyed skinks, distributed from southern Europe and northern Africa to eastern Asia. *Ablepharus alaicus* Elpatjevsky, 1901 inhabits Central Asia and, according to historical literature, was once recorded in north-western China. However, there are no voucher records of this species from China. Some populations of a subspecies of *A. alaicus* have been elevated to new species, for example, *A. eremchenkoi* (Panfilov, 1999). However, no detailed studies have been conducted. In August and September 2023, we captured sixteen and fourteen skink specimens from Wuqia County and Qapqal Xibe Autonomous County, respectively, in Xinjiang, northwest China. Morphological and phylogenetic comparisons showed that the skinks collected from these two locations belong to *A. eremchenkoi* and *A. alaicus*, respectively. In this study, we confirmed the first record of *A. eremchenkoi* in China, rediscovered *A. alaicus*, reported voucher records for these two skinks and reviewed the taxonomic history of *Ablepharus* in Xinjiang, northwest China.

Key Words

Ablepharine skink**,** Kyrgyzstan, Kazakhstan, voucher records, Xinjiang

Introduction

The Ablepharine is a widespread skink lineage distributed from Europe to Asia (Vaissi et al. 2023). *Asymblepharus* Eremchenko & Shcherbak, 1986 and *Himalblepharus* Eremchenko, 1987 were established as separate genera from *Ablepharus* Lichtenstein, 1823, based on morphological divergence. In a recent revision of Ablepharine skinks, Mirza et al. (2022) proposed treating *Asymblepharus* and *Himalblepharus* as subjective junior synonyms of *Ablepharus* because both are embedded within *Ablepharus*, which does not have a movable eyelid. This returns species belonging to these two genera to *Ablepharus sensu lato*, including *Asymblepharus alaicus*, which are mainly distributed in Central Asia in Kyrgyzstan, Uzbekistan, Tajikistan, Kazakhstan and China.

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[*] These authors contributed equally to this study.

There are three subspecies of *A. alaicus*: *A. a. alaicus* Elpatjevsky, 1901; *A. a. kucenkoi* Nikolsky, 1902; and *A. a. yakovlevae* Eremchenko, 1983. Panfilov (1999) evaluated some populations of *A. a. yakovlevae* as *A. eremchenkoi*, based on variations in reproductive traits. Two subspecies were thought to be distributed in China (Zhao et al. 1999). Of these, *A. a. kucenkoi* was distributed in the Ili Region of Xinjiang; this record was based on two specimens not preserved in China (Eremchenko and Shcherbak 1986). *A. a. alaicus* was thought to be distributed near the south-western Xinjiang border close to Kyrgyzstan and Tajikistan, but no voucher specimens are available to support this distribution. Notably, historical records of *A. alaicus* in China have not been confirmed.

In 2022 and 2023, we observed skinks in Wuqia County, Kizilsu Kirgiz Autonomous Prefecture (KKAP) and Qapqal Xibe Autonomous County, Ili Kazakh Autonomous Prefecture (IKAP), Xinjiang, China, locations in which we had not previously observed them. We subsequently captured skinks from these two locations and conducted morphological and phylogenetic analyses. The results suggest that the skinks from KKAP belonged to *A. eremchenkoi*, a new record for this species in China. The skinks from IKAP were *A. alaicus*, confirming the distribution of *A. alaicus* in Xinjiang, China. In this study, we present the first known records of *A. eremchenkoi* and *A. alaicus* with voucher specimens from within China. In addition, we provide detailed descriptions of the morphology, phylogeny and natural history notes.

Materials and methods

Species delimitation

On 8 and 9 August 2023, we collected sixteen specimens from three locations in Yuqitashi (40.1527°N, 74.6310°E, 3040 m elev.; 40.1495°N, 74.6335°E, 3032 m elev.; 40.13°N, 74.64°E, 3014 m elev.), Wuqia County, KKAP and Xinjiang, China (Fig. 1). On 27 September 2023, we collected fourteen specimens close to Baishifeng (43.43°N, 81.05°E, 2466 m elev.), Qapqal Xibe Autonomous County (IKAP) (Fig. 1). All thirty specimens were transported to Xinjiang Agricultural University.

All specimens were euthanised and muscle or liver samples were dissected from the specimens, preserved in 95% ethanol and stored at –20 °C. All specimens were fixed in 10% buffered formalin and transferred to 75% ethanol for preservation. Liver and muscle tissues used for molecular analysis were preserved in 95% alcohol at –20 °C. All specimens were deposited at the Herpetological Museum, Xinjiang Agricultural University (XJAU), Urumuqi, Xinjiang, China.

Sequence extraction

Total DNA was extracted from tissue samples by using the Foregene Animal Tissue Genomic DNA Extraction Kit per the manufacturer's instructions. We referred to

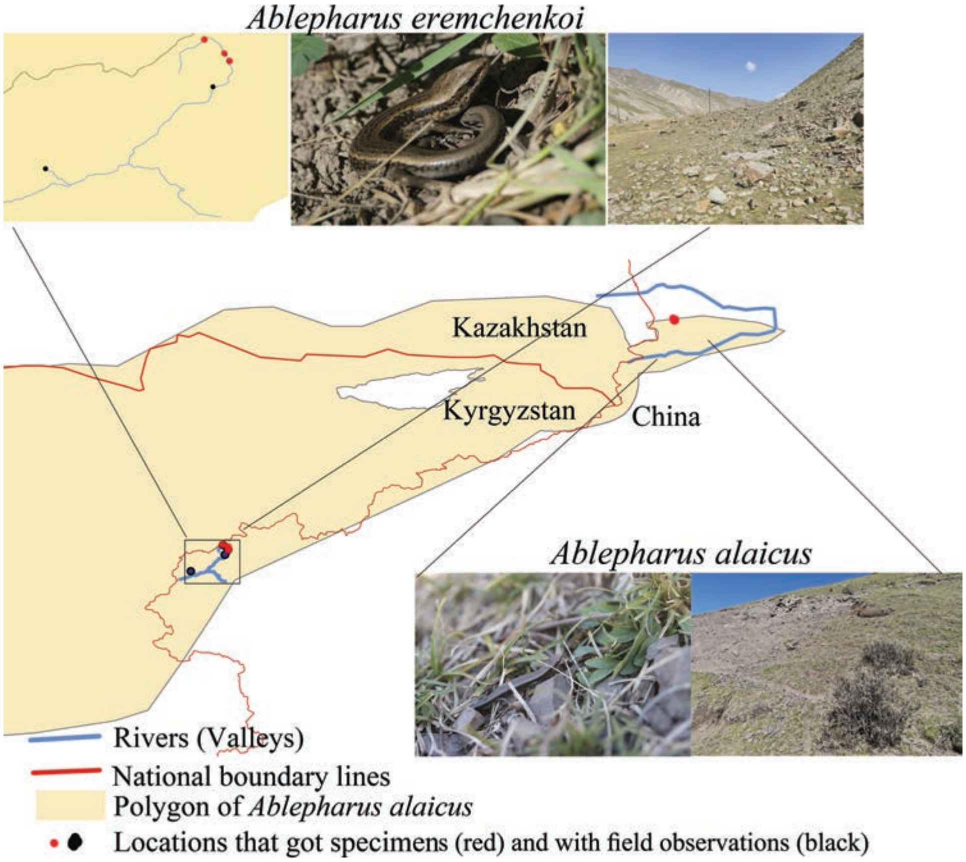

Figure 1. Map showing the distribution of *A. eremchenkoi* and *A. alaicus* within China. The distribution map of *A. alaicus* was obtained from the IUCN Red List (Accessed on 17 September 2023). Photos by Lei Shi, Lin Leng and Tao Liang.

the primer sequences used by (Mirza et al. 2022). Additionally, we sequenced partial segments of the mitochondrial 16S rRNA (16S), 12S rRNA (12S) and NADH dehydrogenase subunit 2 (ND2) (Tables 1, 2). The PCR reaction volume was 25 µl, containing 1 µl template DNA, 1 µl each of upstream and downstream primers, 12.5 µl 2× Taq PCR Mix and 9.5 µl ddH_2O. The PCR protocol used for amplification was as follows: 95 °C for 3 min, (denaturation temperature 95 °C for 30 s, annealing time ranged from 40 to 50 s, elongation temperature 72 °C for 1 min) × 36 cycles, 72 °C for 10 min, hold at 4 °C (Mirza et al. 2022). Gel electrophoresis was performed using 0.5% TBE solution and agarose to verify successful amplification of the samples. Subsequently, the successfully amplified PCR stock solution was sent to Sangon Biotechnology for purification and sequencing.

Molecular data and analyses

All sequences were aligned and manually edited using SeqMan in DNASTAR (Burland 1999). Sequences were aligned in Mega 7.0 (Kumar et al. 2016) using ClustalW (Thompson et al. 1994) with default settings. Ultimately, a 12S sequence of approximately 356 bp, a 16S sequence of 389 bp, an ND2 sequence of 509 bp and a spliced sequence of 1323 bp were obtained. We collected reference sequences of the same genus from GenBank, with *Scincella reevesii* as the outgroup and constructed a phylogenetic tree of *Ablepharus* and its species within the genus by using Bayesian Inference (BI) and Maximum Likelihood (ML) to explore the affinities of the sample sequences in *Ablepharus*. The BI and ML analyses were performed using MrBayes and IQ-TREE in PhyloSuite (Zhang et al. 2020) with default parameters, respectively. The resulting phylogenetic tree was visualised using FigTree v.1.4.3 and the effective sample size (ESS) was assessed using Tracer 1.7.2 (Rambaut et al. 2018), with all ESS values for parameters > 200. Finally, the phylogenetic tree was visualised using ITOL v.6 (Letunic and Bork 2021). A posteriori probability (BPP) or ML bootstrap value (BS) > 95% was considered strong support for monophyly.

We used *p*-distance (uncorrected) in MEGE 7.0 (Kumar et al. 2016) to calculate the genetic distances of the three sequences: 12S, 16S and ND2. We divided the sample and reference sequences into groups by using MEGA and *p*-distance to calculate genetic distances amongst the three gene sequences.

Table 1. Localities and GenBank accession numbers for all species used in this study.

Species	Country	ND2	16S	12S	(ID) References
Ablepharus					
A. eremchenkoi	China	OR687189	OR681490	OR677923	(XND20230808001) This study
A. eremchenkoi	China	OR687188	OR681492	OR677924	(XND20230809010) This study
A. eremchenkoi	China	OR687190	OR681491	OR677925	(XND20230808007) This study
A. eremchenkoi	China	OR687191	OR681493	OR677926	(XND20230808023) This study
A.alaicus	China	OR687182	OR681482	OR677917	(XND2023092701) This study
A.alaicus	China	OR687183	OR681483	OR677918	(XND2023092706) This study
A.alaicus	China	OR687184	OR681484	OR677919	(XND2023092711) This study
A.alaicus	China	OR687185	OR681485	OR677920	(XND2023092712) This study
A.alaicus	China	OR687186	OR681486	OR677921	(XND2023092713) This study
A.alaicus	China	OR687187	OR681487	OR677922	(XND2023092714) This study
A. alaicus	Kyrgyzstan	MZ820276	MZ790578	MZ790566	(Mirza et al. 2022)
A. anatolicus	Turkey	MZ848096	–	MZ827906	(Mirza et al. 2022)
A. bivittatus	Iran	–	MZ707375	MZ707427	(Karamiani et al. 2021)
A. budaki	Syria	–	AY561427	MZ827907	(Poulakakis et al. 2005; Mirza et al. 2022)
A. chernovi	Turkey	–	JX847534	–	(Poulakakis et al. 2013)
A. deserti	Kyrgyzstan	MZ820278	MZ790580	MZ790568	(Mirza et al. 2022)
A. deserti	China	–			This study
A. eremchenkoi	Kyrgyzstan	MZ820277	MZ790579	MZ790567	(Mirza et al. 2022)
A. grayanus	–	–	MZ707422	MZ707474	Karamiani et al. 2021
A. himalayanus	China	MN885892	MN885892	MN885892	
A. kitaibelii	Greece	MZ848097	AY561380	MZ827908	(Mirza et al. 2022)
A. ladacensis	China	MW111453	–	MZ790569	(Xu et al. 2021; Mirza et al. 2022)
A. mahabharatus	Nepal	MZ820282	MZ790598	MZ790570	(Mirza et al. 2022)
A. nepalensis	Nepal	MZ820286	MZ790602	MZ790574	(Mirza et al. 2022)
A. pannonicus	Uzbekistan	MZ820287	MZ790584	MZ790575	(Mirza et al. 2022)
A. rueppellii	Israel	MZ848098	KX591472	MZ827909	(Skourtanioti et al. 2016; Mirza et al. 2022)
A. sikimmensis	Nepal	MZ820283	MZ790601	MZ790573	(Mirza et al. 2022)
Protoblepharus					
P. apatani	India	MZ820288	MZ790586	MZ790576	(Mirza et al. 2022)
P. medogensis	China	MW111454	–	–	(Che et al. 2020)
P. nyingchiensis	China	MW183282	–	–	(Che et al. 2020)
Outgroup					
Scincella reevesii	China	NC054206	NC054206	NC054206	(Zhong et al. 2021)

Table 2. Details of the primers used in the study for PCR amplification and sequencing (Mirza et al. 2022).

Gene	Primer name	Primer sequence (5′-3′)
16SrRNA	16Sa	CGCCTGTTTATCAAAAACAT
	16Sb	CCGGTCTGAACTCAGATCACGT
12S rRNA	12Sa	AAACTGGGATTAGATACCCCACTAT
	12Sb	GAGGGTGACGGGCGGTGTGT
ND2	H4980_edite	ATTTTGCGTGTTTGTGTTTGGT
	L4437	AAGCTTTCGGGCCCATACC

Morphological data and analyses

Measurements of seventeen morphological characteristics, selected from published literature, were recorded to the nearest 0.1 mm using digital calipers from Jiang-miao Ran (Zhao et al. 1999; Qi et al. 2022). These characteristics were: snout-vent length (SVL), distance from tip of snout to vent; head length (HL), distance from the tip of the snout to the posterior border of the collar; head width (HW), distance across the widest point of the head; head depth (HD), highest point of the head; axilla-groin distance (AG), distance between posterior edge of fore-limb insertion and anterior edge of hind-limb insertion; fore-limb length (FLL), distance from fore-limb insertion to the longest digit; hind-limb length (HLL), distance from hind-limb insertion to the longest digit; tail length (TL), distance between the cloaca and the tail top; eye diameter (ED); eye-narial distance (END), from anterior margin of eye to posterior margin of nares; internarial distance (IND), distance between the nares, supraocular count (SC); supralabial count; (SL), supralabial count before eyes; ventral count (VC), number of latitudinal scale columns from the midpoint of the fore-limb base to the cloaca; toe IV lamellae count (T4lam), number of enlarged, undivided lamellae beneath Toe IV; mid-body scale-row count (MBSR), number of longitudinal scale rows measured around the mid-body; and neck scale-row count (NSR), number of longitudinal scale rows measured around the neck.

Data availability statement

All data used in this note can be found in the supporting information.

Results

ML and BI phylogenetic trees were constructed, based on three mitochondrial genes (12S, 356 bp; 16S, 457 bp; ND2, 509 bp) from twenty species, with a total length of 1323 bp. The ML and BI analyses resulted in largely identical topologies (Fig. 2).

Ablepharus was monophyletic (BI/ML:1/98) in the phylogenetic tree, forming a sister clade with *Protoblepharus*. The sample sequences of Groups IKAP and KKAP were clustered into a single clade, forming a strong monophyletic group, Group IKAP, BI/ML:1/100; Group KKAP, BI/ML:1/100. Group KKAP clustered with *A. eremchenkoi* with strong support (BI/ML:1/100). The *A. alaicus* clade formed a sister clade with *A. nepalensis*, *A. mahabharatus* and *A. sikimmensis* (BI/ML:0.58/60).

The uncorrected *p*-distance, based on 12S sequences, was up to 20%; based on 16S sequences, it was up to 20%; and, based on ND2 sequences, it was up to 27%. The uncorrected *p*-distance between Group KKAP and *A. eremchenkoi* was 1%/2%/2% in the 12S/16S/ND2 sequences, respectively and was less than 10% for *A. alaicus* 1, from Kyrgyzstan, 12S/16S/ND2: 4%/4%/9%. The uncorrected *p*-distance between Group IKAP and *A. alaicus* 2, from Kazakhstan, was 4% for the ND2 sequences. The results of the genetic distances (Suppl. material 2) were consistent with the results of the phylogenetic analyses (Fig. 2), where the Groups IKAP and KKAP were clustered into a branch with *A. eremchenkoi* and *A. alaicus* and the genetic distances of all four (Groups IKAP, Groups KKAP, *A. eremchenkoi* and *A. alaicus*) were lower than those of other species of the same genus, indicating the closeness of the relationship between them. All newly-collected specimens were largely similar to specimens of the original description of *A. eremchenkoi* and *A. alaicus* (Tables 3–5). Thus, we report the rediscovery of *A. alaicus* and specimens from the KKAP (*A. eremchenkoi*) as a new record in China.

Description of specimens from China

Ablepharus eremchenkoi (Panfilov, 1999)

Chinese names. 叶氏泛蜥 (Yè Shì Fàn Xī).

Description of specimens from China. The sample size comprised 16 specimens, all collected by Lei Shi, Jing An and Tao Liang. The main description of this species is based on the male specimen (XND0808007; Figs 3, 4) whose tail had been naturally regenerated. Data and descriptions of the three female specimens (XND0808001, 002 and 005) are provided in parentheses in the following text (if different). The data available for the four voucher specimens are listed in Table 3.

Morphologies of the remaining specimens were similar to those four adult specimens; these data are in Suppl. material 3.

The recorded characteristics of the specimen were as follows: body was small, nearly uniform in thickness, with SVL 46.5 mm and mass 2.04 g and slender (BW/SVL ratio 0.11) with an elongated trunk (AG/SVL ratio 0.53); imbricate scales were smooth and glossy; snout was slightly pointed; head was small and longer than it was wide (HL 9.6 mm, HW 6.6 mm, HD 4.9 mm); eyes were small; ED external ear opening was small with obviously projecting lobules; END was 3.4 mm; fore-limbs and hind-limbs were relatively short, the fore-limb was shorter than the hind-limb (FLL/HLL ratio 0.79) and the tips of the digits of the fore-limb and hind-limb met when

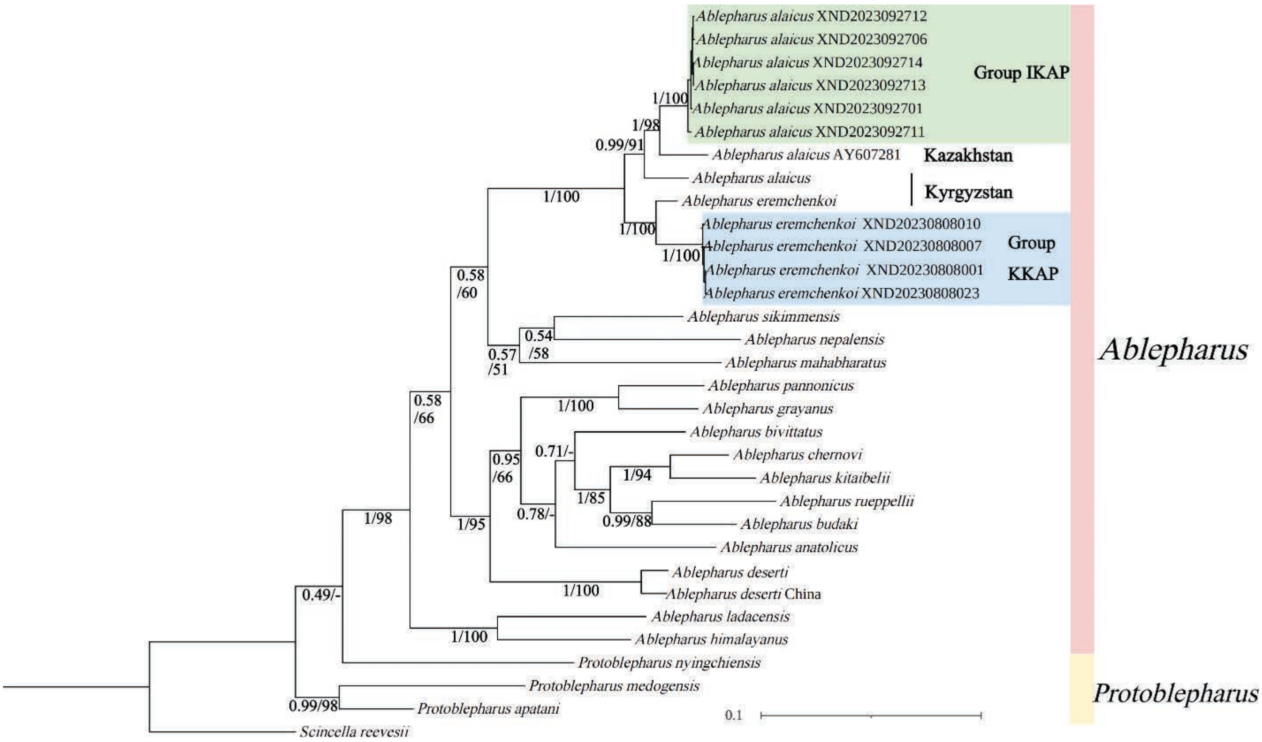

Figure 2. Bayesian phylogenetic trees depicting the relationships amongst *Ablepharus* species using tandem sequences (12S, 16S, ND2). (Note: The values of nodes nearby indicate BI/ML).

Table 3. Measurements (mm) and scale counts of adult *Ablepharus eremchenkoi* from Xinjiang, China. See Materials and Methods for abbreviations. * indicates dropped or regenerated tail.

ID	Sex	BM	SVL	HL	HW	HD	MW	ED	END	IND	AG
XND0808001	Female	3.173	54.2	9.3	6.1	4.2	5.8	1.6	2.1	1.7	29.4
XND0808002	Female	2.348	47.9	9.2	5.1	3. 9	4. 9	1.4	2.8	1.2	29.3
XND0808005	Female	2.381	47.6	8.2	5.5	3.6	5.2	1.3	3.2	1.4	27.9
XND0808007	Male	2.041	46.5	9.6	6.6	4.9	5.9	1.5	3.4	1.5	24.6
	AW	**FLL**	**HLL**	**TL**	**SC**	**VC**	**SL**	**T4lam**	**MBSR**	**NSR**	
XND0808001	9.45	11.59	15.11	40.5	2	46	4	19	26	29	
XND0808002	8.8	12.28	16.01	44.2	2	46	4	19	26	25	
XND0808005	7.96	11.16	15.97	39	2	48	4	16	26	30	
XND0808007	6.23	12.66	15.94	35.3+	2	43	4/5	17	26	26	

Table 4. Descriptive statistics for female reproductive traits of *Ablepharus eremchenkoi*.

ID	Date	Post-oviposition Body Mass (g)	Litter Size	All Litter Mass (g)
XND0808001	20230811	2.13	4	1.04
XND0808002	20230824	1.62	2	0.52
XND0808005	20230825	1.84	2	0.52

the limbs were adpressed against each other along the body axis (except for XND20230808001); the tail was broken, but had regenerated and the regenerated tail was narrower than the body (4.6 mm cf. 6.2 mm) and was shorter than SVL (35.3 mm cf. 46.5 mm) despite tails generally being longer than SVL.

The width of the rostral was greater than its height and it was in contact with the first supralabials, nasals,and fronto-nasal. Nostrils were circular and located at the centre of the nasal cavity. Frontal, fronto-nasal and a pair of prefrontals were connected to a point (seven of sixteen individuals); four of sixteen individuals' prefrontals were widely in contact with each other and frontal and frontal-nasal were separate from each other; three of sixteen individuals' frontal and frontal-nasasl were widely in contact with each other, prefrontals were separate from each other; two of sixteen individuals had three prefrontals, which made frontal and frontal-nasal separate from each other. Prefrontal fan-shaped, a pair of prefrontals were in contact with the postnasal, loreal and first supraocular. A large single frontal, irregularly wedge-shaped, was in broad contact with the third and fourth supraoculars and a pair of frontoparietal posterolaterally. Frontoparietal were widely in contact with parietal and interparietal scales and third and fourth supraoculars. The interparietal rhomboid was posteriorly in contact with parietals. Parietals were anteriorly in contact with frontoparietal, interparietal and fifth supraocular and were laterally touching

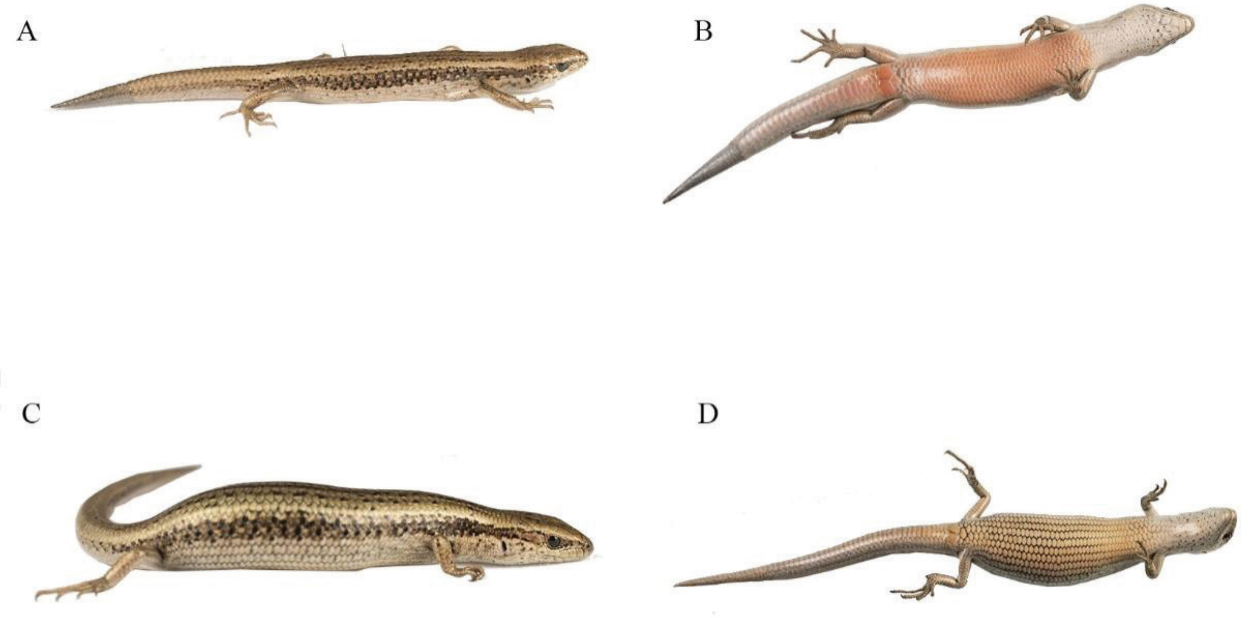

Figure 3. *Ablepharus eremchenkoi* male (**A, B**) and female (**C, D**) in life. Photos by: Tao Liang.

Table 5. Measurements (mm) and scale counts of juveniles of *Ablepharus eremchenkoi*. See Materials and Methods for abbreviations.

ID		BM	SVL	HL	HW	HD	MW	ED	END	IND	AG
XND0808001-1		0.27	22.7	5.7	3.8	2.4	3.5	1.2	1.7	0.97	11.1
XND0808001-2		0.26	22.2	6.1	3.5	2.6	3.2	1.1	1.7	1.04	12.7
XND0808001-3		0.26	23	5.5	3.7	2.5	3.2	1.1	2.1	1.16	13.5
XND0808001-4		0.25	21.7	6.3	3.7	2.4	3.3	1.3	1.9	1.1	11.6
XND0808002-1		0.26	22.5	5.4	4.2	2.7	3.6	0.9	1.3	1.09	12.7
XND0808002-2		0.27	24.1	5.5	3.7	2.5	3.2	1.1	1.5	1.01	12.3
XND0808005-2		0.27	23.5	5.2	3.5	2.4	3.4	1.1	1.9	1.01	11.4
XND0808005-1		0.25	23.4	5.3	3.7	2.2	3.4	1.1	1.9	1.03	11.1
	AW	**FLL**	**HLL**	**TL**	**SC**	**VC**	**SL**	**T4lam**	**MBSR**	**NSR**	
XND0808001-1	2.6	7.5	8.9	25.0	2	45	4	19	29	27	
XND0808001-2	2.8	6.5	8.7	25.8	2	42	4	18	27	28	
XND0808001-3	2.9	7.7	9.8	25.6	2	45	4	19	27	26	
XND0808001-4	3.2	7.9	9.7	26.7	2	43	4	19	29	28	
XND0808002-1	2.8	7.9	10.1	26.3	2	48	4	17	28	27	
XND0808002-2	3.2	7.4	10.2	19.4	2	46	4	19	28	28	
XND0808005-1	3.3	8.1	9.7	26.7	2	40	4	19	28	28	
XND0808005-2	3.4	8.1	9.3	25.3	2	42	4	19	27	28	

the upper posterior temporals. Three supraoculars and the eyes were surrounded by a circle of tiny irregular scales. There were four scales between the nasal cavity and eyes and one individual (XND20230808019) had five scales. For seven supralabials, there was a tiny supraocular between the second and third scales (Figs 3, 4) on the right side (Fig. 4) and seven infralabials. The mental was wider than it was long and was in contact with the first infralabial laterally, postmental posteriorly. Postmental was large and single; four pairs of large chin-shields were present, with the first pair in contact and the second pair narrowly separated by a single medial scale. Dorsal scalation was homogeneous with four columns; longitudinal scale rows were at mid-body 26 (25–29). Twenty-six scales were around the middle of the neck. The number of ventral scales was 43 (46–48). The lengths of the digits (measurements in mm in parentheses) were as follows: left manus IV (2.84) > III (2.69) > II (1.82) > V (1.46) > I (0.98); left pes IV (4.99) > III (3.46) > V (2.31) > II (1.96) > I (1.07). Toe IV lamellae 17.

Colouration in life: Overall, in the one male, the dorsal was coppery brown; dark longitudinal spots were present on the edges of scales and generated three irregular black lines continuing on to the tail. White dots were grouped into six irregular lines along the back of them; the two external dots merged into light lines on the dorsal sides (Fig. 4). The lines on the dorsal sides began at the nasal base until the tail base and they were filled with rare light dots (Fig. 4). The bottom half of the dorsal side was white. The male abdomen was orange-red to the tail, but not the regenerated tail. Females and males were coloured similarly, but the abdomen was paler for females than males;

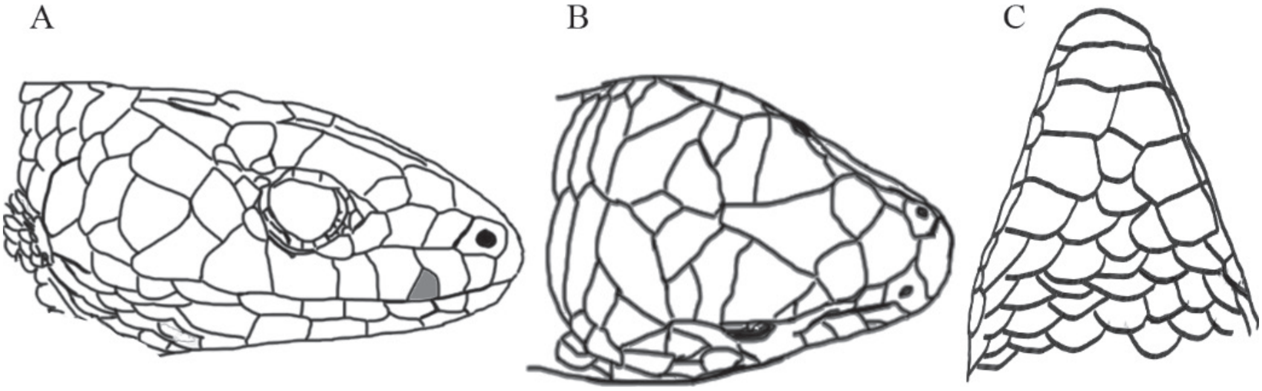

Figure 4. Schematic representation of head scalation of *Ablepharus eremchenkoi*. **A**. Lateral view; **B**. Dorsal view; **C**. Ventral view. The plots were based on the male (XND0808007), the grey scale was the mutational scale between the second and third scales on the right side.

the outline of ventrals was black; and subadults and juveniles had abdomens similar to females, but without the orange-red colour.

Activity, habitats and distribution. All 16 specimens were collected during the day: 16:00–18:00 h and 11:30–13:00 h. According to the residents, these regions receive snow from September to May; thus, the activity times ranged probably from May to August. These individuals were collected at the bottom of a hill, from under rocks and some individuals were collected from riverbeds, 40.20°N, 74.56°E, 3133 m elev., (observations from Ya-hui Huang). This species was observed in Wuqia County, China. Except for Yuqitashi, where we obtained the specimen, this species has been observed in Kalatashi, at 40.0559°N, 74.5941°E and 3004 m elev. and in Jigen Village, at 39.82°N, 74.1069°E and 2709 m elev., identified by images provided by Ya-hui Huang and Jin-Xin Gu, respectively. All individuals were located in a continuous valley (Fig. 1), with altitude ranging from 2709 m to 3133 m (Fig. 1).

Reproduction and diet. Viviparity. Of these individuals, three were gravid females, one female (XND0808001) laid four litters on the morning of 11 August 2023 and two females (XND0808002 and XND0808005) laid two litters on 23 and 24 August (Tables 4, 5) in the laboratory. On average, for these young, weight was 0.26 g, SVL was 22.9 mm and TL was 25.2 mm (Suppl. material 3). Juveniles were coloured and morphologically similar to adults, but had no orange-red colour on their abdomens. The diet of this species remains poorly understood, but they are thought to be carnivorous.

Ablepharus alaicus Elpatjevsky, 1901

Chinese names. 阿赖山泛蜥 (Ā Lài Shān Fàn Xī).

Description of specimens from China. The sample size comprised 14 specimens, all specimens were collected by Peng Ding, Lin Leng and Ke-fan Wu. The main descriptions of this species were based on one specimen (XND2023092704). Additional descriptions were based on the other 13 specimens (in parentheses). All specimen morphological data is in the Suppl. material 3.

The body was small and nearly uniform in thickness, SVL 60.4 mm (26.1–51.1 mm); body mass was 2.93 g (0.29–2.24 g); eyes were small, ED 1.77 mm (1–1.5 mm); END 2.9 mm (1.7–3.3 mm); IND 2.37 mm (1.1–2.3 mm); the head was small, but longer than its width or depth, HL 11.97 mm (6.2–12.5 mm); HW 7.28 mm (3.8–6.9 mm); HD 6.5 mm (2.5–4.7 mm); AG 34.13 mm (14.1–33.1 mm); body was slender (BW/SVL ratio 0.17, 0.12–0.17) with an elongated trunk (AG/SVL ratio 0.56, 0.45–0.64); tail, broken or regenerated tails were excluded, was not as wide (TBW, 5.2 mm, 2.3–5.2 mm) as the trunk, but was longer (64.5 mm, 24.7–55.4 mm) than SVL (TL/SVL ratio 1.06, 0.94–1.17). Limbs were short, FLL 12.53 mm (8.1–12.7 mm) and HLL 16.45 mm (9.5–15.8 mm); the tips of the digits of the fore-limb and hind-limb did not meet each other when the limbs were adpressed against each other along the body axis, but for twelve of fourteen individuals they did meet each other. The lengths of the digits (measurements in parentheses) were as follows: left manus IV (2.68) > III (2.65) > II (1.91) > V (1.6) > I (1); left pes IV (5.07) > III (3.26) > V (2.8) > II (2.15) > I (0.95).

The rostral was single; wider than it was high; and was in contact with the first supralabials, nasals and frontonasals. The nasal rhomboid comprised circular nostrils, located at the centre of the nasal cavity. Fronto-nasal was fan-shaped and connected to the prefrontals. Prefrontals were pentagonal, a pair of prefrontals were connected with a border between them, located between the fronto-nasal and frontal (seven of fourteen individuals); three of the 14 individuals had frontals, fronto-nasals and a pair of prefrontals connected by a point; 3 of the 14 individuals had the frontal and frontal-nasal widely in contact with each other and the prefrontals were separate from each other. The frontal was wedge-shaped, which contacts with the prefrontals, the third and fourth supraoculars and a pair of frontoparietals posterolaterally. Six of the 14 individuals had frontals in contact with fronto-nasals, the prefrontals were not in contact with each

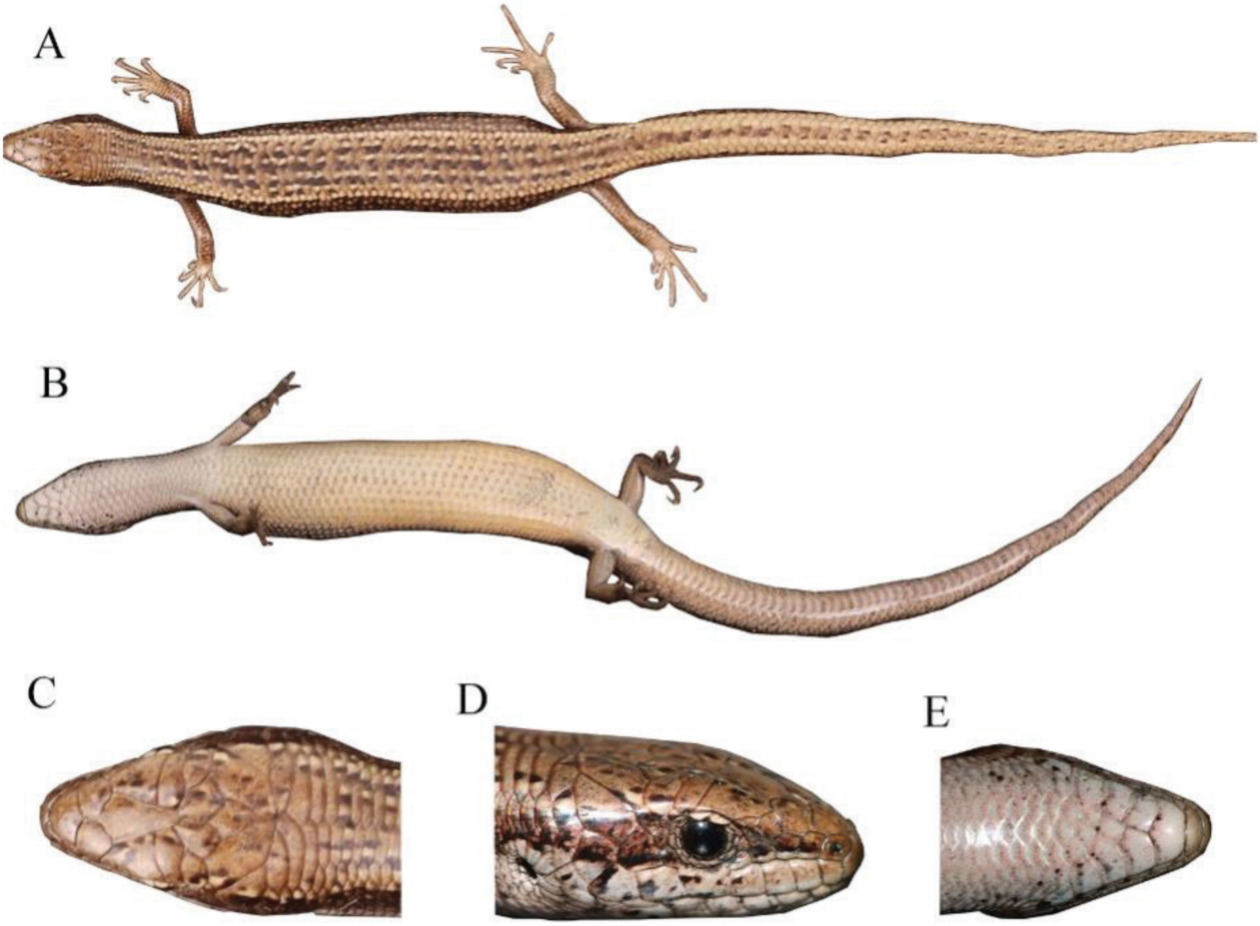

Figure 5. The general aspect and close-up views of *Ablepharus alaicus* (XND2023092704) in life from Qapqal Xibe Autonomous County, Ili Kazakh Autonomous Prefecture, Xinjiang, China. **A.** Dorsolateral view of body; **B.** Ventral view of body; **C.** Dorsal view of head; **D.** Right side view of head; **E.** Ventral view of head. Photos by: Wei-Zhen Gao.

other. The second supraocular region, in contact with the frontal and prefrontal regions, was a single tiny supracular hexagon, between the second supraocular and prefrontal regions. A pair of frontoparietals were in broad contact with each other; besides each frontoparietal was in contact with the frontal, third, fourth supraoculars, the parietal and interparietal. The interparietal rhomboid, in contrast with the frontoparietals, was posteriorly in contact with the parietals. A pair of parietals contact each other; additionally, each parietal was in contact with the interparietal, frontoparietal, fourth supracular and temporals. There were three scales between the nasal cavity and eyes; 10 of the individuals had at least four scales on one side. There were seven supralabials, four loreals between the nasal and eyes and a fourth tiny loreal. The specimen had seven infralabials; three individuals had six infralabials on each side. The temporal 1+2 and the second subtemporal were large and trapezoidal. The mental was wider than long, in contact with the first infralabial laterally and postmental posteriorly. There was a single, large postmental with four pairs of large chin-shields; the first pair was in contact with the second pair narrowly separated by a single medial scale. Dorsal scalation was homogeneous with four columns; there were longitudinal scale rows at mid-body 25 (25–28). There were 28 (25–29) scales around the middle of the neck and 50 (42 –51) ventral scales. There were 17 toe IV lamellae.

Colouration in life. The back was coppery brown, with dark longitudinal spots on the edges of the scales, which generated three black lines continuing on-to the tail; there were white longitudinal spots in the middle of the scales, generating three irregular lines continuing to the back of the tail base. A dark sooty area on each side was sharply defined above, but faded below the belly. The dark sooty area began after the nasal cavity and ended at the middle of the tail (Fig. 5). The abdomen of the females was slightly orange-red during the breeding seasons.

Reproduction, activity, habitats, diet and distribution. Viviparity. All fourteen specimens were collected during the day, from 12:00 to 18:00 h; therefore, this species appears to be diurnal. These individuals were collected at the bottom of a hill at an altitude of 2466 m and the microhabitats were covered with shrubs and gravel. Their diet remains poorly understood, but they are thought to be carnivorous. This species has been observed in Qapqal Xibe Autonomous County, China and probably in adjacent Zhaosu County, which, along with Tianshan, has populations that are connected to those in Kazakhstan (Fig. 1).

Discussion

There have been long-standing questions on the generic taxonomy of Ablepharine skinks (Grismer et al. 2019; Mirza et al. 2022). Nineteen species were included in the genus *Ablepharus* (Uetz et al. 2023). Without molecular data, the genera *Ablepharus*, *Asymblepharus* and *Himalblepharus*, were distinguished using morphological variation which was widely accepted. Our results showed that the latter two genera are embedded within *Ablepharus*, which is consistent with Mirza et al. (2022). The distribution of *A. alaicus* in China was uncertain, although studies and the IUCN have suggested it is distributed in the western Xinjiang border regions (Zhao et al. 1999; Shestopal et al. 2019). Based on the phylogenetic tree of the three sequences (12S, 16S and ND2), our samples, Groups IKAP and KKAP, were close to *A. eremchenkoi* and *A. alaicus*, respectively. Additionally, per the results of the morphological comparison, we identified these two groups as *A. eremchenkoi* and *A. alaicus*.

The distribution of *A. a. kucenkoi* is around north-eastern Tianshan, including south-eastern Kazakhstan, north-eastern Kyrgyzstan and the Ili Valley of Xinjiang (Eremchenko and Shcherbak 1986; Zhao et al. 1999). Group IKAP clustered with *A. alaicus* (AY607281), which was collected from Kazakhstan (BI/ML:1/98, Fig. 2) and with samples from similar altitudes (2466 m vs. 2000 m, respectively). Therefore, the taxon collected from IKAP is likely *A. a. kucenkoi*. However, we found intrapopulation variations in the positions of the prefrontal, fronto-nasal and frontal regions. Six of fourteen (~ 42%) specimens' frontal and fronto-nasal borders were connected. Eight of fourteen (52%) of the specimens were not connected because the prefrontals were connected at the border, but the middle temporal was large and trapezoidal, consistent with *A. a. kucenkoi*. Eremchenko and Shcherbak (1986) demonstrated that approximately 9% of the specimens had prefrontals connected at the border. Such intrapopulation variations in the positions of these scales were also observed in *A. deserti* (Shi et al. 2006). Populations from south-western Xinjiang were assumed to be *A. a. alaicus*; the frontal and fronto-nasal regions of this subspecies were not connected (Zhao et al. 1999). However, based on all voucher specimens in this study, these two scales were connected for most individuals and Group KKAP clustered with *A. eremchenkoi* (BI/ML: 1/100, Fig. 2), which excluded this subspecies. Therefore, whether *A. a. alaicus* is distributed in southern Xinjiang requires further exploration. Notably, we discovered new records of *A. deserti* and substantiated the presence of this species north of the Ili River (Liang et al. 2021). Therefore, at least two skinks are distributed in Ili Valley and further research is necessary to determine whether these two species have a sympatric distribution in China (Kolbintzev et al. 1999).

Xinjiang covers one-sixth of China's territory; however, it remains the least studied area in China for reptiles. For example, the checklist of lizards in China has increased by almost 60 species since 2015 (Cai et al. 2015; Wang et al. 2020; Liang and Meiri 2023), of which only three (two new national records and two newly-described species) are from Xinjiang. The record of *A. eremchenkoi* reported in this study represents the fifth species added to the lizard checklist of Xinjiang. Hence, further research should aim to document the biodiversity of the region.

Conclusion

In summary, we identified the distribution of *A. alaicus* and A. *eremchenkoi* in Xinjiang, northwest China. These records indicate that the number of skink species in Xinjiang ranges from two to three. We also reported the phylogeny, morphology and natural historical notes of these two species.

Acknowledgements

We thank Philipp Wagner and two anonymous referees for their helpful comments on a previous draft of this manuscript, Anna Zimin for providing and translating Russian references. We also thank Editage for language editing service and Ju-xin Gu for providing his field observation. This work was supported by the Third Xinjiang Scientific Expedition Program [2022xjkk1200] and the National Natural Science Foundation of China [32260527 and 31660613].

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Supplementary material 1

Best models for the three sequences

Authors: Tao Liang, Qian-ru Liang, Jiang-miao Ran, Jing An, Ya-hui Huang, Peng Ding, Lei Shi

Data type: docx

Link: https://doi.org/10.3897/herpetozoa.37.e116071.suppl1

Supplementary material 2

Average uncorrected *p*-distances (percentages) between *Ablepharus*

Authors: Tao Liang, Qian-ru Liang, Jiang-miao Ran, Jing An, Ya-hui Huang, Peng Ding, Lei Shi

Data type: xlsx

Link: https://doi.org/10.3897/herpetozoa.37.e116071.suppl2

Supplementary material 3

Morphological traits of all individuals included in this study

Authors: Tao Liang, Qian-ru Liang, Jiang-miao Ran, Jing An, Ya-hui Huang, Peng Ding, Lei Shi

Data type: xlsx

Link: https://doi.org/10.3897/herpetozoa.37.e116071.suppl3

ÖGH Herpetozoa
Austrian Herpetological Society

Herpetozoa 37: 107–110 (2024)
DOI 10.3897/herpetozoa.37.e123770

Extending the known vertical distribution for the highly adaptive *Triturus macedonicus* (Karaman, 1922)

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Academic editor: Ben Wielstra ♦ **Received** 22 March 2024 ♦ **Accepted** 31 March 2024 ♦ **Published** 23 April 2024

Abstract

Current knowledge of the vertical distribution of *Triturus macedonicus* places the species at altitudes up to 2140 m. Herein, we report its presence in the alpine lake Gistova at 2360 m on Mt. Grammos, Greece, thus extending the highest altitudinal point for the species, as well as for the *Triturus* genus. This record showcases the adaptive capacity of the Macedonian newt, which allows it to occupy markedly different environments. More populations may exist at similar or even higher altitudes and their discovery could positively impact conservation efforts.

Key Words

adaptability, alpine, Gistova, Grammos, Macedonian newt, plasticity, vertical distribution

Introduction

Triturus macedonicus (Karaman, 1922) is a newt species endemic to the Balkan Peninsula that belongs to the wider *Triturus cristatus* complex (Wielstra and Arntzen 2016; Wielstra et al. 2019) and its distribution spans through seven countries in the southern part of the European continent: Bosnia & Herzegovina, Montenegro, Serbia, Albania, North Macedonia, Greece (Wielstra and Arntzen 2011) and Bulgaria (Naumov and Tzankov 2008). Previously referred to as *Triturus carnifex macedonicus* and later raised to species level as *Triturus macedonicus* (Arntzen et al. 2007), the Macedonian newt has distinct morphological features from the other *Triturus* species (Arntzen and Wallis 1999; Arntzen 2003; Wielstra et al. 2013). Records to date have placed its vertical distribution at elevations up to 1900 m (Speybroeck et al. 2016), 2000 m (Valakos et al. 2008) and 2140 m on Mt. Smolikas, Greece (Denoël 2004). In the current work, we provide evidence for the presence of *Triturus macedonicus* in Lake Gistova (Fig. 1), the highest alpine lake in Greece at an altitude of 2360 m a.s.l. on Mt. Grammos (Kastoria Prefecture, Greece, 40°21.905'N, 20°47.419'E) (Fig. 2), thus extending the known altitudinal point for the species; as well as for the *Triturus* genus, under which the closely-related species often demonstrate parapatric distributions (Wielstra et al. 2014; Wielstra and Arntzen 2020). In parallel, we confirmed the presence of *Triturus macedonicus* at sea level within the estuaries of the River Pineios (Larissa Prefecture, Greece, 39°52.947'N, 22°43.096'E) at the southern limit of the genus' distribution in Europe (Fig. 3).

There has been only one targeted survey in Lake Gistova, where researchers collected hundreds of alpine newts (*Ichthyosaura alpestris*) for sampling purposes (Papaioannou et al. 2015), but did not record the Macedonian newt. Earlier bibliographical knowledge highlights the presence of *T. macedonicus* on Mt. Grammos when placing a limit on its altitudinal distribution at 1950 m (Sotiropoulos et al. 1995). The question arises whether the present record is a novel discovery of a pre-existing

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Figure 1. *Triturus macedonicus* male in the alpine Lake Gistova.

Figure 2. Alpine lake Gistova, Kastoria, Greece.

population or if a lower population has shifted its dispersal upwards and only recently started occupying this alpine lake. Interestingly, *Triturus* species have been shown to be directly affected by increased temperature (Walther et al. 2002). Studies have also underlined that climate warming has forced a variety of amphibian taxa (Hickling et al. 2006; Chen et al. 2011) including newts of the *Triturus* genus (Tiberti et al. 2021), to shift their distribution towards higher elevations.

Whichever the case may be, the highlight of this record is the impressive adaptive capability of the Macedonian newt, spanning 2360 m of vertical distribution within various environmental conditions. Between sea-level wetlands that suffer scorching summer temperatures and almost permanently frozen alpine lakes, the Macedonian newt can effectively adapt to an impressive range of heterogeneous and even microclimatically fluctuating ecological niches. This is a critical determinant of species

Figure 3. *Triturus macedonicus* female at Pineios Delta, Greece.

survival and an undeniable advantage especially under unstable climatic conditions that can affect hydrological regimens in the Mediterranean Basin (Escoriza and Ben Hassine 2023), including Greece (Panagoulia and Dimou 1995). A recent study conducted by Degani and Meerson (2024) on the newt species *Ommatotriton vittatus*, showed extensive gene expression shifts and transcriptomic remodelling between terrestrial and aquatic stages in a population occupying an extreme habitat, confirming that phenotypic plasticity is the unsurpassed tool that allows amphibians to re-adjust to rapid environmental shifts (Diamond and Martin 2016). Considering the flexible responses that *T. macedonicus* also seems to possess, it may be another great candidate to investigate transcriptomic remodelling in populations occupying the two extreme habitats of its vertical range. Furthering our understanding of these mechanisms can have important implications for conservation and site preservation, because they may act as a buffer against serious threats under climate change (Urban et al. 2014).

Another significant implication of the current work is the possibility of more isolated populations of the Macedonian newt or even other species of the genus existing at higher altitudes. Subject to the available datasets, species distribution modelling approaches can be employed to facilitate the discovery of new populations within their range. A course of action that becomes more imperative after taking into consideration the direct threats through an ever-changing habitat (e.g. climate change, overgrazing, bioaccumulation of agricultural chemicals) and the status of the species. *T. macedonicus* is designated as “Vulnerable” (VU) by IUCN due to a multitude of factors, including aquatic habitat loss (Romano et al. 2009) and is also included in Annex II of the Directive 92/43/EEC which dictates the establishment of special conservation areas (Danelis et al. 2023). A better understanding of its distribution throughout its range would assist in more efficient conservation actions.

Acknowledgements

We would like to thank Dionisios Iakovidis and Nikolaos Gogolos for their assistance with fieldwork.

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ÖGH Herpetozoa
Austrian Herpetological Society

Herpetozoa 37: 111–121 (2024)
DOI 10.3897/herpetozoa.37.e120524

Range extension and expanded description of *Micryletta hekouensis* Liu, Hou, Mo & Rao, 2021 (Amphibia, Anura, Microhylidae), with comments on *Micryletta* of Northern Vietnam

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Academic editor: Günter Gollmann ♦ **Received** 8 February 2024 ♦ **Accepted** 3 April 2024 ♦ **Published** 23 April 2024

Abstract

The Paddy frog species *Micryletta hekouensis* was described based on only two specimens from Nanxi Village, Nanxi Town, Hekou County, Honghe Prefecture, Yunnan Province, China. Herein, we report on new findings and a range extension of this species based on a re-examination of preserved specimens deposited in Duy Tan University (DTU) and Zoological Museum of Lomonosov Moscow State University (ZMMU) collected from Vietnam. All new specimens were previously identified as *Micryletta* cf. *inornata* or *M.* cf. *steinegeri*. Molecular analyses based on mitochondrial DNA supported the morphological findings. The newly identified specimens have a pairwise divergence of only 0.7% from those of the type series of *M. hekouensis*, based on the 16s rRNA mtDNA gene. Based on the new information, we confirm the presence of *M. hekouensis* in Vietnam and update the diagnostic characters of this species and its distribution. We suggest the species should be considered as Near Threatened (NT) following the IUCN's Red List categories. Further studies reassessing the populations of the *Micryletta inornata* complex in the Indochinese Region (including Vietnam, Laos, Cambodia, and Thailand) are required.

Key Words

Cat Ba National Park, Cuc Phuong National Park, *Micryletta inornata*, *Micryletta steinegeri*, morphology, mtDNA, new record, phylogeny, redescription

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Introduction

The Paddy frogs of the genus *Micryletta* Dubois, 1987 are a little-known group of microhylids, with 13 nominal species currently recognized, namely: *M. aishani* Das, Garg, Hamidy, Smith & Biju, 2019; *M. dissimulans* Suwannapoom, Nguyen, Pawangkhanant, Gorin, Chomdej, Che & Poyarkov, 2020; *M. erythropoda* (Tarkhnishvili, 1994); *M. hekouensis* Liu, Hou, Mo & Rao, 2021; *M. immaculata* Yang & Poyarkov, 2021; *M. inornata* (Boulenger, 1890); *M. lineata* (Taylor, 1962); *M. melanops* Poyarkov, Nguyen, Yang & Gorin, 2021; *M. menglienica* (Yang & Su, 1980); *M. nigromaculata* Poyarkov, Nguyen, Duong, Gorin & Yang, 2018; *M. steinegeri* (Boulenger, 1909); *M. subaraji* Sankar, Law, Law, Shivaram, Abraham & Chan, 2022; and *M. sumatrana* Munir, Hamidy, Matsui, Kusrini & Nishikawa, 2020 (Sankar et al. 2022; Frost 2024). However, the small body size, elusive habits, as well as the remarkable morphological similarity of some of the *Micryletta* species complicates taxonomic studies of this group. Therefore, the taxonomic diversity of the genus *Micryletta* is not yet fully realized and requires additional studies. Presently, four nominal *Micryletta* species are recorded from Vietnam, including *Micryletta erythropoda*, *M. melanops*, *M. menglienica*, and *M. nigromaculata*. Two other species, namely *Micryletta inornata* (restricted to Sumatra Island and Southern Myanmar) and *M. steinegeri* (restricted to the Taiwan Island of China), have been reported from Vietnam in previous works (Nguyen et al. 2009; Poyarkov et al. 2018, 2021b), but recent phylogenetic studies suggest that these records were likely based on misidentifications with either *Micryletta* cf. *immaculata* or *M. menglienica* or *M. hekouensis* (Miller et al. 2021; Poyarkov et al. 2021b; Sankar et al. 2022; see Discussion).

The Hekou Paddy Frog, *Micryletta hekouensis* was described based on one male and one female specimen, both originating from Nanxi Village, Nanxi Town, Hekou County, Honghe Prefecture, Yunnan Province, China (Liu et al. 2021a). To date, this species was known only from its type locality in China. The species is characterized by: comparatively small body size (SVL 20.5 mm in male, 20.8 mm in female); areas above the canthus rostralis, upper eyelids, areas posterior to eyelids, and dorsum of upper arms golden, other parts of the dorsum are almost solid black or yellowish grey with brownish black stripes; lateral sides of head and body black or yellowish grey, a white stripe from lower front of eye along upper lip back to anterior forelimb insertion; ventral side of body and limbs is pink brown, chin region in adult males brownish black, small and irregular white marbling patterns on chest and lateral belly; supratympanic fold indistinct; outer metatarsal tubercle absent; webbing between toes absent; tibiotarsal articulation adpressed limb reaching level of front of eye (Liu et al. 2021a).

The recent molecular results of Miller et al. (2021) and Sankar et al. (2022) revealed that populations previously identified as *Micryletta* cf. *inornata* or *M.* cf. *steinegeri* in the Ninh Binh and Hai Phong in Northern Vietnam reported by Poyarkov et al. (2018) were nested within the same lineage as the holotype and paratype of *Micryletta hekouensis*, implying that this species has a more extensive distribution than currently recognized. To address this question, we re-examined specimens previously identified as *Micryletta* cf. *inornata* or *M.* cf. *steinegeri* from Ninh Binh and Hai Phong Provinces deposited in the zoological collections of the Duy Tan University (**DTU**, Vietnam) and Zoological Museum of Lomonosov Moscow State University (**ZMMU**, Russia), respectively. Our results confirm that the specimens from Ninh Binh Province as well as Hai Phong Province belong to *Micryletta hekouensis*. We herein formally confirm the occurrence of *Micryletta hekouensis* in Vietnam, update the distribution of this species, provide additional data on its natural history and revise its diagnostic characters.

Materials and methods

Material examined

We examined twelve specimens that were previously registered as *M.* cf. *inornata* or *M.* cf. *steinegeri* from Cuc Phuong National Park (hereafter **NP**), Ninh Binh Province and Cat Ba NP, Hai Phong Province, Northern Vietnam (Poyarkov et al. 2018) (see Table 1). Morphological comparisons were based on literature data from: Boulenger (1890); Das et al. (2019); Liu et al. (2021a,b); Miller et al. (2021); Munir et al. (2020); Poyarkov et al. (2018, 2021b); Sankar et al. (2022); Suwannapoom et al. (2020); Tarkhnishvili (1994); Taylor (1962); Yang and Poyarkov (2021) (see Suppl. material 1).

Morphological analyses

Measurements were taken using a digital caliper under a light dissecting microscope to the nearest 0.01 mm, subsequently rounded to 0.1 mm. The morphometrics of adults and character terminology followed Nguyen et al. (2020), including **SVL**: snout-vent length, **HL**: head length (from the back of mandible to tip of snout), **HW**: maximum head width (across angles of jaws), **SL**: snout length (from anterior corner of eye to tip of snout), **NSD**: distance from nostril to the tip of snout, **END**: distance from anterior corner of eye to the nostril, **IND**: internarial distance, **IOD**: interorbital distance, **ED**: eye diameter, **UEW**: maximum width of upper eyelid, **TD**: tympanum diameter, **TYED**: distance from anterior margin of tympanum to posterior corner of the eye, **FLL**: forearm length (from axilla to elbow), **HAL**: hand length (from elbow to the tip of third finger), **FL1–4**:

finger length I–IV, **OPTL**: outer palmar tubercle length, **IPTL**: inner palmar tubercle length, **NPL**: nuptial pad length, **FeL**: femur length (from vent to knee), **TbL**: tibia length (from knee to tarsus), **TbW**: maximum tibia width, **FoL**: foot length (from tarsus to the tip of fourth toe), **TL 1–5**: toe length I–V, **IMTL**: inner metatarsal tubercle length, **FD3D**: maximal diameter of disk of finger III, and **TD4D**: maximal diameter of disk toe IV. Terminology for describing eye colouration in living individuals followed Glaw and Vences (1997); subarticular tubercle formulas and webbing formula followed those of Savage (1975). All measurements were taken on the right side of the examined specimen. Sex was determined by gonadal inspection following dissection.

Table 1. Sequences (16S rRNA) used in molecular analyses of this study.

No.	Previously taxon	Proposed taxon	Voucher	Locality	Accession	Reference
1	*M. aishani*	*M. aishani*	SDBDU 3920	India: Assam, Cachar, Subhong	MK889218	Das et al. (2019)
2	*M. aishani*	*M. aishani*	CAS 231526	Myanmar: Kachin, Indawgyi WS	MW035599	Miller et al. (2021)
3	*M. dissimulans*	*M. dissimulans*	AUP 01690	Thailand: Songkla, Saba Yoi	MT573414	Suwannapoon et al. (2020)
4	*M. dissimulans*	*M. dissimulans*	AUP 01691	Thailand: Songkla, Saba Yoi	MT573415	Suwannapoon et al. (2020)
5	*M. dissimulans*	*M. dissimulans*	AUP 01696	Thailand: Songkla, Saba Yoi	MT573416	Suwannapoon et al. (2020)
6	*M. dissimulans*	*M. dissimulans*	AUP 01698	Thailand: Songkla, Saba Yoi	MT573413	Suwannapoon et al. (2020)
7	*M. erythropoda*	*M. erythropoda*	ZMMU A4721-1533	Vietnam: Dong Nai, Ma Da N.R.	MH756146	Poyarkov et al. (2018)
8	*M. erythropoda*	*M. erythropoda*	ZMMU A4721-1542	Vietnam: Dong Nai, Ma Da N.R.	MH756147	Poyarkov et al. (2018)
9	*M. hekouensis*	*M. hekouensis*	KIZ 20210510	China: Yunnan, Honghe, Hekou	MZ536627	Liu et al. (2021a)
10	*M. hekouensis*	*M. hekouensis*	KIZ 20210511	China: Yunnan, Honghe, Hekou	MZ536628	Liu et al. (2021a)
11	*M.* cf. *inornata*	*M. hekouensis*	DTU 310	Vietnam: Ninh Binh, Cuc Phuong N.P.	PP264232	This study
12	*M.* cf. *inornata*	*M. hekouensis*	DTU 311	Vietnam: Ninh Binh, Cuc Phuong N.P.	PP264231	This study
13	*M.* cf. *inornata*	*M. hekouensis*	DTU 312	Vietnam: Ninh Binh, Cuc Phuong N.P.	PP264230	This study
14	*M.* cf. *inornata*	*M. hekouensis*	ZMMU NAP-3352-1	Vietnam: Hai Phong, Cat Ba N.P.	MH879843	Poyarkov et al. (2018)
15	*M.* cf. *inornata*	*M. hekouensis*	ZMMU NAP-3352-2	Vietnam: Hai Phong, Cat Ba N.P.	MH879844	Poyarkov et al. (2018)
16	*M.* cf. *inornata*	*M. hekouensis*	ZMMU NAP-3580	Vietnam: Hai Phong, Cat Ba N.P.	MH879845	Poyarkov et al. (2018)
17	*M. immaculata*	*M. immaculata*	KFBG 14270	China: Hainan, Exian	MW376736	Yang and Poyarkov (2021)
18	*M. immaculata*	*M. immaculata*	KFBG 14271	China: Hainan, Exian	MW376737	Yang and Poyarkov (2021)
19	*M. inornata*	*M. immaculata*	FMNH 255121	Laos: Khammouan, Boualapha	KC179997	de Sa et al. (2012)
20	*M. inornata*	*M. immaculata*	TZ9892	Vietnam: Ha Tinh, Ke Go	AF285206	Ziegler (2002)
21	*M. inornata*	*M. inornata*	MZB 23949	Indonesia: Sumatra, Deli Serdang	LC208135	Alhadi et al. (2019)
22	*M. inornata*	*M. inornata*	MZB 23947	Indonesia: Sumatra, Deli Serdang	LC208136	Alhadi et al. (2019)
23	*M. inornata*	*M. inornata*	MZB 23948	Indonesia: Sumatra, Deli Serdang	LC208137	Alhadi et al. (2019)
24	*M. inornata*	*M. inornata*	MZB 27242	Indonesia: Sumatra, Aceh	LC208138	Alhadi et al. (2019)
25	*M. inornata*	*M. inornata*	USNM 587625	Myanmar: Tanintharyi	MT609033	Miller et al. (2021)
26	*M. inornata*	*M. inornata*	USNM 587901	Myanmar: Tanintharyi	MT609034	Miller et al. (2021)
27	*M. inornata*	*M. lineata*	KUHE 23858	Thailand: Ranong	AB634695	Matsui et al. (2011)
28	*M. inornata*	*M. lineata*	CAS 247206	Myanmar: Tanintharyi, Kawthaung	KM509167	Peloso et al. (2015)
29	*M. melanops*	*M. melanops*	ZMMU NAP-00449	Vietnam: Lam Dong, Biduop-Nui Ba N.P.	MZ474684	Poyarkov et al. (2021b)
30	*M. melanops*	*M. melanops*	ZMMU NAP-01381	Vietnam: Lam Dong, Biduop-Nui Ba N.P.	MZ474685	Poyarkov et al. (2021b)
31	*M. menglienica*	*M. menglienica*	KIZ 20210708	China: Yunnan, Pu'er, Menglian	OK335183	Liu et al. (2021b)
32	*M. menglienica*	*M. menglienica*	KIZ 20210709	China: Yunnan, Pu'er, Menglian	OK335184	Liu et al. (2021b)
33	*M. menglienica*	*M. menglienica*	KFBGF 14653	China: Yunnan, Xishuangbanna, Mengla	OR053962	Yeung et al. (2023)
34	*M. inornata*	*M. menglienica*	KUHE 20497	Thailand: Phrae, Mae Yom	AB598341	Matsui et al. (2011)
35	*M. inornata*	*M. menglienica*	K 3068	Thailand: Chiang Mai, Doi Chiang Dao	KR827953	Grosjean et al. (2015)
36	*M. inornata*	*M. menglienica*	K 3246	Laos: Luangprabang, Ban Sop Chuna	KC180027	Grosjean et al. (2015)
37	*M. nigromaculata*	*M. nigromaculata*	ZMMU A5947	Vietnam: Hai Phong, Cat Ba N.P.	MH756148	Poyarkov et al. (2018)
38	*M. nigromaculata*	*M. nigromaculata*	ZMMU A5937	Vietnam: Hai Phong, Cat Ba N.P.	MH756149	Poyarkov et al. (2018)
39	*M. nigromaculata*	*M. nigromaculata*	ZMMU A5946	Vietnam: Hai Phong, Cat Ba N.P.	MH756151	Poyarkov et al. (2018)
40	*M. nigromaculata*	*M. nigromaculata*	DTU 301	Vietnam: Ninh Binh, Cuc Phuong N.P.	MH756154	Poyarkov et al. (2018)
41	*M. steinegeri*	*M. steinegeri*	KUHE 35937	China: Taiwan, Yunlin	AB634696	Matsui et al. (2011)
42	*M. steinegeri*	*M. steinegeri*	ZMMU A5336-1	China: Taiwan, Kaohsiung	MW376732	Poyarkov et al. (2018)
43	*M. steinegeri*	*M. steinegeri*	ZMMU A5336-2	China: Taiwan, Kaohsiung	MW376733	Poyarkov et al. (2018)
44	*M. steinegeri*	*M. steinegeri*	ZMMU A5336-3	China: Taiwan, Kaohsiung	MW376734	Poyarkov et al. (2018)
45	*M. subaraji*	*M. subaraji*	ZRC1.13370	Singapore: Kranji Marshes	ON026065	Sankar et al. (2022)
46	*M. subaraji*	*M. subaraji*	ZRC 1.13369	Singapore: Kranji Marshes	ON026064	Sankar et al. (2022)
47	*M. subaraji*	*M. subaraji*	ZRC 1.13389	Singapore: Kranji Marshes	ON026066	Sankar et al. (2022)
48	*M. subaraji*	*M. subaraji*	ZRC 1.13323	Singapore: Kranji Marshes	ON026063	Sankar et al. (2022)
49	*M. sumatrana*	*M. sumatrana*	MZB 30594	Indonesia: Sumatra Selatan	MN727065	Munir et al. (2020)
	Our group					
50	*Kaloula pulchra*	*Kaloula pulchra*	NMNS 3208	China	KC822614	Blackburn et al. (2013)
51	*Kaloula pulchra*	*Mysticellus franki*	ZSI/WGRC/V/A/967	India: Kerala, Wayand	MK285340	Garg and Biju (2019)
52	*Kaloula pulchra*	*Uperodon systoma*	SDBDU 2005.4723	India: Tamil Nadu: Kunnapattu	MG557949	Garg and Biju (2019)

Molecular phylogeny

We synthesized previously published sequences of the *Micryletta steinegeri* members from GenBank to estimate the phylogenetic relationships of the genus *Micryletta* and genetically identity samples referable to *M. hekouensis*. We focused on sequences for the mitochondrial 16S rRNA gene as it is phylogenetically informative for most Paddy frogs and has the largest availability of any gene for *Micryletta*. As the sequences of the three specimens (DTU 310-12) provided by Poyarkov et al. (2018) are too short to obtain a stable phylogenetic position, we obtained longer sequences of these three specimens and uploaded them to GenBank. We aligned the 16S sequences of 13 species of *Micryletta*; we used the sequences of *Kaloula pulchra* Gray, 1831; *Mysticellus franki* Garg & Biju, 2019; and *Uperodon systoma* (Schneider, 1799) to root the tree (GenBank accession numbers, voucher specimens, locality, and source information are summarized in Table 1).

Sequences were aligned using MUSCLE (Edgar 2004) integrated in MEGA 11 (Tamura et al. 2021) with default parameters. Genetic divergences (uncorrected p-distance) were calculated in MEGA 11. The best substitution models were selected using the Akaike Information Criterion (AIC) in ModelFinder (Kalyaanamoorthy et al. 2017). Maximum likelihood phylogenetic analysis was performed in IQ-TREE 1.6.12 (Nguyen et al. 2015) based on the TIM2+F+I+G4 model, and nodal support was estimated by 1,000 ultrafast bootstrap (UFB) replicates. Nodes with UFB values of 95 and above were considered significantly supported (Minh et al. 2013). Bayesian Inference was performed in MrBayes 3.2.7 (Ronquist et al. 2012) based on the GTR+F+I+G4 model. Two runs were performed simultaneously with four Markov chains starting from a random tree. The chains were run for 5,000,000 generations and sampled every 100 generations. The first 25% of the sampled trees were discarded as burn-in and then the remaining trees were used to estimate Bayesian posterior probabilities (BPPs). Nodes were considered well-supported if they had BPPs of 0.95 or higher (Huelsenbeck et al. 2001; Wilcox et al. 2002).

Results

The ML and BI analyses of 16S rRNA sequences recovered trees with similar topologies (Fig. 1). With respect to the position of *M. hekouensis*, our phylogenetic results largely conform to those of Poyarkov et al. (2018), Miller et al. (2021), Liu et al. (2021a) and Sankar et al. (2022). The reconstructed phylogenetic relationship indicates that four species, namely *M. steinegeri*, *M. menglienica*, *M. immaculata*, and *M. hekouensis* are nested within a single monophyletic clade *M. steinegeri* complex (Fig. 1).

The uncorrected *p*-distances of the 16s gene fragment among examined members of the *M. steinegeri* species groups are presented in Table 2. Six sequences of *Micryletta* spp (PP264232 [DTU 310], PP264231 [DTU 311], PP264230 [DTU 312], respectively, reported from Cuc Phuong NP., Ninh Binh Province as well as MH879843 [ZMMU NAP-3352-1], MH879844 [ZMMU NAP-33521–2], MH879845 [ZMMU NAP-3580], respectively, reported from Cat Ba NP., Hai Phong Province, Vietnam clustered with the type series of *M. hekouensis* from Nanxi Town, Hekou County, Honghe Prefecture, Yunnan Province, China, and were only 0.7% divergent from the type series of *M. hekouensis*.

Furthermore, we also examined the morphology of other *Micryletta* specimens previously registered as *Micryletta* cf. *inornata* or *M.* cf. *steinegeri* from Ninh Binh and Hai Phong including five specimens DTU 310–312 as well as ZMMU NAP-3580, ZMMU NAP-3580 and found morphological similarities with *M. hekouensis*. These results support our hypothesis that previous records of *M. inornata* (restricted to Sumatra Island in Indonesia, southern Myanmar) and *M. steinegeri* (restricted to Taiwan Island in China) in Northern Vietnam (Ninh Binh and Hai Phong province), should be referred to as *M. hekouensis*. Therefore, we extend the distribution of *Micryletta hekouensis* to Vietnam and provide an expanded diagnosis and description.

Table 2. Uncorrected p-distances (%) of 16S rRNA sequences between the species of *Micryletta*.

Species	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**
1 *Micryletta aishani*													
2 *Micryletta dissimulans*	4.4												
3 *Micryletta erythropoda*	4.8	7.4											
4 *Micryletta hekouensis* (China)	3.6	5.0	6.5										
5 *Micryletta hekouensis* (Vietnam)	3.0	4.7	6.3	0.7									
6 *Micryletta immaculata*	4.3	6.3	6.8	3.9	3.5								
7 *Micryletta inornata*	5.0	5.9	7.6	5.5	5.1	7.0							
8 *Micryletta lineata*	3.4	6.0	3.1	4.6	4.4	5.4	6.3						
9 *Micryletta melanops*	3.0	4.4	7.1	5.9	5.6	5.9	5.6	5.5					
10 *Micryletta menglienica*	3.6	5.4	6.4	3.2	2.8	2.6	6.6	4.8	5.4				
11 *Micryletta nigromaculata*	4.7	5.2	8.3	8.1	7.6	7.5	6.6	7.2	6.8	7.1			
12 *Micryletta steinegeri*	3.6	4.8	6.7	3.1	2.8	3.6	5.5	5.1	6.5	2.8	7.2		
13 *Micryletta subaraji*	4.6	5.2	7.5	6.6	6.3	6.9	2.9	6.4	7.1	6.5	7.9	7.1	
14 *Micryletta sumatrana*	5.9	5.1	9.1	6.7	6.3	7.8	8.1	7.4	6.2	7.1	5.5	6.0	8.9

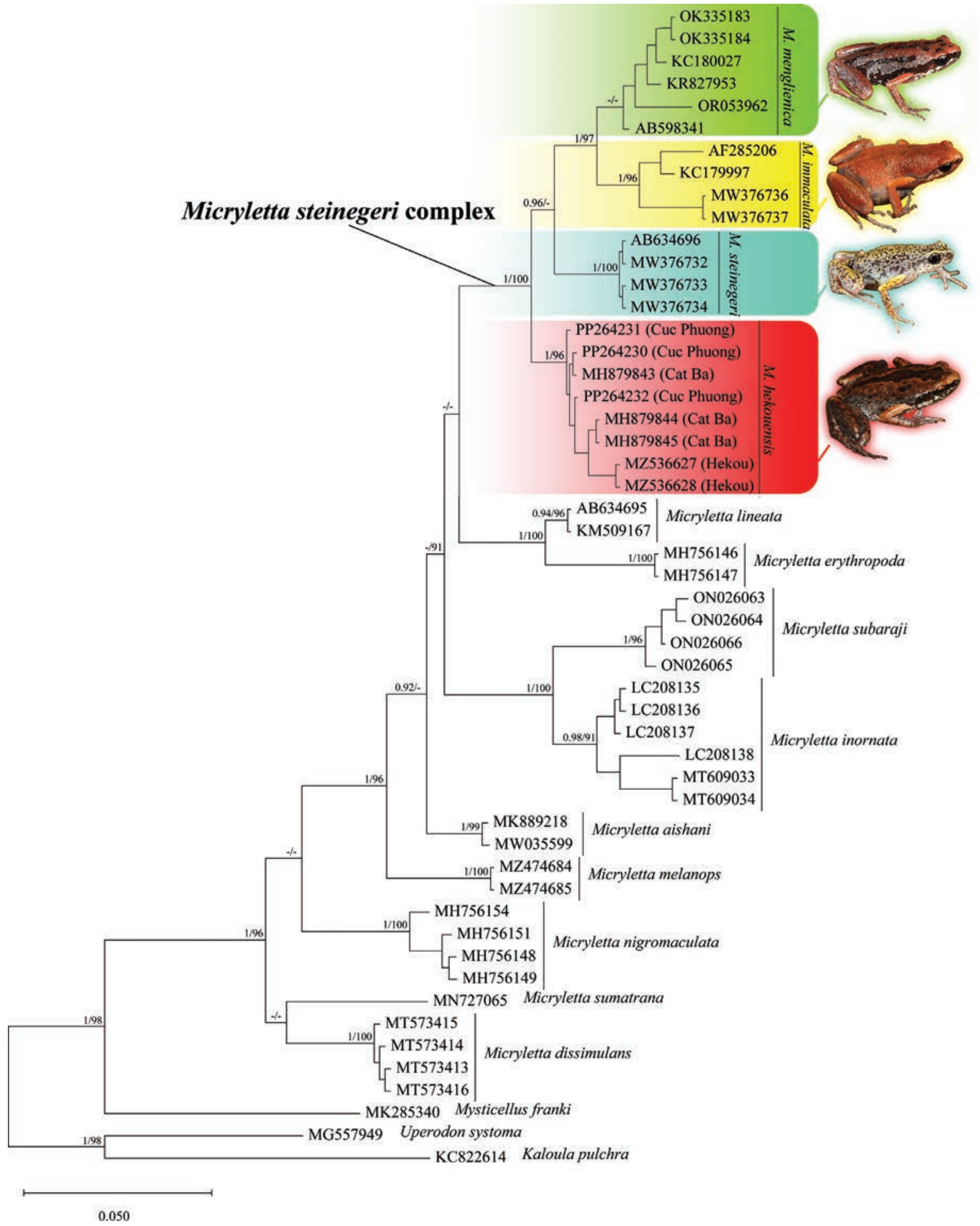

Figure 1. Maximum Likelihood (ML) tree of *Micryletta* derived from the analysis of 16s mitochondrial DNA gene sequences. For voucher specimen information and GenBank accession numbers see Table 1. Numbers at tree nodes correspond to ML UFBS/BI PP support values, respectively. Photos by J.B Zhao, J.H. Yang, C.W. You, and N.A. Poyarkov.

Taxonomic account

Micryletta hekouensis

Table 3, Fig. 2

Micryletta cf. *inornata* – Poyarkov et al. (2018: 1–27, in part); Miller et al. (2021: 248, in part).

Micryletta cf. *steinegeri* – Poyarkov et al. (2021a: 42–43, in part).

Micryletta hekouensis – Liu, Hou, Mo & Rao (2021: 133).

Micryletta hekouensis – Sankar et al. (2022: 462).

Holotype. KIZ 20210510, adult male collected by Shuo Liu on 15 May 2021. **Type locality:** Nanxi Village, Nanxi Town, Hekou County, Honghe Prefecture, Yunnan Province, China (22°38'17"N, 103°59'8"E; elevation 350 m a.s.l.).

Suggested name in Vietnamese. Nhái bầu Hà khẩu.

Specimens examined (n = 12). Two adult males DTU 310, 316 and four adult females DTU 309, 311–312, 317 from Cuc Phuong NP, Ninh Binh Province, Vietnam (ca. 20.2594°N, 105.6928°E; elevation of 160–215 m a.s.l) collected by Tan Van Nguyen on 3 June 2018. Three adult males ZMMU NAP-2176, ZMMU NAP-3580, ZMMU NAP-5572 and three adult females ZMMU NAP-3352, ZMMU NAP-3574, ZMMU NAP-5574 from Cat Ba NP, Hai Phong Province, Vietnam (ca. 20.8123°N, 106.9988°E; elevation of 90 m a.s.l.) collected by Nikolay A. Poyarkov in October 2013.

Variation based on examined specimens from Vietnam (n = 12). According to the original description of Liu et al. (2021a) the species *M. hekouensis* is a small-sized species of *Micryletta* with SVL 20.5 mm in adult male (Holotype, KIZ 20210510) and SVL 20.8 mm in adult female (Paratype, KIZ 20210511). However, the specimens from Vietnam exhibit generally larger body sizes: SVL 20.0–24.1 mm in males (n = 5), 25.4–29.5 mm in females (n = 7). Therefore, we propose that the paratype specimen should be considered a subadut female, and that *M. hekouensis* in fact represents a medium-sized species within its genus. Moreover, the TbL/SVL ratio in female specimens from Vietnam (0.43–0.55) was also slightly larger than in the paratype female from China (0.40). Furthermore, Liu et al. (2021a) reported this species to have a small rounded and distinct tympanum; however, the specimens from Vietnam had the tympanum hidden. It is not clear if the observed morphological differences between the type series of *M. hekouensis* from China and the series from Vietnam represent the actual intraspecific variation, or might result from the different state of specimen preservation, the reproductive condition of the specimens, or the observer effect. However, though we acknowledge the limitations of mtDNA markers for species delimitation in amphibians (e.g., Velo-Antón et al. 2023), the overall similarity of the Chinese and Vietnamese specimens of *M. hekouensis* in external morphological traits and coloration strongly suggest that they belong to the same species.

Additionally, the Vietnamese specimens examined were characterized by: habitus relatively slender; head small and triangular, width approximately equal to length (HW/HL 0.93–1.11 in males, 0.97–1.22 in females); snout abruptly rounded in dorsal view and slightly acuminate in profile, projecting beyond margin of lower jaw; eyes relatively small, slightly protuberant, pupil oval, transverse, eye diameter slightly equal to snout length (ED/SL 0.75–1.20 in males, 0.83–1.18 in females). Top of the head flat, canthus rostralis rounded and distinct; loreal region weakly concave; nostril round, closer to tip of snout than to eye; interorbital distance greater than internarial distance (IOD/

Figure 2. The *Micryletta hekouensis* alive: from Cuc Phuong NP., Ninh Binh, Vietnam. **A, B.** DTU 316, adult male; **C.** DTU 311, adult male; **D, E.** DTU 317, adult female); from Cat Ba NP, Hai Phong, Vietnam; **F, G.** ZMMU NAP-3580, adult female); from Hekou, Yunnan, China; **H.** KIZ 20210510, holotype, adult male; **I.** KIZ 20210511, paratype, subadult female). Photos by: TV. Nguyen (**A–E**), NA. Poyarkov (**F, G**), and L. Shuo (**H–K**).

IN 1.26–2.16 in males, 1.20–1.71 in females) and upper eyelid width (IOD/UEW 1.45–1.91 in males, 1.30–1.89 in females). Tympanum and supratympanic fold indistinct. Choanae rounded; vomerine teeth absent; opening of vocal sac long cleft; tongue oval, with no notch at posterior tip.

***Forelimbs*:** Forearm length ca. three times shorter than hand length (FLL/HAL 0.43–0.62 in males, 0.40–0.53 in females). Fingers slender with no webbing, rounded in cross-section, no lateral fringes; relative finger lengths: I<II<IV<III; tips of fingers round and not dilated; subarticular tubercles on fingers distinct, rounded and prominent, formula: 1, 1, 2, 2; supernumerary tubercles on palm present and developed; three metacarpal tubercles, inner one rounded and smallest, median one rounded and almost directly in front of oval outer one; two rounded and one elongated prominent supernumerary palmar tubercles on the base of fingers II–IV, respectively; nuptial pad absent.

***Hindlimbs*:** Tibia slightly longer than thigh (FeL/TbL 0.80–0.97 in males, 0.82–1.02), approximately three times longer than wide (TbL/TbW 3.27–5.50 in males, 3.08–5.19 in females); tibiotarsal articulation of adpressed limb reaching eye; foot longer than tibia (TbL/FoL 0.60–0.67 in males, 0.59–0.68 in females). Relative toe lengths: I<II<V<III<IV; tarsal fold absent; tips of toes round and not dilated, slightly wider than those of fingers; webbing between toes absent; subarticular tubercles on toes oval and prominent, formula: 1, 1, 2, 3, 2; dermal ridges present under 2nd to 4th toes but indistinct; inner metatarsal tubercle rounded, prominent, and small; outer metatarsal tubercle absent.

Dorsal skin scattered with small tubercles on dorsum of body, flanks, and hindlimbs, dorsal skin of forelimbs smooth; subtle longitudinal median ridge present on dorsum; dorsolateral fold absent; lateral sides of head smooth; ventral skin of body and limbs smooth.

Colouration in life. Coloration varies greatly, dorsum of body purple brown, blueish grey, or dark brown with two indistinct parallel longitudinal grey stripes on back. Dorsum of forelimbs light yellow, dorsum of hindlimbs the same color as dorsum of body, no bands on dorsum of limbs. Upper lip white. Ventral side of head, body, and limbs greyish brown, purple grey or pinkish brown. Chin region brownish black, males usually have a darker one than females, white marbling patterns on chest and belly, some white spots on chin region and ventral side of limbs. Iris bicolored, with upper third bronze and lower two-thirds brownish black.

Revised diagnosis. Medium-sized within genus *Micryletta* (SVL 20.0–24.1 mm in males, 25.4–29.5 mm in females); areas above canthus rostralis, upper eyelids, areas posterior to eyelids, and dorsum of upper arms golden, other parts of dorsum almost solid black or yellowish grey with brownish black stripes; lateral sides of the head and body black or yellowish grey, a white stripe from lower front of eye along upper lip back to anterior forelimb insertion; ventral side of body and limbs pink brown, chin region in adult males brownish black, small and irregular white marbling patterns on chest and lateral belly; tympanum indistinct; supratympanic fold indistinct; outer metatarsal tubercle absent; webbing between toes absent; tibiotarsal articulation adpressed limb reaching level of eye (data from Liu et al. (2021) and this study).

Comparisons. We summarize the main characters separating *Micryletta hekouensis* from the other twelve species of the genus *Micryletta* in Suppl. material 1. In Vietnam, previously this species was often recorded under the names *M. steinegeri* or *M. inornata*, therefore we focused on comparing the morphological characteristics of *Micryletta hekouensis* with these two species. *Micryletta hekouensis* differs from *M. steinegeri* by having:venter

Table 3. Measurements (in mm) of the specimens of *Micryletta hekouensis* in Vietnam.

Specimen number	Sex	SVL	HW	HL	SL	ED	NSD	END	UEW	IOD	IND	FLL	HAL	fd3D	FeL	TbL	FoL	TbW	td4D	IMTL
DTU 310	**M**	22.2	6.9	6.4	2.5	3.0	1.3	1.6	2.0	2.9	2.3	5.1	11.4	0.7	9.5	9.8	16.3	3.0	0.6	0.9
DTU 316	**M**	24.1	7.2	7.2	2.9	3.0	1.3	1.9	2.0	3.1	2.3	6.0	14.0	0.5	11.6	12.5	20.3	3.3	0.5	0.9
ZMMU NAP-2176	**M**	21.6	6.7	7.2	2.8	2.1	1.8	1.6	1.8	2.7	1.6	5.5	10.8	0.5	8.4	10.5	16.2	2.5	0.5	0.7
ZMMU NAP-3580	**M**	20.0	6.2	6.6	2.7	2.5	1.0	1.4	1.6	3.0	2.2	6.1	9.8	0.5	8.9	9.9	14.9	1.9	0.6	0.6
ZMMU NAP-5572	**M**	23.1	7.9	7.1	3.7	3.0	0.8	2.2	1.3	4.1	1.9	7.4	12.6	0.5	11.6	13.2	19.6	2.4	0.6	0.5
	Min	20.0	6.2	6.4	2.5	2.1	0.8	1.4	1.3	2.7	1.6	5.1	9.8	0.5	8.4	9.8	14.9	1.9	0.5	0.5
	Max	22.2	6.9	6.4	2.5	3.0	1.3	1.6	2.0	2.9	2.3	5.1	11.4	0.7	9.5	9.8	16.3	3.0	0.6	0.9
	Mean	22.2	7.0	6.9	2.9	2.7	1.2	1.7	1.7	3.1	2.1	6.0	11.7	0.5	10.0	11.2	17.5	2.6	0.6	0.7
	SD	1.54	0.63	0.36	0.47	0.40	0.37	0.33	0.33	0.53	0.28	0.85	1.62	0.07	1.49	1.56	2.34	0.52	0.07	0.17
DTU 309	**F (gravid)**	28.9	7.9	7.7	3.2	3.2	1.3	1.9	2.2	3.6	2.5	6.5	14.6	0.7	12.5	13.0	19.8	3.1	0.5	1.6
DTU 311	**F (gravid)**	27.1	7.4	7.6	2.7	3.1	1.3	1.7	1.8	3.4	2.3	5.8	13.3	0.7	11.2	11.7	19.7	3.8	0.8	1.1
DTU 312	**F (gravid)**	29.5	7.6	7.1	2.8	3.3	1.3	1.8	1.8	3.3	2.6	5.3	13.4	0.7	12.4	12.6	19.3	3.6	0.6	1.0
DTU 317	**F (gravid)**	28.7	9.4	8.8	3.3	3.2	1.6	2.1	2.3	3.0	2.5	6.9	15.0	0.6	13.6	13.3	21.7	3.6	0.7	1.0
ZMMU NAP-3352	**F**	25.4	8.9	8.0	3.5	2.9	1.5	2.3	1.7	3.5	2.6	6.5	14.0	0.4	11.5	14.0	20.6	2.7	0.4	0.6
ZMMU NAP-3574	**F**	26.0	9.6	7.9	3.6	3.1	1.4	2.0	2.0	3.9	2.4	7.4	14.0	0.4	12.9	13.0	20.5	2.9	0.4	0.8
ZMMU NAP-5574	**F**	26.8	9.5	8.5	3.3	3.2	0.9	1.7	1.6	4.1	2.4	7.5	15.7	0.6	11.6	13.1	21.5	2.8	0.5	0.4
	Min	25.4	7.4	7.1	2.7	2.9	0.9	1.7	1.6	3.0	2.3	5.3	13.3	0.4	11.2	11.7	19.3	2.7	0.4	0.4
	Max	29.5	9.6	8.8	3.6	3.3	1.6	2.3	2.3	4.1	2.6	7.5	15.7	0.7	13.6	14.0	21.7	3.8	0.8	1.6
	Mean	27.5	8.6	8.0	3.2	3.1	1.3	1.9	1.9	3.6	2.5	6.6	14.3	0.6	12.3	13.0	20.4	3.2	0.6	0.9
	SD	1.55	0.96	0.58	0.34	0.13	0.22	0.23	0.26	0.38	0.11	0.80	0.88	0.14	0.86	0.68	0.92	0.46	0.14	0.37

without dark patterns (vs. with greyish white and brown spots); webbing between toes absent (vs. rudimentary webbing); tibiotarsal articulation adpressed limb reaching level of eye (vs. reaching to tympanum). *Micryletta hekouensis* differs from *M. inornata* sensu stricto by having: lager body size in females (SVL 25.4–29.5 mm vs. 19.5 mm); ventral side of body and limbs pinkish brown or pinkish grey with small and irregular white marbling patterns on chest and lateral belly (vs. reddish grey without mottling, nearly immaculate, or chin, chest, and lateral belly with a few dark marbling patterns).

Distribution. *Micryletta hekouensis* was previously known only from Nanxi Town, Hekou County, Honghe Prefecture, Yunnan Province, China (Liu et al. 2021). We here add further records of this species in Vietnam (Ninh Binh and Hai Phong provinces). The new location in Cat Ba NP is situated ca. 335 airline kilometers southeast of the type locality. Given the geographic proximity and distribution patterns of limestone-associated herpetofauna in Vietnam (see Poyarkov et al. 2021a, 2023), *M. hekouensis* likely occurs in several limestone karst massifs of Northern Vietnam; in particular, records from Quang Ninh, Lang Son and Bac Giang provinces of Northeastern Vietnam, as well as from Hoa Binh, Ha Nam, and Thanh Hoa provinces of Northwestern Vietnam are anticipated**.**

Natural history notes. Prior to this study, biological data of *Micryletta hekouensis* were very limited; it was only reported from an altitude of 350 m a.s.l. (Liu et al. 2021). The species appears to be closely associated with karstic habitats (Poyarkov et al. 2018; Liu et al. 2021). In Cuc Phuong NP, the frogs were observed from 16:00 to 20:00 h under the dead leaves on the ground. Other species of microhylids recorded syntopically with *Micryletta hekouensis* in Cuc Phuong NP included *Kalophrynus interlineatus* (Blyth, 1855), *Glyphoglossus* cf. *yunnanensis* (Boulenger, 1919), *Kaloula pulchra* Gray, 1831, *Microhyla berdmorei* (Blyth, 1856), *M. butleri* Boulenger, 1900, *M.* cf. *heymonsi* Vogt, 1911, *M. mukhlesuri* Hasan, Islam, Kuramoto, Kurabayashi & Sumida, 2014, *M. pulchra* (Hallowell, 1861), *Micryletta nigromaculata* Poyakov, Nguyen, Duong, Gorin & Yang, 2017, and *Vietnamophryne* cf. *orlovi* Poyarkov, Suwannapoom, Pawangkhanant, Aksornneam, Duong, Korost & Che, 2018. In Cat Ba NP, the frogs were observed from 16:00 to 20:00 h hiding between small pieces of limestone rocks. Other species of microhylids recorded syntopically with *Micryletta hekouensis* in Cat Ba NP included *Kaloula pulchra*, *Microhyla butleri*, *M. fissipes* Boulenger 1884, *M.* cf. *heymonsi*, *M. pulchra*, *Micryletta nigromaculata*. We also recorded gravid females of *M. hekouensis* in June in Cuc Phuong, but other reproduction biology data, such as advertisement call, tadpole morphology, as well as diet of the species remains unknown. It is remarkable that in both localities of *Micryletta hekouensis* in Vietnam, this species was recorded in syntopy with *M. nigromaculata*, another species of the genus *Micryletta* strongly associated with limestone karst formations of northern Vietnam.

Discussion

In this study we re-examined specimens reported by Poyakov et al. (2018, 2021) from karstic habitats in northern Vietnam (Ninh Binh and Hai Phong Provinces) that had previously been assigned to *Micryletta* cf. *inornata* or *M.* cf. *steinegeri* and found that all of these should be assigned to the recently described *Micryletta hekouensis*, representing the first record of this species in Vietnam. The discovery of *Micryletta hekouensis* in this study raises the known number of *Micryletta* species in Vietnam to five, with two species endemic to this country, namely *M. melanops* and *M. nigromaculata*. Consequently, we suggest to remove *Micryletta steinegeri* from the fauna of Vietnam.

Future studies reassessing the taxonomy and distribution of populations of *Micryletta inornata* sensu lato in Vietnam as well as other countries of the Indochinese Region (Laos, Cambodia, Thailand) are required for clarification. Until this work, two species of *Micryletta*, previously confused with *M. inornata* were considered to be endemic to China: *M. menglienica* (until now known only from two locations in Menglian and Mengla counties, Yunnan Province, China) and *Micryletta immaculata* (presently considered to be endemic to Hainan Island, China) (Liu et al. 2021; Poyarkov et al. 2021b; Yang and Poyarkov 2021; Yeung et al. 2023). In our molecular study, we found that populations of *M.* cf. *inornata* reported from Ha Tinh Province, Vietnam and Khammouan Province, Laos, were clustered with *M. immaculata* with minimal genetic distance among them (p = 0.9–2.3%). Meanwhile, populations of *M.* cf. *inornata* from Phrae and Chiang Mai provinces, Thailand and Luangprabang Province, Laos were grouped with *M. menglienica* and were also found to be genetically very closely related to it (p = 0.2–1.5%). Our results suggest that these two species may have a wider distribution than currently recorded. *Micryletta immaculata* is likely distributed in southern China, in northern and central Vietnam, central Laos, while *M. menglienica* likely inhabits southern Yunnan, northern Vietnam, northern Laos, northern Thailand, and possibly also can be found in eastern Myanmar (see Fig. 3). Therefore, re-examination of specimens previously reported as *M. inornata* as well as additional specimens from other locations within the Indochinese Region are required. As noted above, mtDNA markers alone cannot serve as a solid basis for species identification (Solovyeva et al. 2023; Velo-Antón et al. 2023); therefore a thorough examination of phenotypic and multilocus nuclear data is required to obtain a more comprehensive understanding of *Micryletta* diversity and distribution in Indochina.

Micryletta hekouensis is to date known only from two national parks in Northern Vietnam and one region in southern Yunnan; in all three localities these frogs were recorded from a very specific limestone-associated habitat. It is important to notice that karst massifs in Vietnam, as well as in other parts of Southeast Asia, are facing ongoing severe threats from progressing deforestation and

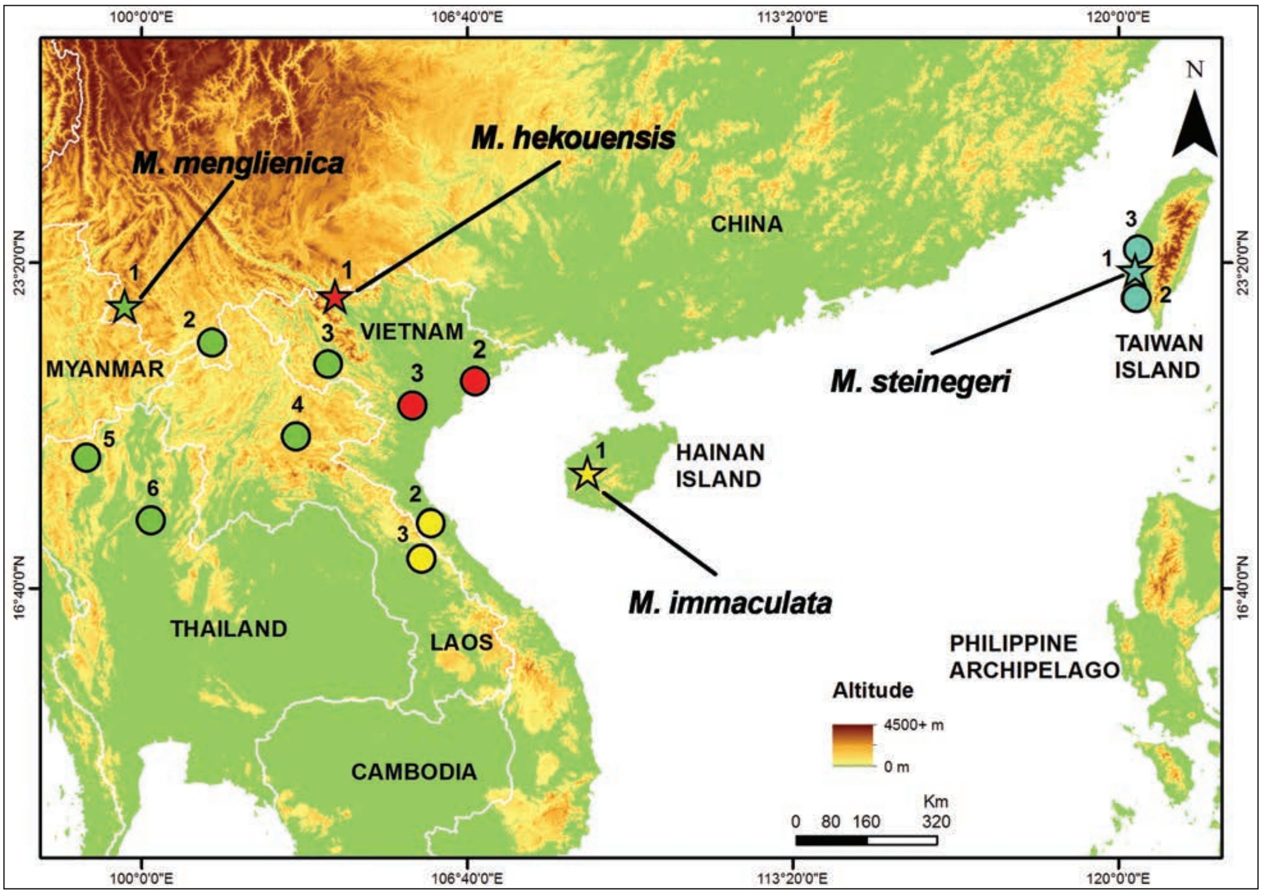

Figure 3. Distribution ranges of the species of the *Micryletta steinegeri* complex. Notes: numbers indicate different localities where the species have been recorded (see Suppl. material 2 for the details of localities)

destruction for cement manufacturing purposes; their continued exploitation for limestone cannot be stopped (Clements et al. 2006; Poyarkov et al. 2021a, 2023). Uncontrolled destruction of limestone massifs may represent the major threatening factor for the species. Although the actual distribution and population status of *Micryletta hekouensis* remains unknown, it is obvious that the species is restricted to isolated highly endangered limestone karst massifs of northern Vietnam and southern Yunnan. Additional surveys in other limestone areas of Northern Vietnam as well as southern Yunnan are essential for elucidating the biology of the species. Our new findings of additional populations of *M. hekouensis* in Vietnam expanded its extent of occurrence (EOO) to 23374 km^2. Given the available information, we suggest *Micryletta hekouensis* to be considered Near Threatened (NT) following IUCN's Red List categories (IUCN 2019).

We also take this opportunity to comment on a recent paper by Pham et al. (2023), who reported on range extension and dietary ecology of *Micryletta nigromaculata* from Son La Province of Northwestern Vietnam. Though the authors claim that the morphological characteristics of the specimens from Son La Province they examined "match well with the diagnosis of *Micryletta nigromaculata*" (Pham et al. 2023: p. 134) this statement is largely misleading, as the photos presented in their paper allow to unambiguously identify this population as *M. menglienica*. For example, in two specimens depicted in Fig. 1 of Pham et al. (2023) white markings are distinctly visible on their upper lips (a feature never observed in *M. nigromaculata*), a light hour-glass shaped pattern on dorsum is absent (always present in *M. nigromaculata*), and body flanks have wide continuous dark bands (vs. black blotches of irregular shape in *M. nigromaculata*) (see Poyarkov et al. 2018). Therefore, even by judging the published photos of both species, the Son La population can hardly be identified as *M. nigromaculata*. Herein, we would like to report that the Son La population described as "*M. nigromaculata*" by Pham et al. (2023) was misidentified and further stress the necessity of accurate comparison of specimens and diagnostic characters with museum vouchers and published information. Furthermore, as *Micryletta* species are often hard to identify by morphological data alone (though not in the case of *M. nigromaculata*), the authors should verify their identification by the means of DNA barcoding, especially in cases when they are not sure about the species identification.

Acknowledgements

We are deeply grateful to Jiangbo Zhao, Jian-Huan Yang (China) and Chung-Wei You (Taiwan) for providing photos of *Micryletta* spp. We also warmly thank Ngoc Quynh

Nguyen (SIFASV, Vietnam) for help in the preparation of the figures. We are grateful to Christopher Oldnall for English proofreading and linguistic help. We thank Günter Gollmann and three anonymous reviewers for kindly reviewing a previous version of the manuscript. NAP and AVT are grateful to Andrei N. Kuznetsov, Svetlana P. Kuznetsova (JVRT STRC), Hoi Dang Nguyen (JVRT STRC), and Leonid P. Korzoun (MSU) for support and organization of fieldwork. This research is supported in part by the Rufford Foundation (Grant No. 39897-1) and the Russian Science Foundation (RSF grant No. 22-14-00037, molecular analyses).

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Supplementary material 1

Morphological comparison of *Micryletta hekouensis* with 13 currently recognized species of the genus *Micryletta*

Authors: Tan Van Nguyen, Shuo Liu, Vy The Tran, Thinh Gia Tran, Alexei V. Trofimets, Vinh Quang Dau, Nikolay A. Poyarkov

Data type: docx

Explanation note: Symbols: (1): SVL (Males, mm); (2) SVL (Females, mm); (3) Iris color; (4) Snout in dorsal view; (5) Foot webbing; (6) Outer metatarsal tubercle; (7) Tibiotarsal articulation of adpressed limb reaching up to; (8) Supratympanic fold; (9) Dorsal skin texture; (10) Dorsal coloration; (11) Dorsal color pattern; (12) Coloration of lateral sides of the head; (13) Coloration of flanks; (14) Ventral coloration.

Link: https://doi.org/10.3897/herpetozoa.37.e120524.suppl1

Supplementary material 2

List of localities of the *Micryletta steinegeri* group complex appearing on Fig. 3

Authors: Tan Van Nguyen, Shuo Liu, Vy The Tran, Thinh Gia Tran, Alexei V. Trofimets, Vinh Quang Dau, Nikolay A. Poyarkov

Data type: docx

Link: https://doi.org/10.3897/herpetozoa.37.e120524.suppl2

ÖGH Herpetozoa
Austrian Herpetological Society

Herpetozoa 37: 123–136 (2024)
DOI 10.3897/herpetozoa.37.e122317

Redescription and a significant range extension of *Rhacophorus napoensis* Li, Liu, Yu & Sun, 2022 (Amphibia, Anura, Rhacophorinae) with the first record from Vietnam

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https://zoobank.org/BF6A5C69-4EDC-444A-8D53-9A95FF0961D9

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Academic editor: Ben Wielstra ♦ **Received** 5 March 2024 ♦ **Accepted** 2 April 2024 ♦ **Published** 25 April 2024

Abstract

The Napo Treefrog *Rhacophorus napoensis* was described based on five male specimens from Napo County, Baise City, Guangxi Autonomous Region, China. Herein, we report on new findings and a range extension of this species based on an examination of preserved specimens collected from Bac Giang and Nghe An provinces, Vietnam. Futhermore, molecular analyses of specimens *Rhacophorus* spp. previously identified as *R. rhodopus* that were reported in Bac Giang, Nghe An as well as Thanh Hoa, and Ha Tinh provinces based on mitochondrial DNA supported the morphological findings. The Vietnam specimens have a pairwise divergence of less than 2% from those of the type series of *Rhacophorus napoensis* (based on the 16s rRNA mtDNA gene). Based on the new information, we confirm the presence of *R. napoensis* in Vietnam and update the diagnostic characters of this species and distribution. We suggest the species should be considered as of Least Concern (LC) following the IUCN's Red List categories. Further studies reassessing the populations of the *Rhacophorus bipunctatus* complex are required.

Key Words

morphology, mtDNA, new record, phylogeny, redescription, *Rhacophorus bipunctatus* complex, Vietnam

Introduction

Currently, the Treefrog of the genus *Rhacophorus* Kuhl & Van Hasselt, 1822 contains 44 nominal species recognized, distributed widely across South and Southeast Asia, including India, Bangladesh, Myanmar, Indochina, Malaysia, Indonesia, and Brunei, as well as extreme Southern and southwestern China (Jiang et al. 2019; Poyarkov et al. 2021; Frost 2024). Even so, the species diversity in the genus was indicated to be much underestimated and many cryptic species have not been described until now and this underscores the need for additional studies (Kropachev et al. 2022; Li et al. 2022). Presently, 16 nominal *Rhacophorus* species are recorded from Vietnam, including: *R. annamensis* Smith; *R. calcaneus* Smith; *R. exechopygus* Inger, Orlov & Darevsky; *R. helenae* Rowley, Tran, Hoang & Le; *R. hoabinhensis* Nguyen, Pham, Nguyen, Ninh & Ziegler; *R. hoanglienensis* Orlov, Lathrop, Murphy & Ho; *R. kio* Ohler & Delorme; *R. larissae* Ostroshabov, Orlov & Nguyen; *R. marmoridorsum* Orlov; *R. orlovi* Ziegler & Köhler; *R. rhodopus* Liu & Hu; *R. robertingeri* Orlov, Poyarkov, Vassilieva, Ananjeva, Nguyen, Nguyen & Geissler; *R. trangdinhensis* Kropachev, Evsyunin, Orlov & Nguyen; *R. vanbanicus* Kropachev, Orlov, Ninh & Nguyen; *R. vampyrus* Rowley, Le, Tran, Stuart & Hoang; and *R. viridimaculatus* Ostroshabov, Orlov & Nguyen (Poyarkov et al. 2021; Kropachev et al. 2022; Frost 2024).

Among the Treefrogs occurring in the Indochinese region of South-East Asia, the *Rhacophorus bipunctatus* complex is characterized by red web on feet, green or reddish-brown dorsal colour, presence of black spots at axillary region and skin folds above the anus, currenly including three species: *R. bipunctatus* Ahl, *R. rhodopus*, and *R. napoensis* Li, Liu, Yu & Sun (Bordoloi et al. 2007; Li et al. 2022). In the past few decades the phylogenetic relationship between *R. bipunctatus* and *R. rhodopus* has attracted considerable controversy (eg. Yu et al. 2008a; Li et al. 2012; Nguyen et al. 2014; Chan et al. 2018; Chen et al. 2022). The distribution of *R. bipunctatus* has now been restricted to Northeastern India, Bangladesh, Bhutan, and Northern and Western Myanmar, whereas *R. rhodopus* is known from Southern China, Central and Southern Myanmar, Thailand, Laos, Northern southwards Langbian Plateau Vietnam, Cambodia, and Peninsular Malaysia (Chan et al. 2018; Nguyen et al. 2020a; Poyarkov et al. 2021; this study).

The Napo Treefrog, *Rhacophorus napoensis* was recently described based on five males originating from Napo County, Baise City, Guangxi Autonomous Region, China (type locality) belonging to the *R. bipunctatus* complex (Li et al. 2022). To date, this species was known only from its type locality, which is approximately four kilometers northeast (straight line) of the Chinese/Vietnamese border. The species is characterized by: comparatively medium body size (SVL 38.6–43.6 mm in males); head width greater than head length, snout pointed, loreal region oblique, tympanum distinct, maxillary teeth distinct, tongue cordiform, external single subgular vocal sac, tibiotarsal articulation reaches the snout, tibia length is greater than foot length and slightly greater than half of snout-vent length, and single outer metatarsal tubercle is flat (Li et al. 2022).

Futhermore, the Red-webbed Treefrog *Rhacophorus rhodopus* was reported to be widely distributed in the Northern southwards Langbian Plateau of Vietnam, including the following provinces: Ha Giang (Bac Me District [hereafter – Dist.]), Bac Giang (Tay Yen Tu Nature Reserve [hereafter – NR]), Ha Noi (Soc Son Dist.), Dien Bien [Muong Nhe NR], Lai Chau (Sin Ho Dist.), Lao Cai (Van Ban NR), Son La (Coipa NR, Sop Cop NR, Xuan Nha NR), Phu Tho (Xuan Son National Park [hereafter – NP]) Thanh Hoa (Xuan Lien NR), Nghe An (Pu Hoat NR, Pu Huong NR, Pu Mat NP), Ha Tinh (Vu Quang NP), Quang Binh (Phong Nha-Ke Bang NP), Quang Tri (Bac Huong Hoa NR), Thua Thien-Hue (Sao La NR), Quang Nam (Tay Giang Dist.), Kon Tum (Kon Plong Dist.), Gia Lai (Kon Ka Kinh NP, Kon Chu Rang NR), Lam Dong (Biduop-Nui Ba NP, Cat Tien NP, Di Linh Dist.), Binh Thuan (Nui Ong NR) (Nguyen et al. 2009; Hecht et al. 2013; Nguyen et al. 2014; Rowley et al. 2014; Nguyen et al. 2015b; Pham et al. 2016; Pham et al. 2017a,b; Luong et al. 2021; our data). However, the records of the *Rhacophorus rhodopus* in Vietnam are mainly based on morphological data, with very few molecular phylogeny investigations being examined. According to Nguyen et al. (2014) the Vietnamese populations from Bac Giang, Thanh Hoa, and Nghe An provinces are not conspecific with true *Rhacophorus rhodopus* from Yunnan Province, China (the province of the type locality of the species).

To address this, we examined specimens *Rhacophorus* spp. resembling *R. rhodopus* from Nghe An Province deposited in the Zoological Collections of Duy Tan University (DTU, Vietnam), Hong Duc University (HDU, Vietnam), and Zoological Museum of Lomonosov Moscow State University (ZMMU, Russia). Our results confirm that the specimens previously identified as *R. rhodopus* from Bac Giang and Nghe An provinces based both on morphological and molecular data, as well as the specimens from Thanh Hoa and Ha Tinh pronvices based on molecular data only, should be re-identified as *Rhacophorus napoensis*. We herein formally confirm the occurrence of *R. napoensis* in Vietnam, update the distribution of this species, provide additional data on its natural history and revise its diagnostic characters.

Materials and methods

Material examined

We examined 39 specimens that were previously registered as *Rhacophorus bipunctatus*, *R. napoensis*, and *R. rhodopus* from Northern and Central Vietnam, Northern and Central Myanmar, and Southern China (see Table 1, Fig. 1, and Suppl. material 1: table S1). Morphological

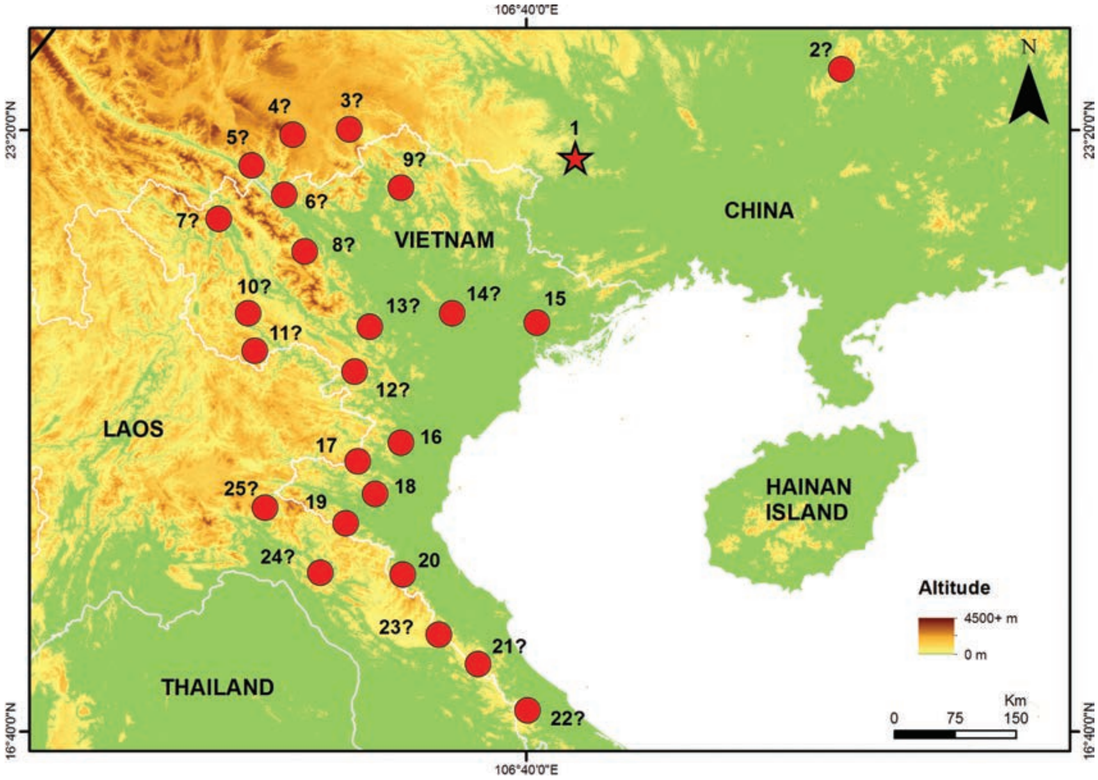

Figure 1. Distribution ranges of the species of the *Rhacophorus napoensis* in southern China and Vietnam. Notes: numbers indicate different localities where the species have been recorded (see Suppl. material 1: table S3 for the details of localities).

Table 1. Measurements (in mm) of the specimens of *Rhacophorus napoensis* from Vietnam.

Specimen number	Sex	SVL	HW	HL	SL	ED	NS	EN	TYD	TYE	UEW	IOD	IN	FLL	HAL	FeL	TbL	FoL	TbW
HDU 1179	M	39.2	14.5	14.1	6.1	5.6	3.6	3.1	3.1	1.2	4.5	5.3	5.0	8.6	20.5	20.1	19.0	27.3	3.5
HDU 1180	M	42.0	15.1	14.7	7.1	6.3	4.1	3.1	2.9	1.2	4.6	4.9	4.5	8.5	21.4	20.7	20.4	28.4	4.6
DTU 84	M	39.5	14.2	14.1	6.1	5.9	3.5	2.7	3.0	1.0	4.5	4.8	4.7	8.3	19.1	19.0	18.6	25.4	2.9
DTU 85	M	39.9	15.3	15.0	6.4	6.4	3.4	3.0	3.4	1.0	4.2	5.5	4.8	7.9	17.9	17.9	17.9	24.5	3.5
DTU 87	M	38.6	14.2	14.1	5.7	5.4	3.3	2.5	4.2	1.3	4.1	4.8	4.2	8.0	19.4	18.6	18.5	27.1	3.8
DTU 88	M	41.3	14.4	13.7	6.4	5.5	3.3	3.5	2.5	1.0	3.9	5.1	4.1	8.7	18.8	19.4	19.2	26.7	4.0
DTU 89	M	43.2	15.9	15.8	6.6	5.9	3.4	3.3	3.1	1.2	4.2	4.9	4.7	9.6	20.4	19.9	19.6	27.0	3.9
DTU 90	M	39.0	13.4	13.4	5.3	5.0	2.9	2.4	2.3	1.4	4.0	4.0	3.9	8.9	18.5	19.7	19.0	26.6	3.2
ZMMU NAP-07414	M	40.9	14.8	14.6	6.3	5.9	3.5	3.1	3.2	1.2	4.4	5.0	4.6	8.7	19.7	19.6	19.2	26.8	3.7
	Min	***38.6***	***13.4***	***13.4***	***5.3***	***5***	***2.9***	***2.4***	***2.3***	***1.0***	***3.9***	***4.0***	***3.9***	***7.9***	***17.9***	***17.9***	***17.9***	***24.5***	***2.9***
	Max	***43.2***	***15.9***	***15.8***	***7.1***	***6.4***	***4.1***	***3.5***	***4.2***	***1.4***	***4.6***	***5.5***	***5***	***9.6***	***21.4***	***20.7***	***20.4***	***28.4***	***4.6***
	Mean	***40.4***	***14.6***	***14.4***	***6.2***	***5.8***	***3.4***	***3.0***	***3.1***	***1.2***	***4.3***	***4.9***	***4.5***	***8.6***	***19.5***	***19.4***	***19.0***	***26.6***	***3.7***
	SD	***1.5***	***0.7***	***0.7***	***0.5***	***0.4***	***0.3***	***0.4***	***0.5***	***0.1***	***0.2***	***0.4***	***0.4***	***0.5***	***1.1***	***0.8***	***0.7***	***1.1***	***0.5***
HDU 1130	F	54.9	19.7	19.2	8.6	6.2	4.5	4.4	3.1	1.7	5.2	6.2	5.8	10.0	25.2	24.0	23.8	34.9	5.9
DTU 86	F	55.8	20.0	19.0	7.9	6.4	4.3	4.0	3.5	1.9	5.2	6.1	5.6	11.4	25.4	25.2	25.1	35.7	4.8
	Mean	***55.4***	***19.8***	***19.1***	***8.3***	***6.3***	***4.4***	***4.2***	***3.3***	***1.8***	***5.2***	***6.2***	***5.7***	***10.7***	***25.3***	***24.6***	***24.4***	***35.3***	***5.3***
	SD	***0.6***	***0.2***	***0.1***	***0.5***	***0.2***	***0.2***	***0.3***	***0.3***	***0.1***	***0.0***	***0.1***	***0.2***	***1.0***	***0.2***	***0.9***	***0.9***	***0.6***	***0.7***

comparisons were based on literature data from: Wilkinson et al. (2005); Ohler and Delorme (2006); Bordoloi et al. (2007); Chan and Grismer (2010); Fei et al. (2009, 2012); Rowley et al. (2012); Hecht et al. (2013); Pham et al. (2016); Nguyen et al. (2020a); Li et al. (2022); and Fajri et al. (2023) (see Suppl. material 1: table S2).

Morphological analyses

Measurements were taken using digital calipers under a light dissecting microscope to the nearest 0.01 mm, subsequently rounded to 0.1 mm. The morphometrics of adults and character terminology follow Nguyen et al. (2020b) include SVL: snout-vent length, HL: head length (from back of mandible to tip of snout), HW: maximum head width (across angles of jaws), SL: snout length (from anterior corner of eye to tip of snout), NS: distance from nostril to the tip of snout, EN: distance from anterior corner of eye to nostril, IND: internarial distance, IOD: interorbital distance, ED: eye diameter, UEW: maximum width of upper eyelid, TD: tympanum diameter, TYE: distance from anterior margin of tympanum to posterior corner of eye, FLL: forearm length (from axilla to elbow), HAL: hand length (from elbow to tip of third finger), FL1–4: finger length I–IV, OPT: outer palmar tubercle length, IPT: inner palmar tubercle length, NPL: nuptial pad length, FeL: femur length (from vent to knee), TbL: tibia length (from knee to tarsus), TbW: maximum tibia width, FoL: foot length (from tarsus to tip of fourth toe), TL 1–5: toe length I–V, IMT: inner metatarsal tubercle length, FD3: maximal diameter of disk of finger III, and TD4: maximal diameter of disk of toe IV. Terminology for describing eye colouration in living individuals followed Glaw and Vences (1997); subarticular tubercle formulas and webbing formula followed those of Savage (1975). All measurements were taken on the right side of the examined specimen. Sex was determined by gonadal inspection following dissection.

Molecular phylogeny

We focused on sequences for the mitochondrial 16S rRNA gene as it is phylogenetically informative for most tree frogs and has the largest availability of any gene for *Rhacophorus*. We synthesized previously published sequences of the *Rhacophorus bipunctatus* complex members from GenBank to estimate the phylogenetic relationships of the genus *Rhacophorus* and genetically identify samples referrable to *R. napoensis*. In addition, we sequenced a specimen (ZMMU NAP-07414) from Tay Yen Tu NR, Bac Giang Province, Vietnam. The new sequence has been deposited in GenBank under Accession No. PP391368. We used the sequences of *Zhangixalus dennysi* (Blanford) and *Z. dugritei* (David) to root the tree (GenBank accession numbers, voucher specimens, locality, and source information are summarized in Table 2).

Sequences were aligned using MAFFT 7.471 (Katoh and Standley 2013) with default parameters. Genetic divergences (uncorrected p-distance) were calculated in MEGA 11 (Tamura et al. 2021). The best substitution models were selected using the Akaike Information Criterion (AIC) in ModelFinder (Kalyaanamoorthy et al. 2017). Maximum likelihood (ML) phylogenetic analysis was performed in IQ-TREE 1.6.12 (Nguyen et al. 2015a) based on the TIM2+F+R4 model, and nodal support was estimated by 1,000 ultrafast bootstrap (UFB) replicates. Nodes with UFB values of 95 and above were considered significantly supported (Minh et al. 2013). Bayesian Inference (BI) was performed in MrBayes 3.2.7 (Ronquist et al. 2012) based on the GTR+F+I+G4 model. Two runs were performed simultaneously with four Markov chains starting from a random tree. The chains were run for 1,000,000 generations and sampled every 100 generations. The first 25% of the sampled trees were discarded as burn-in and then the remaining trees were used to estimate Bayesian posterior probabilities (BPPs). Nodes were considered well-supported if they had BPPs of 0.95 or higher (Huelsenbeck et al. 2001; Wilcox et al. 2002).

Other abbreviations. Dist.: District; Mt.: Mount; NP: National Park; NR: Natural Reserve.

Institutions and museums acronyms

CAS: California Academy of Sciences, San Francisco, USA; **CIB**: Herpetological Museum, Chengdu Institute of Biology, Chinese Academy of Sciences, Sichuan, China; **DTU**: Duy Tan University, Da Nang, Vietnam; **GXNU**: Guangxi Normal University, Guangxi, China; HDU: Hong Duc University, Thanh Hoa, Vietnam; **IEBR**: Institute of Ecology and Biological Resources, Hanoi, Vietnam; **KIZ**: Kunming Natural History Museum of Zoology, Kunming Institute of Zoology, Chinese Academy of Sciences, Yunnan, China; **NHMUK** (formerly BMNH): The Natural History Museum, London, UK; **NCSM**: North Carolina Museum of Natural Sciences, North Carolina, USA; **USNM**: National Museum of Natural History, Smithsonian Institution, Washington D.C., USA; **ZMMU**: Zoological Museum of Lomonosov Moscow State University, Moscow, Russia.

Results

The ML and BI analyses recovered trees with similar topologies (Fig. 2). With respect to the position of *R. napoensis*, our phylogenetic results largely conform to those of Nguyen et al. (2014), Chan et al. (2018), Chen et al. (2022), Li et al. (2022). The sequences related to the *R. bipunctatus* complex formed six highly divergent lineages as follows: the lineage from Southern Yunnan, China represents the true *R. rhodopus*; the lineage from Northern Myanmar and Eastern India represents the true *R. bipunctatus*; the lineage from Guangxi, China and Northeastern Vietnam represents *R. napoensis*; and the other three lineages from Western Yunnan, China; Xizang, China; and Hainan, China as well as Gia Lai, Vietnam, respectively, represent three unnamed species (Fig. 2).

The sequences from Bac Giang, Thanh Hoa, Nghe An, and Ha Tinh provinces, Vietnam and the sequences of the type series of *R. napoensis* formed a highly supported clade, and there are two sub-clades within this clade. One sub-clade contains the sequences of the type series of *R. napoensis* and the sequences from Bac Giang Province; the other sub-clade contains the sequences from Thanh Hoa, Nghe An, and Ha Tinh provinces. The uncorrected pairwise distance between these two sub-clades was 1.8% (Table 3).

Furthermore, we also examined the morphology of *Rhacophorus* spp. specimens from Tay Yen Tu NR in Bac Giang Province as well as Pu Hoat NR Pu Huong NR, and Pu Mat NP in Nghe An Province and found morphological similarities with *R. napoensis*. These results support our hypothesis that previous records of *R. rhodopus* in Northern and North-Central Vietnam (Bac Giang, Thanh Hoa, Nghe An, and Ha Tinh provinces), should be referred to as *R. napoensis*. Therefore, we extend the distribution *Rhacophorus napoensis* to Vietnam and provide an expanded diagnosis and description.

Table 2. Sequences (16S) used in molecular analyses of this study. **Remark**: *R*= *Rhacophorus*, Z= *Zhangixalus*.

Previously taxonomy	Proposed taxonnomy	Voucher No.	Locality	GenBank No.	Reference
R. annamensis	*R. annamensis*	AMNH 161414	Phong Nha-Ke Bang NP, Quang Binh, Vietnam	DQ283047	Frost et al. (2006)
R. baluensis	*R. baluensis*	FM 235958	Sabah, Borneo, Malaysia	KC961089	Hertwig et al. (2013)
R. bipunctatus	*R. bipunctatus*	PUCZM/IX/SL 360	Mizoram, India	MH087073	Lalronunga & Ramliana unpublished
R. bipunctatus	*R. bipunctatus*	PUCZM/IX/SL 312	Mizoram, India	MH087076	Lalronunga & Ramliana unpublished
R. bipunctatus	*R. bipunctatus*	CAS 229913	Putao, Kachin, Myanmar	JX219445	Li et al. (2012)
R. bipunctatus	*R. bipunctatus*	CAS 235303	Mindat, Chin, Myanmar	JX219444	Li et al. (2012)
R. borneensis	*R. borneensis*	BORN 22411	Maliau Basin, Sabah, Borneo, Malaysia	AB781694	Matsui et al. (2013)
R. chuyangsinensis	*R. calcaneus*	KIZ 746	Bidoup-Nui Ba NP, Lam Dong, Vietnam	JX219451	Li et al. (2012)
R. catamitus	*R. catamitus*	ENS 14726	Pesagi, Sumatra, Indonesia	KX398877	O'Connell et al. (2018)
R. exechopygus	*R. exechopygus*	ZFMK 86409	Phong Nha-Ke Bang NP, Quang Binh, Vietnam	GQ469980	Unpublished
R. helenae	*R. helenae*	ZFMK 92544	Tan Phu, Dong Nai, Vietnam	JQ288091	Rowley et al. (2012)
R. hoabinhensis	*R. hoabinhensis*	VNMN A.2016.16	Hang Kia-Pa Co NR, Hoa Binh, Vietnam	LC331097	Nguyen et al. (2017)
R. indonesiensis	*R. indonesiensis*	MZB 23619	Indonesia	AB983367	Hamidy and Kurniati (2015)
R. kio	*R. kio*	VNMN 4110	Kon Ka Kinh NP, Gia Lai, Vietnam	LC010589	Nguyen et al. (2014)
R. laoshan	*R. laoshan*	1705014	Tianlin, Guangxi, China	MW149528	Yuan et al. (2021)
R. lateralis	*R. lateralis*	SDB 2010.330	Bygoor, Karnataka, India	KC571277	Biju et al. (2013)
R. malabaricus	*R. malabaricus*	/	Madikeri, Karnataka, India	AB530549	Hasan et al. (2014)
R. margaritifer	*R. margaritifer*	ENS 16162	Tilu, Java, Indonesia	KX398889	O'Connell et al. (2018)
R. modestus	*R. modestus*	ENS 16853	Utara, Sumatra, Indonesia	KX398904	O'Connell et al. (2018)
R. napoensis	*R. napoensis*	GXNU YU000169	Napo, Guangxi, China	ON217794	Li et al. (2022)
R. napoensis	*R. napoensis*	GXNU YU000170	Napo, Guangxi, China	ON217795	Li et al. (2022)
R. napoensis	*R. napoensis*	GXNU YU000171	Napo, Guangxi, China	ON217796	Li et al. (2022)
R. napoensis	*R. napoensis*	GXNU YU000172	Napo, Guangxi, China	ON217797	Li et al. (2022)
R. napoensis	*R. napoensis*	GXNU YU000173	Napo, Guangxi, China	ON217798	Li et al. (2022)
R. rhodopus	*R. napoensis*	VNMN 4118	Tay Yen Tu NR, Bac Giang, Vietnam	LC010605	Nguyen et al. (2014)
R. rhodopus	*R. napoensis*	VNMN 4119	Pu Huong NR, Nghe An, Vietnam	LC010606	Nguyen et al. (2014)
R. rhodopus	*R. napoensis*	VNMN 4122	Xuan Lien NR, Thanh Hoa, Vietnam	LC010607	Nguyen et al. (2014)
R. rhodopus	*R. napoensis*	VNMN 4121	Xuan Lien NR, Thanh Hoa, Vietnam	LC010608	Nguyen et al. (2014)
R. rhodopus	*R. napoensis*	VNMN 4120	Pu Huong NR, Nghe An, Vietnam	LC010609	Nguyen et al. (2014)
R. bipunctatus	*R. napoensis*	AMNH 161418	Huong Son, Vu Quang NP, Ha Tinh, Vietnam	AY843750	Faivovich et al. (2005)
R. napoensis	*R. napoensis*	ZMMU NAP-07414	Tay Yen Tu NR, Bac Giang, Vietnam	PP391368	This study
R. nigropalmatus	*R. nigropalmatus*	KUHE 53935	Sarawak, Borneo, Malaysia	AB781701	Matsui et al. (2013)
R. norhayatiae	*R. norhayatiae*	KUHE UNL	Endau Rompin, Johor, Malaysia	AB728191	Kuraishi et al. (2013)
R. orlovi	*R. orlovi*	VNMN 3067	Huong Son, Vu Quang NP, Ha Tinh, Vietnam	LC010598	Nguyen et al. (2014)
R. pardalis	*R. pardalis*	FMNH 273243	Sarawak, Bintulu, Malaysia	JX219454	Li et al. (2012)
R. poecilonotus	*R. poecilonotus*	ENS 16480	Utara, Sumatra, Indonesia	KX398920	O'Connell et al. (2018)
R. pseudomalabaricus	*R. pseudomalabaricus*	SDB.2011.1010	Kerala, Kadalar, India	KC593855	Biju et al. (2013)
R. reinwardtii	*R. reinwardtii*	ENS 16447 (UTA)	Bandung, Sumatra, Indonesia	KY886335	O'Connell et al. (2018)
R. rhodopus	*R. rhodopus*	SCUM 060692L	Mengyang, Yunnan, China	EU215531	Li et al. (2008)
R. rhodopus	*R. rhodopus*	KUHE 47794	Jinghong, Yunnan, China	LC386573	Matsui et al. (2019)
R. rhodopus	*R. rhodopus*	Lc0805109	Lvchun, Yunnan, China	JX219440	Li et al. (2012)
R. rhodopus	*R. rhodopus*	KIZ 60821037	Puer, Yunnan, China	EF564572	Yu et al. (2008)
R. robertingeri	*R. robertingeri*	VNMN 3446	Kon Plong, Kon Tum, Vietnam	LC010615	Nguyen et al. (2014)
R. spelaeus	*R. spelaeus*	IEBR A.2011.1	Khammouan, Lao	LC331095	Nguyen et al. (2017)
R. translineatus	*R. translineatus*	Rao 6237	Motuo, Xizang, China	JX219449	Li et al. (2012)
R. tuberculatus	*R. tuberculatus*	KIZ 14154	Motuo, Xizang, China	MW111522	Wu et al. (2021)
R. vampyrus	*R. vampyrus*	VNMN 4125	Hon Ba NR, Khanh Hoa, Vietnam	LC010616	Nguyen et al. (2014)
R. rhodopus	*Rhacophorus* sp1	KIZ 060821175	Yongde, Yunnan, China	EF564573	Yu et al. (2008)
R. rhodopus	*Rhacophorus* sp1	KIZ 589	Longling, Yunnan, China	EF564578	Yu et al. (2008)
R. rhodopus	*Rhacophorus* sp1	KIZ 060821248	Jindong, Yunnan, China	EF564575	Yu et al. (2008)
R. rhodopus	*Rhacophorus* sp1	Loc 08007018	Longchuan, Yunnan, China	JX219439	Li et al. (2012)
R. rhodopus	*Rhacophorus* sp2	L06245	Motuo, Xizang, China	JX219441	Li et al. (2012)
R. rhodopus	*Rhacophorus* sp2	L062456	Motuo, Xizang, China	JX219442	Li et al. (2012)
R. rhodopus	*Rhacophorus* sp2	/	Motuo, Xizang, China	OK165559	Chen et al. (2022)
R. rhodopus	*Rhacophorus* sp2	/	Motuo, Xizang, China	OK181853	Chen et al. (2022)
R. rhodopus	*Rhacophorus* sp3	SN030035	Hainan, China	EF564579	Yu et al. (2008)
R. rhodopus	*Rhacophorus* sp3	SN030036	Hainan, China	EF564580	Yu et al. (2008)
R. rhodopus	*Rhacophorus* sp3	SN 030035	Hainan, China	EU215529	Li et al. (2008)
R. rhodopus	*Rhacophorus* sp3	/	Limu Mt., Hainan, China	EF646369	Yu et al. 2007
R. rhodopus	*Rhacophorus* sp3	/	Limu Mt., Hainan, China	EF646370	Yu et al. 2007
R. bipunctatus	*Rhacophorus* sp3	ROM 29944	Tran Lap, Gia Lai, Vietnam	AF458144	Wilkinson et al. (2002)
R. bipunctatus	*Rhacophorus* sp3	FMNH 253114	An Khe, Gia Lai, Vietnam	GQ204716	Meegaskumbura et al. (2010)
R. rhodopus	*Rhacophorus* sp3	VNMN 4117	K'Bang, Gia Lai, Vietnam	LC010604	Nguyen et al. (2014)
Outgroup					
Z. dennysi	*Z. dennysi*	SCUM 060401L	Shaoguan, Guangdong, China	EU215545	Li et al. (2008)
Z. dugritei	*Z. dugritei*	SCUM 051001L	Baoxing, Sichuan, China	EU215541	Li et al. (2008)

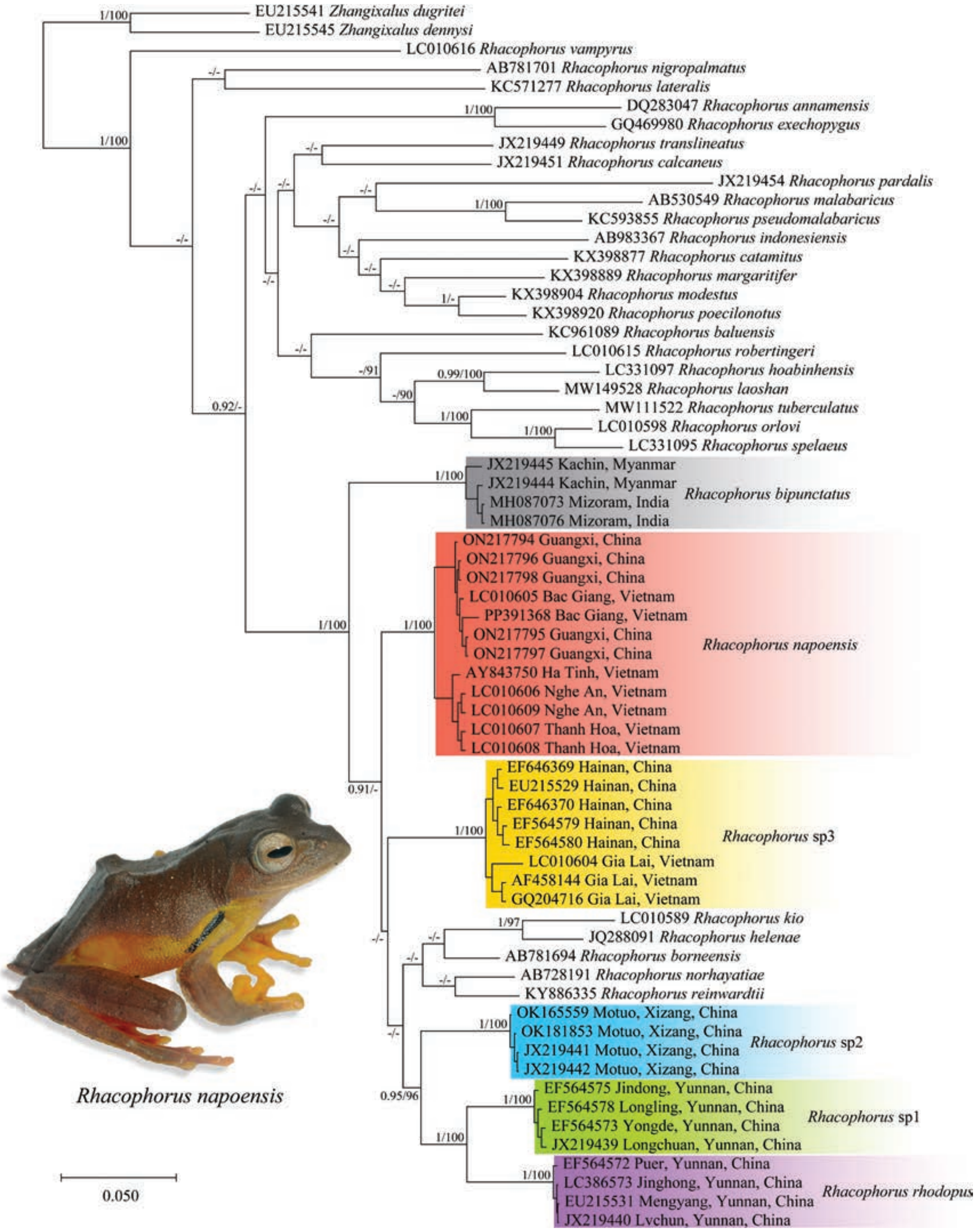

Figure 2. Maximum Likelihood (ML) tree of species in the genus *Rhacophorus* derived from an analysis of 16s mitochondrial DNA gene sequences. For voucher specimen information and GenBank accession numbers see Table 2. Numbers at tree nodes correspond to BI PP support values/ML UFBS, respectively. Photograph by N.A. Poyarkov.

Table 3. Uncorrected p-distances (%) of the 16S rRNA sequences within the *Rhacophorus bipunctatus* species complex.

No	Species	1	2	3	4	5	6
1	*Rhacophorus napoensis* (Guangxi in China & Bac Giang in Vietnam)						
2	*Rhacophorus napoensis* (Ha Tinh, Nghe An, Thanh Hoa in Vietnam)	1.8					
3	*Rhacophorus bipunctatus*	7.3	6.5				
4	*Rhacophorus rhodopus*	7.8	7.0	9.0			
5	*Rhacophorus* sp1	7.1	7.0	7.6	5.0		
6	*Rhacophorus* sp2	6.4	4.9	8.8	7.2	6.0	
7	*Rhacophorus* sp3	5.8	5.5	8.3	6.7	7.4	6.2

Taxonomic account

Rhacophorus napoensis Li, Liu, Yu & Sun, 2022

Table 1, Figs 3, 4

Holotype. GXNU YU000172, adult male collected by Shuo Liu on 25 March 2019.

Type locality. Napo County, Baise City, Guangxi Autonomous Region, China (23°1'20"N, 105°50'58"E; elevation 1032 m a.s.l.).

Chresonymy. *Rhacophorus rhodopus* (non *Rhacophorus rhodopus* Liu & Hu, 1969) – Nguyen et al. (2008: 364, in part); Nguyen et al. (2009: 181, in part); Hecht et al. (2013: 526–527, in part); Nguyen et al. (2014: 276–277, in part); Pham et al. (2016: 37–38, in part); Poyarkov et al. (2021: 61–62, in part).

Rhacophorus napoensis Li, Liu, Yu & Sun (2022: 130).

Suggested name in Vietnamese. Ếch cây Na pha

Specimens examined (n= 11). Two adult males HDU 1179–80 from Pu Hoat NR, Que Phong Dist., Nghe An Province, Vietnam (ca. 19.66313°N, 104.78185°E; elevation 1360 m a.s.l) collected by V.Q. Dau et al. on 12 April 2011. One adult female HDU 1130 from Khe Co area in Pu Huong NR, Chau Cuong Dist., Nghe An Province, Vietnam (19.33155°N, 105.0016°E, elevation 645 m a.s.l.) collected by V.Q. Dau on 22 July 2013. One adult male ZMMU NAP-07414 from Khe Cam I area within Tay Yen Tu NR, Son Dong Dist., Bac Giang Province, Vietnam (21.18058°N, 106.72683°E, elevation 181 m a.s.l.) collected by N.A. Poyarkov on 07 July 2017. Four adult males DTU 84–85, 87–88 and one adult female DTU 86 from Khe Moi area within Pu Mat NP, Con Cuong Dist., Nghe An Province, Vietnam (ca. 18.917446°N, 104.773233°E; elevation 900 m a.s.l) collected by T.V. Nguyen on 27 August 2018. Two adult males DTU 89–90 from Khe Bu area within Pu Mat NP, Tam Quang Dist., Nghe An Province, Vietnam (ca. 19.037120°N, 104.605944°E; elevation 760 m a.s.l) collected by T.V. Nguyen on 12 February 2019.

Revised diagnosis. Medium-sized within genus *Rhacophorus* (SVL 38.6–43.2 mm in males, 54.9–55.8 mm in females); snout pointed, projecting beyond margin of lower jaw in ventral view, and the tip has a distinct bulge; tympanum distinct, rounded; maxillary teeth distinct; tongue cordiform, notably notched posteriorly; external single subgular vocal sac; the tibiotarsal articulation reaches the snout; entire web between fingers and toes; single inner metatarsal tubercle, flat; banding exists in dorsal surface of limbs posterior part of dorsum; two to three black spots at axillary region; webbing not black; and dorsum reddish brown with or without small black spots (dorsum hoary in color with numerous black spots after preservation per original diagnosis); ventral yellowish white (data from Li et al. (2022) and this study).

Comparisons. We summarize the main characters separating *Rhacophorus napoensis* from the *R. bipunctatus* and *R. rhodopus* in Suppl. material 1: table S2. *Rhacophorus napoensis* differs from *R. bipunctatus* by having: lower maximum SVL in both sexes (43.2 mm in males, 55.8 mm in females vs. 50.4 mm in males, 59.1 mm in females), absent yellow pigmentation at the upper and lower portions of iris (vs. present, see Suppl. material 1: fig. S1), ventral coloration yellowish white (vs. bright yellow), dorsal coloration brown or reddish brown (vs. green or brown), snout pointed, and the tip has a distinct bulge (vs. broad and pointed), loreal region oblique (vs. concave), tympanum distinct (vs. slight indistinct), tongue cordiform, notably notched posteriorly (vs. round, slight notched posteriorly), two to three black spots at axillary region (vs. one big and one small black spot at axillary region). *Rhacophorus napoensis* differs from *R. rohdopus* by having: larger body size in males (SVL 38.6–43.2 mm [avg. 41.41 mm] vs. 31.2–38.0 mm [avg. 35.16 mm]), slightly lower ratio TbL/SVL in both sexes (0.45–0.51 [avg. 0.48] in males, 0.43–0.46 [avg. 0.45] in females vs. 0.47–0.55 [avg. 0.51] in males, 0.44–0.50 [avg. 0.47], ventral coloration yellowish white (vs. bright yellow), tongue cordiform, notably notched posteriorly (vs. narrow and long, deeply notched posteriorly), external single subgular vocal sac (vs. internal single), the tibiotarsal articulation reaches the snout (vs. reaches the eye), two to three black spots at axillary region (vs. one black or dark round spot at axillary region).

Futhemore, *Rhacophorus napoensis* can be rapidly distinguished from most of the closely-related species of the *R. reinwardtii* complex (includes: *R. borneensis* Matsui, Shimada & Sudin, *R. helenae* Rowley, Tran, Hoang & Le, *R. kio* Ohler & Delorme, *R. norhayatiae* Chan & Grismer, and *R. reinwardtii* (Schlegel)) by having a distinctly smaller body size in both sexes (SVL 38.6–43.2 mm in males, 54.9–55.8 in females vs. 50.1 mm male, 62 mm in females in *R. borneensis* (data from Matsui et al. 2013); 72.3–85.5 mm [avg. 77.7 mm] in males, 89.4–90.7 mm [avg. 90.1 mm] in females of the *R. helenae* (data from Rowley et al. 2014); 66.6–69.5 mm [avg. 68.2 mm] in males, 78.0–88.9 mm [avg. 84.6 mm] in females of *R. kio* (data from Ohler & Delorme, 2006; Fajri et al. 2023); 60.6–64.7 mm [avg. 61.9 mm] in males, 75.7–83.0 mm [avg. 80.3 mm] in females of the *R. norhayatiae* (data from Chan and Grismer 2010); and 41.6–52.5 mm [avg. 46.4 mm] in males, 66.6–74.8 mm [avg. 70.7 mm] in females of the *R. reinwardtii* (data from Fajri et al. 2023); the absence of black spots/pigmentation on webs (vs. present in all five species), brown dorsum coloration in life (vs. green in all five species), and a significantly different color pattern on flanks.

Description based on examined specimens from Vietnam (n=11). SVL 38.6–43.6 mm in males (n=9), 54.9–55.8 mm in females (n=2); habitus relatively slender; head almost equal or slight width greater than long (HW/HL 1.00–1.05 in males, 1.04–1.05 in females); snout pointed in dorsal view and slightly acuminate in profile, protruding from the margin of the lower jaw; eyes relatively larger, slightly protuberant, pupil horizontal, eye diameter slightly equal to snout length (ED/SL 0.86–1.00 in males, 0.72–0.82 in females), pineal ocellus absent. Top of head flat, canthus rostralis rounded and distinct; loreal region oblique; nostril round, small, closer to eye than to eye tip of snout (NS/EN 0.94–1.32 in males, 1.02–1.07 in females); interorbital distance slightly greater than internarial distance (IOD/IN 1.02–1.24 in males, 1.07–1.10 in females) and upper eyelid width (IOD/UEW 1.00–1.31 in males, 1.18–1.19 in females). Tympanum and supratympanic fold distinct. Vomerine teeth present; tongue cordiform, attached anteriorly, notably notched posteriorly; choanae oval; external single subgular vocal sac, vocal sac openings at bottom of the mouth on both sides.

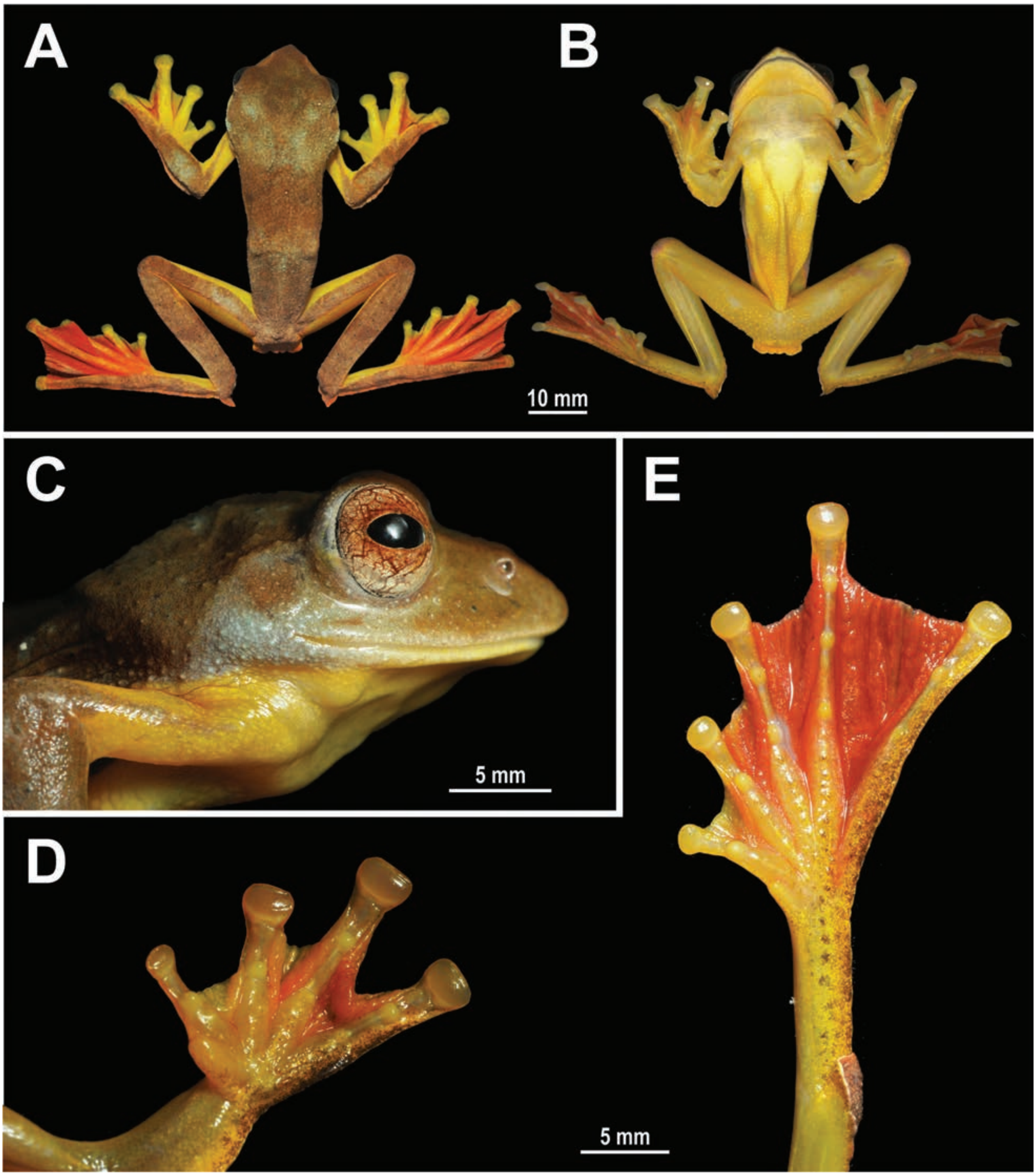

Figure 3. Morphological details of *Rhacophorus napoensis* from Tay Yen Tu NR, Bac Giang, Vietnam (ZMMU NAP-07414, adult male) **A**. Dorsal aspect; **B**. Ventral aspect; **C**. Head in lateral aspect; **D**. Volar view of left hand; **E**. Plantar view of left foot. Photographs by N. A. Poyarkov.

***Forelimbs*:** Forearm length ca. two times shorter than hand length (FLL/HAL 0.40–0.48 in males, 0.40–0.45 in females). Fingers short, tips of all fingers expanded into discs; entire web between fingers; relative finger lengths: I<II<IV<III; subarticular tubercles on fingers distinct, rounded and prominent, formula: 1, 1, 2, 2; supernumerary tubercles below the base of finger absent; single thenar (inner metacarpal) tubercle large, oval, distinct; nuptial pad present on finger I in adult males.

***Hindlimbs*:** thigh slightly longer than tibia (FeL/TbL 1.00–1.06 in males, 1.00–1.01 in females), approximately four to five times longer than wide (TbL/TbW 4.43–6.41 in males, 4.06–5.20 in females); tibiotarsal articulation of adpressed limb reaching snout; foot longer than tibia (TbL/FoL 0.68–0.73 in males, 0.68–0.70 in females). Relative toe lengths: I<II<III<V<IV; tarsal fold present; tips of all toes expanded into discs; entire webbing between toes; subarticular tubercles on toes oval and promi-

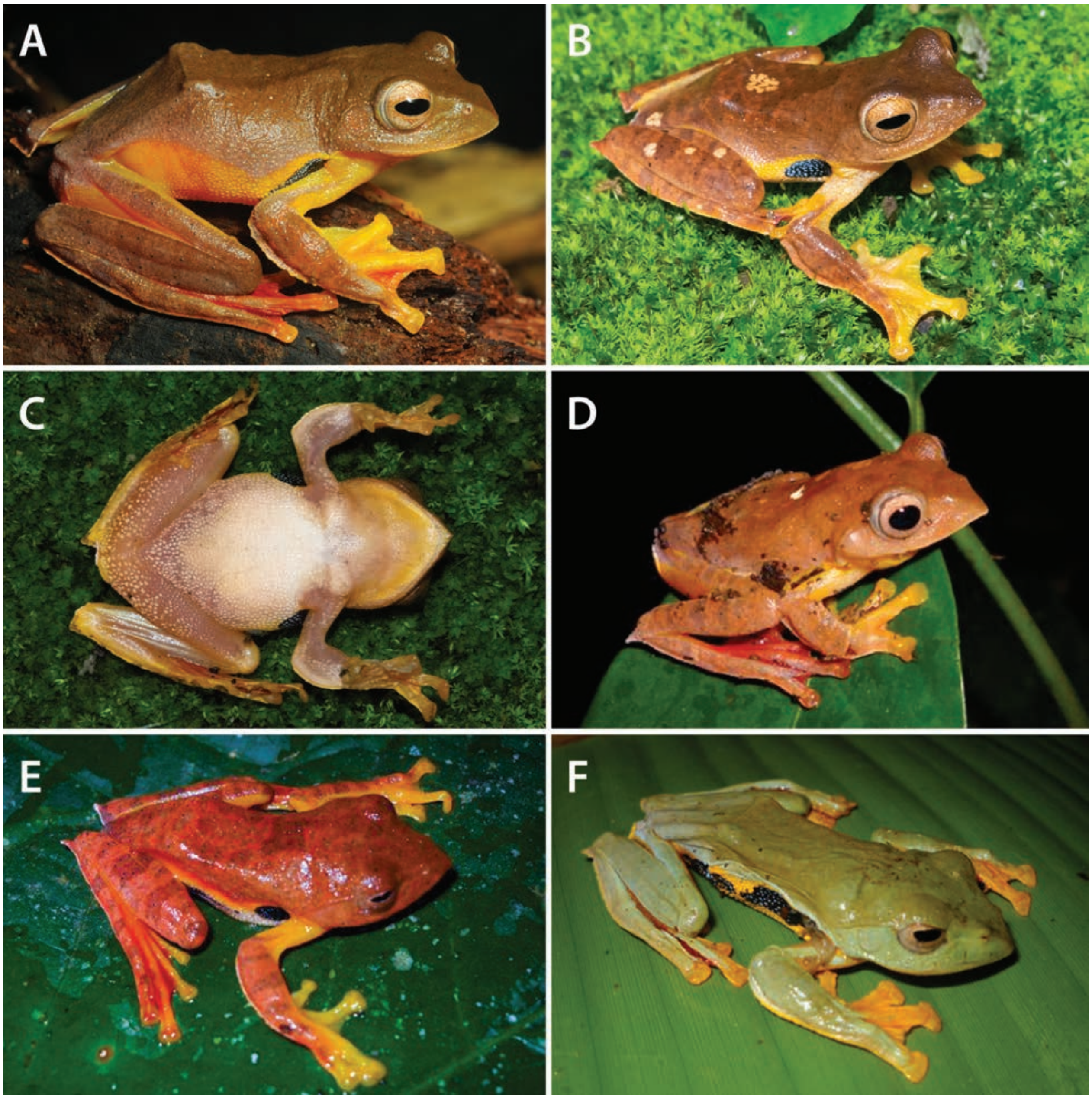

Figure 4. Alive specimens of *Rhacophorus napoensis*: from Tay Yen Tu NR, Bac Giang, Vietnam (**A**. ZMMU NAP-07414, adult male); from Pu Mat NP, Nghe An, Vietnam (**B, C**. DTU 89; adult male); from Pu Hoat NR, Nghe An, Vietnam (**D, E**. HDU 1179-1180, two adult males, respectively); from Pu Huong NR, Nghe An, Vietnam (**F**. HDU 1130, adult female). Photographs by: N. A. Poyarkov (**A**), T.V. Nguyen (**B**, **C**), and V.Q. Dau (**D, F**).

nent, formula: 1, 1, 2, 3, 2; supernumerary tubercle below the base of toe absent; inner metatarsal tubercle rounded, prominent, and small; outer metatarsal tubercle absent.

The skin of dorsal throat, ventral part of tibia, foot and tarsus smooth; the skin of chest, venter, vent and thigh rough and granular; some warts are found around the vent and flanks; dermal fringe along joint, vent and the outer sides of limbs. Cloacal dermal fringe present.

Colouration in life (Figs 3, 4): Dorsum reddish brown or gray brown with small black spots; two or three large black blotches on axilla and flanks present; transverse bands on hind limbs absent or indistinct; ventral yellowish white; webbing of fingers and toes reddish orange without black spotted. Iris copper or pale yellow.

Distribution (Fig. 1). *Rhacophorus napoensis* was previously known only from Napo County, Baise City, Guangxi Autonomous Region, China (Li et al. 2022). We here report on the first records for Vietnam including provinces, namely: Bac Giang (Tay Yen Tu NR), Thanh Hoa (Xuan Lien NP), Nghe An (Pu Hoat NR, Pu Huong NR, and Pu Mat NP), and Ha Tinh (Vu Quang NP) previously reported as *Rhacophorus rhodopus*. The new location in Huong Son area within Vu Quang NP. is ca. 520 airline kilometers south of the type locality. Given its geographic proximity, it likely occurs in several secondary and evergreen forests of Northern and North-Central Vietnam, Central Laos as well as Southern China; in particular, the records previously reported as *Rhacophorus rhodopus* from Ha Giang (Bac Me Dist.), Ha Noi (Soc Son Dist.), Lai Chau (Sin Ho Dist.), Lao Cai (Van Ban NR), Son La (Copia NR, Sop Cop NR, Xuan Nha NR); Phu Tho (Xuan Son NP); Quang Binh (Phong Nha-Ke Bang NP); Quang Tri (Bac Huong Hoa NR) provinces of Northern and North-Central Vietnam; Khammouan, Bolikhamxay, and Xiangkhoang provinces of Central Laos, as well as from southeastern Yunnan Province (Wenshan Prefecture, and Pingbian and Hekou counties) and other parts of Giangxi Autonomous Region (Jinxiu County) of China are anticipated**.**

Natural history notes. Prior to this study, the biological data of *Rhacophorus napoensis* were very limited; for example, it was only reported from an altitude of 1.032 m a.s.l. (Li et al. 2022). The species appears to be closely associated with secondary and evergreen forests with a wide range of altitudes of 400–430 mm a.s.l. from Bac Giang Province, at 870 m a.s.l. from Thanh Hoa Province, and 654–900 m a.s.l. from Nghe An Province (Hecht et al. 2013; Pham et al. 2016; this study). In Tay Yen Tu NR, Bac Giang Province, the frogs were found on trees and bushes, ca. 0.5–1.5 m above ground, near ponded water, in secondary forest. Other species of rhacophorids were recorded syntopically and included: *Kurixalus* cf. *hainanus* (Zhao, Wang & Shi), *Polypedates megacephalus* Hallowell, *Rohanixalus* cf. *hansenae* (Cochran), *Theloderma albopunctatum* (Liu & Hu), *T. corticale* (Boulenger), *T.* cf. *lateriticum* Bain, Nguyen & Doan, *Zhangixalus pachyproctus* Yu, Hui, Hou, Wu, Rao & Yang (Hecth et al. 2013; this study). In Xuan Lien NP., the frogs were found on trees and bushes, ca. 0.5–1.5 m above the ground, near ponded water, between 19:00 and 23:00. The surrounding habitat consisted of secondary forest composed of small to medium and sized hardwoods. Other species of rhacophorid were recorded syntopically and included: *Gracixalus quangi* Rowley, Dau, Nguyen, Cao & Nguyen, *Kurixalus* cf. *hainanus*, *Raorchestes* cf. *malipoensis* Huang, Liu, Du, Bernstein, Liu, Yang, Yu & Wu, *Polypedates megacephalus*, *Rhacophorus kio* Ohler & Delorme, *R. orlovi* Ziegler & Kohler, *Theloderma albopunctatum*, *Zhangixalus pachyproctus* (Pham et al. 2016; our data). In Pu Hoat NR, Nghe An Province, the frogs were found in a tree, ca. 0.5–2.0 m above the ground, more than 10 meters from the nearest stream, adjacent to a puddle in the secondary forest. The call of the frogs recorded in August is comprised of two calls types, 1–2 notes; calls types note 1, with 3–29 pulses, and calls types note 2, with 4–26 pulses of a call. The dominant frequency varied from 1,9–2,3 kHz. Other species of rhacophorids recorded syntopically included: *Gracixalus quangi*, *Gracixalus* sp., *Raorchestes* cf. *malipoensis*, *Rhacophorus kio*, *R. orlovi*, *Theloderma albopunctatum, T. gordoni* Taylor, *Zhangixalus feae* (Boulenger), *Z. pachyproctus* (Dau et al. 2012; our data). In Pu Mat NP, Nghe An Province, the frogs were found calling from leaves or branches 1–2.5 m above ground, near a ponded water, between 19:00 and 21:00. The surrounding habitat consisted of secondary forest composed of medium to larger hardwoods. During the breeding season, which lasted from May-August, this species breeds in rain pools and standing water in streams within forests. Foam nests were created on tree branches that overhang shallow, moving water (TV Nguyen pers. obs.). Other species of rhacophorids recorded syntopically included: *Kurixalus* cf. *hainanus*, *Kurixalus gracilloides* Nguyen, Duong, Luu & Poyarkov, *Polypedates megacephalus* Hallowell, *Rohanixalus* cf. *hansenae*, *Raorchestes* cf. *malipoensis*, *Rhacophorus kio*, *R. orlovi*, *Theloderma albopunctatum*, *T. lateriticum*, *T. gordoni* Taylor, and *Zhangixalus dennysi* (Blanford) (Nguyen et al. 2020a; this study)

Discussion

Rhacophorus napoensis was known previously only from Guangxi Autonomous Region, China. Based on our data and the available literature, this study provides the first records, of *R. napoensis* outside of China, from Vietnam (Bac Giang, Thanh Hoa, Nghe An, and Ha Tinh provinces). These new findings highlight that our knowledge of the herpetofauna of Vietnam, particularly of the border region between China and Vietnam (see Poyarkov et al. 2021, 2023; Nguyen et al. 2024), is still incomplete and that additional field research is required.

Presently, the range of *Rhacophorus bipunctatus* s. str. should be restricted to Northeastern India, Bangladesh, Bhutan, and Northern and Western Myanmar, while the range of *R. rhodopus* s. str. should be restricted to Southern China, Central and Southern Myanmar, Thailand, Northern and Northwestern Laos, Northwestern Vietnam, Cambodia, and Western (Peninsular) Malaysia (Bordoloi et al. 2007; Chan et al. 2018; Chen et al. 2022; this study). Consequently, herein we speculate that the records of these two species from the southeastern part of Yunnan Province and areas except Napo County in Guangxi Autonomous Region of China as reported by Yu et al. (2008a), Fei et al. (2009, 2012), AmphibiaChina (2024); Northern and North-Central Vietnam, including Ha Giang, Ha Noi, Lai Chau, Lao Cai, Son La, Phu Tho, Quang Binh, and Quang Tri provinces, which as reported by Nguyen et al. (2008), Nguyen et al. (2009), Hecht et al. (2013), Nguyen et al. (2014), Pham et al. (2016), Pham et al. (2017a,b), Tran & Le (2019, 2021), and Luong et al. (2021), and from central Laos in Nakai-Nam Theun NP, Khammuoan Province as reported based on specimen NCSM 85400 (http://portal.vertnet.org/o/ncsm/ncsm-herp?id=2d83b252-923a-4689-866b-2253f1821e00); in Viengthong, Bolikhamxay Province as reported based on specimen NCSM 80909 (http://portal.vertnet.org/o/ncsm/ncsm-herp?id=22b8590b-786d-4bab-8cbc-a2761263dc4e); and Phou Samsoum Mt. in Xiangkhoang Province as reported by Nguyen et al. (2020a) should be assigned to *R. napoensis*. Re-examinations of specimens of *R. bipunctatus* and/or *R. rhodopus* from Southeastern region of Yunnan Province and areas except Napo County of Guangxi in China, Northern and North-Central Vietnam as well as from Central Laos are required to verify these distributions.

On other hand, although reported to be widely distributed in Vietnam,the actual distribution of *R. rhodopus* in Vietnam is still quite vague. According to literature, we only confirm the record *Rhacophorus rhodopus* in Northwest Vietnam based on morphological evidence by Nguyen et al. (2015b). Nguyen et al. (2015b) reported on the *R. rhodopus* from Muong Nhe NR, Dien Bien Province with SVL 33.3–36.4 mm (n=5) in males, 45.7–49.0 mm (n=3) in females, TbL/SVL 0.52–0.53 in males, 0.51–0.53 in females. These ratios are consistent with the identification characteristics of the species *R. rhodopus* (see Suppl. material 1: table S2) and this location Muong Nhe NR is very close to the type locality of *R. rhodopus* (123 km from northwest of Mengyang Town, Yunnan Province, China). Therefore, we recommend that the population at Muong Nhe NR, Dien Bien Province, Vietnam is still assigned to the species *R. rhodopus*, although future molecular studies are required to verify this.

Futhermore, the *Rhacophorus namdaphaensis* Sarkar & Sanyal, 1985 (type locality: Miao, Tirap District, Arunachal Pradesh Sate, India) was subsequently considered as a junior synonym of *R. rhodopus* according to Bordoloi et al. (2007). However, the individuals were found in Arunachal Pradesh State, in northeastern India, far from the type locality of *R. rhodopus* in southwestern China, and according to our observations, the morphology of the individuals of this species from Arunachal Pradesh seems to be more closely related to *R. bipunctatus* than to *R. rhodopus* (see Suppl. material 1: fig. S1D) with the presence of yellow pigmentation at the upper and lower portions of the iris, which are considered diagnostic characteristics of *R. bipunctatus* (see Suppl. material 1: figs. S1, S2). In addition, the area of distribution of *R. namdaphaensis* is within the range of *R. bipunctatus* (Arunachal Pradesh Sate, India). Therefore, additional sampling of *R. namdaphaensis* at the type locality, with subsequent molecular and morphological analyses should be conducted to elucidate its taxonomic status. Futhermore, our molecular results confirm that previous findings of populations of *R.* cf. *rhodopus* from the Kon Tum-Gia Lai Plateu, Vietnam together with those from Hainan, China, as well as *R.* cf. *rhodopus* from Xizang, China, likely represent cryptic species in this complex, as revealed by other recent studies (Nguyen et al. 2014; Chan et al. 2018; Chen et al. 2022; Li et al. 2022). Therefore, future molecular and morphological studies and phylogenetic studies are required to clarify the taxonomy of the *R. bipunctatus* and *R. rhodopus* species complexes.

According to the original description of *Rhacophorus napoensis* by Li et al. (2022), this species is characterized by head width greater than head length (HW>HL), tibia length is slightly greater than a half of snout-vent length (TbL/SVL 0.50–0.51 [avg. 0.50, n=5] in males), and by the absence of nuptial pad in males. However, the specimens from Vietnam examined in this study have head width almost equal to head length (HW≥HL), tibia length less than a half of snout-vent length (TbL/SVL 0.45–0.49 [avg. 0.47, n=9] in males, 0.43–0.46 [avg. 0.44, n=2] in females); nuptial pad is present on finger I in males. Our results suggest that these characters appear to be quite variable, so they cannot be used as diagnostic characters of *R. napoensis*.

Rhacophorus napoensis is to date reliably known from seven localities including two national parks, four nature reserves in Northern and North-Central Vietnam, as well as one narrow region in Southern Guangxi Autonomous Region, China. It is an arboreal species recorded from closed-canopy evergreen forests and forest edges in lowland and montane regions of rainforest. The main threats to this species in Vietnam are habitat loss and degradation, and harvesting for food (TV Nguyen pers. obs.). Though the actual extent of distribution, population trends, reproductive behaviour and ecology of this species remain poorly known, given the available information, we suggest *Rhacophorus napoensis* be considered of Least Concern (LC) following IUCN's Red List categories (IUCN 2019).

The new record of *Rhacophorus napoensis* in this study raises the known number of the genus *Rhacophorus* species in Vietnam to 17 with nine species being endemic in this country namely: *R. helenae*, *R. hoabinhensis*, *R. hoanglienensis*. *R. larissae*, *R. marmoridorsum*, *R. trangdinhensis*, *R. vanbanicus*, *R. vampyrus*, and *R. viridimaculatus* (Poyarkov et al. 2021; Frost et al. 2024; this study). Despite recent progress, the taxonomy and distribution of the species of *Rhacophorus* species in Vietnam still remain unclear, especially of the species belonging to *R. hoanglienensis-orlovi* species complex (including: *R. hoanglienensis*. *R. larissae*, *R. marmoridorsum*, *R. trangdinhensis*, *R. vanbanicus*, and *R. viridimaculatus*); many of these species lack molecular data and their taxonomic status remains questionable. Further field surveys and taxonomic efforts both in Vietnam and in the adjacent parts of Southeast Asia and China will likely reveal additional lineages within the widely-distributed and insufficiently sampled species of the genus *Rhacophorus*.

Acknowledgements

The fieldwork in Vietnam was completed within the frameworks and with partial financial support from the research project E-1.2 of the Joint Vietnam-Russia Tropical Science and Technology Research Centre for 2017. Permission to conduct fieldwork in Bac Giang Province was granted by the Bureau of Forestry, Ministry of Agriculture and Rural Development of Vietnam and by local administration (Peoples' Committee of Bac Giang Province: #712/TCLN-BTTN of 17.05.2017). We would like to thank the management board of Tay Yen Tu NR in Bac Giang Province; Pu Hoat NR, Pu Huong NR, and Pu Mat

NP in Nghe An Province, Vietnam for their permission to work in the national reserves and the park. We are deeply grateful to Abhishek Jamalabad, Aditya Satish, Jayanta Kr Roy (India), Paul Freed (USA), Pawangkhanant Parinya (Thailand), Peter Brakels (Laos), Guangyu Liu, Ming Jue, Chenxi Liao, Cui Yu (China), Tim Adriaens (Belgium), Artur Tomaszek (Hongkong), Thomas Bader (Austria), and Bang Van Tran (Vietnam) for providing photos of *Rhacophorus* spp. We also warmly thank Ngoc Quynh Nguyen (SIFASV, Vietnam) for help in the preparation of the figures. We are grateful to Christopher Oldnall for English proofreading and linguistic help. We thank Ben Wielstra, Gabriel Hershman and an anonymous reviewer for kindly reviewing a previous version of the manuscript. NAP and AVT are grateful to Andrei N. Kuznetsov, Svetlana P. Kuznetsova (JVRT STRC), Hoi Dang Nguyen (JVRT STRC), and Leonid P. Korzoun (MSU) for support and organization of fieldwork. We thank Valentina F. Orlova and Roman A. Nazarov (ZMMU) for permission to examine specimens under their care. This research was supported in part by the Rufford Foundation (Grant No. 39897-1) and the Russian Science Foundation (RSF grant No. 22-14-00037, molecular and phylogenetic analyses).

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Supplementary material 1

Supplementary information

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Data type: docx

Explanation note: **table S1.** Measurements (in mm) of the snout-vent length (SVL) and tibia length (TbL) of the *Rhacophorus bipunctatus, R. rhodopus,* and *R. napoensis* from this study and the literature. t**able S2.** Morphological comparisons of *Rhacophorus napoensis* with *R. bipunctatus* and *R. rhodopus*. Remark: N/a= data not available, *= request verification). **table S3.** List of localities of the *Rhacophorus napoensis* appearing on Fig. 1. Remark: ?= verification required. **fig. S1.** *Rhacophorus bipunctatus* complex in life (all not collected except CAS 229893): *R. bipunctatus* – A: Kongthong, Meghalaya, India; B: Nongpoh, Meghalaya, India; C: Shillong, Meghalaya, India; D: Roing, Arunachal Pradesh, India; E: Manas NP, Assam, India; F: Lawachara NP, Sylhet, Bangladesh; G: Putao, Kachin, Myanmar (CAS 229893, Holotype of *R. htunwini*); H: Myitkyina, Kachin, Myanmar; *R.* cf. *bipunctatus* – I: Linzhi, Xizang, China. Photographs by: A. Jamalabad (A), A. Satish (B), A. Sanglyne (C), J.K. Roy (D), S. Harikrishnan (E), P. Freed (F), H. Win (G), P. Pawangkhanant (H), and C. Yu (I). **fig. S2.** *Rhacophorus rhodopus* complex in life (all not collected): *R. rhodopus* – A: Mengla, Yunnan, China; B: Xishuangbanna, Yunnan, China; C: Doi Inthanon NP., Chiangmai, Thailand; D: Suan Phueng, Ratchaburi, Thailand; E: Phou Samsoum Mt., Xiangkhouang, Laos; F: Cameron Highlands, Pahang, Malaysia; G: Fraser's Hill, Pahang, Malaysia; *R.* cf. *rhodopus* – H: Jianfengling NP, Hainan, China, I: Tay Giang, Quang Nam, Vietnam. Photographs by: G.Y. Liu (A), J. Ming (B), T. Adriaens (C), P. Pawangkhanant (D), P. Brakels (E), T. Bader (F), A. Tomaszek (G), C.X. Liao (H), and B.V. Tran (I).

Link: https://doi.org/10.3897/herpetozoa.37.e122317.suppl1

ÖGH Herpetozoa
Austrian Herpetological Society

Herpetozoa 37: 137–140 (2024)
DOI 10.3897/herpetozoa.37.e123069

First evidence of scavenging behaviour in *Ichthyosaura alpestris* (Laurenti, 1768)

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Academic editor: Günter Gollmann ♦ **Received** 14 March 2024 ♦ **Accepted** 1 April 2024 ♦ **Published** 26 April 2024

Abstract

The alpine newt is an opportunistic predator whose diet consists predominantly of aquatic and terrestrial invertebrates with Crustacea, Plecoptera, Collembola, Diptera, Isopoda and Gastropoda being the predominant prey groups. Consumption of amphibian eggs and larvae are another common food source. Here, we report the first documented case of adult *Ichthyosaura alpestris* feeding on a rodent carcass, thus adding to its menu a prey item that belongs to the Class Mammalia, while showcasing the previously unknown scavenging behaviour. This finding suggests that rodent carcasses can serve as an important resource for nutrients in remote, high-altitude populations of the alpine newt.

Key Words

alpine newt, Pindus, predator-prey relationship, Urodela

Scavenging behaviour is typically used to overcome food resource scarcity (Cramer 2008; Amor et al. 2010) and is showcased by almost all carnivorous vertebrates (DeVault et al. 2003; Selva and Fortuna 2007). However, only the vultures are obligate scavengers (Ruxton and Houston 2004; Walker et al. 2021), whereas most others (e.g. coyotes, freshwater turtles etc.) are facultative scavengers (DeVault and Krochmal 2002; DeVault et al. 2003; Selva and Fortuna 2007; Pereira et al. 2014; Santori et al. 2020), thus capitalising on opportunity. In amphibians, there are no known obligate scavengers; nevertheless, facultative scavenging has been recorded in some frog species (Nishikawa and Ochi 2016; Bassett et al. 2023) and in one salamander species (Unger 2018). In newts specifically, one such record exists where several individuals of the species *Triturus cristatus* were observed consuming a fish carcass (Iftime and Iftime 2011). Although scavenging is considered random and opportunistic behaviour, it plays a crucial role in the food web as it enhances nutrient recycling and community stability (Wilson and Wolkovich 2011). In harsh environments, such as high-altitude alpine ecosystems or arid environments where food and prey availability are scarce and unpredictable, such adaptive flexibility in foraging behaviour could be an important feature for the animals' survival (Castilla et al. 2011).

The alpine newt *Ichthyosaura alpestris* (Laurenti 1768) is a small-sized newt species, native to Europe known to occupy permanent or ephemeral waterbodies, such as lakes, troughs or puddles and, within Greece, it can be found in elevations between 659 m and 2,350 m (Tzoras 2023). The species is a polyphagous opportunistic predator exhibiting significant daily (Salvidio 2019), seasonal and spatial plasticity (Heiss et al. 2013) in the predator-prey interaction. Its multiphasic lifestyle, with adults shifting from a terrestrial to an aquatic lifestyle and vice versa (Heiss et al. 2013), its presence in high altitude lakes (Schabetsberger and Jersabek 1995) and its temporal diel activity shifts (Fasola and Canova 1992) have forced it to expand its diet as an adaptive response to the variation of food availability.

PENSOFT.

In alpine karst lakes, the alpine newt can be placed at the higher level of the trophic pyramid as a top predator (Schabetsberger and Jersabek 1995). The alpine newt's diet, feeding habits and predatory behaviour have been studied thoroughly in many European countries including, but not limited to, Romania (Covaciu-Marcov et al. 2010; Kovács et al. 2010; Bogdan et al. 2011), Italy (Fasola and Canova 1992; Joly and Giacoma 1992; Salvidio et al. 2019), Czechia (Kopecký et al. 2011, 2016), Austria (Schabetsberger and Jersabek 1995; Heiss et al. 2013) and Greece (Mettouris and Giokas 2017). Most studies were carried out predominantly through the method of flushing the stomach contents (Solé et al. 2005) of a high number of alpine newt individuals, thus allowing satisfactory results on the diet composition. The alpine newt's diet largely consists of invertebrates, such as Crustacea, Plecoptera, Collembola, Isopoda, Coleoptera, Diptera and Homoptera. Other prey categories include tadpoles (Dimancea et al. 2011) and *Salamandra salamandra* larvae (Bogdan et al. 2011). Non-discriminatory oophagy has also been documented against their own or other amphibian eggs (Kopecký et al. 2011; Mettouris and Giokas 2017). Own and conspecific sloughs were also found to be regularly consumed as classic non-prey items (Griffiths 1986; Schabetsberger and Jersabek 1995; Kopecký et al. 2011). Vegetal items have also been known to be accidentally ingested during the feeding process (Covaciu-Marcov et al. 2010; Kovács et al. 2010; Bogdan et al. 2011; Kopecký et al. 2011), something that has been documented in other amphibian species as well (Dolmen and Koksvik 1983; Sas et al. 2005). Inorganic elements from the substrate sediment have been found in the alpine newt's stomach contents and are also attributed to accidental ingestion (Bogdan et al. 2011; Kopecký et al. 2011). Within the current literature, the opportunistic behaviour of the newts is highlighted (Joly and Giacoma 1992; Denoël and Andreone 2003; Kovács et al. 2010). However, there is no reference to an alpine newt or to any other newt species feeding on an animal prey item that does not belong to any of the previously mentioned categories, but to the Class Mammalia.

Herein, we provide the first record of alpine newts feeding on a rodent carcass (Order Rodentia). The observation was made mid-day in October 2023 during a field survey to assess the presence of newt species in the sub-alpine lake, "Kosmeou" also known as "Tsouka Rossa" (39°51.825'N, 21°2.322'E, 1690 m a.s.l.) in the Province of Metsovo, Ioannina, Greece. Kosmeou Lake spans just over 100 m in length and over 60 m in width with varying depth between half and 2 metres and it is constantly supplied with water from low output springs (Fig. 1).

Towards the end of the survey, a rodent carcass at the shallow end of the lake bank was observed. Upon closer inspection, two male and one female alpine newts were seen nibbling on the carcass, managing to remove and swallow small pieces of it (Fig. 2). To eliminate potential confounding factors affecting the newts' feeding behaviour, like the presence of invertebrates on the carcass, a kick-net with a 0.5 mm - opening mesh was used to bring out the dead rodent. The carcass was thoroughly inspected to identify any hidden aquatic invertebrates or worms that could explain this uncommon newt behaviour. The examination revealed no evidence of invertebrates, forcing us to hypothesise that the newts were indeed lured to the carcass itself. The carcass was then placed back to the water in a different site and closer to the lake's overflowing point. Within minutes three female alpine newts approached it and started eating its decomposing flesh

Figure 1. Lake Kosmeou in Ioannina, Greece.

Figure 2. A female alpine newt feeding on a decomposing rodent carcass.

again. Notably, the sympatric newt *Triturus macedonicus* had no interest in the carcass.

These scavengers revealed the hitherto unknown foraging behaviour in *I. alpestris*, expanding upon previous research on its diet and further indicating that newts are generalists and opportunists, capable of adopting an energy-efficient behaviour to acquire food in demanding conditions. Given the current loss of suitable aquatic habitats and breeding sites due to agriculture and agrochemical pollution (in Greece, the populations of the Peloponnese are classified as Endangered according to Valakos et al. (2008)), dietary research can complement management actions via the identification of suitable habitats. Such research also possesses intrinsic value towards fully comprehending the alpine newt's biology and natural history.

Acknowledgements

We would like to thank Ilias Strachinis for a pre-peer review of the manuscript.

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ÖGH Herpetozoa
Austrian Herpetological Society

Herpetozoa 37: 141–147 (2024)
DOI 10.3897/herpetozoa.37.e108976

Diet, sexual dimorphism, and fertility aspects of *Melanophryniscus fulvoguttatus* (Mertens, 1937) from Central-East Brazil

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Academic editor: Lukas Landler ♦ **Received** 4 July 2023 ♦ **Accepted** 12 April 2024 ♦ **Published** 8 May 2024

Abstract

Melanophryniscus fulvoguttatus is a small toad that inhabits the Cerrado, Atlantic Forest, and humid Chaco of South America. Due to its aposematic coloration and behavior, it is a species that may present chemical defenses such as alkaloids and a restricted diet similar to other species of the genus. Although it was described in the early 20th century, there is still little information on its natural history and ecology. Here, we described the diet, tested the sexual dimorphism, and calculated the fertility index of *M. fulvoguttatus* based on a sample of eight males and 21 females from a population within the Cerrado. Sexual dimorphism was assessed using 15 morphometric measures; diet was calculated as an index of relative importance by prey item and tested for sex differences; fertility was tested for relations between body size and the number of eggs; and we also estimated the reproductive investment. Our results demonstrate that this population's diet consisted mostly of ant species, with *Pheidole radoszkowskii* being the most important, followed by *Crematogaster* sp. and *Cyphomyrmex transversus*. Regarding sexual dimorphism, we observed that females were significantly larger than males, with a positive relationship between female size and the number of eggs. We did not find significant differences in the diets of males and females. Although the diet and reproductive aspects of *M. fulvoguttatus* are similar to those of its congeneric species, further studies are needed in order to address the relationship between the species diet and its chemical defenses.

Key Words

Bufonidae, food webs, natural history

Introduction

Melanoprzyniscus is a South American genus of small-sized toads whose distribution includes Bolivia, Paraguay, Argentina, Brazil, and Uruguay (Frost 2023). The genus is currently composed of 31 species arranged in three species groups based on warts, snout protuberances, and skin coloration (Cruz and Caramaschi 2003; Frost 2023). Furthermore, *Melanophryniscus* is one of the few genera within Bufonidae with known alkaloids, which is a chemical defense against predators (Wells 2007; Grant et al. 2012). In addition, species of this genus typically display the unken reflex, the defensive behavioral response of arching the back and rising the limbs and head, thus exposing aposematic red fingers and palms, presumably to avoid subjugation against predators (Wells 2007; Toledo et al. 2011; Hantak et al. 2013).

Melanophryniscus fulvoguttatus is a relatively medium-sized toad from the *M. stelzneri* species group typically found in the Cerrado, Atlantic Forest, and humid Chaco from Argentina, Paraguay, and Brazil (Weiler et al. 2003; Frost 2023). *M. fulvoguttatus* is classified as Least

Concern (Baldo and Langone 2004). However, species within this genus are reported to be illegally commercialized; the last conservation assessment took place more than 15 years ago (Baldo and Langone 2004), and it inhabits biomes that face intensive habitat losses due to anthropogenic pressures (Carvalho et al. 2009; Mereles and Rodas 2014; Mohebalian 2022).

Individuals of *M. fulvoguttatus* species present skin spines in their head, dorsum, and flanks, whereas body spines are located in shallow warts (Cruz and Caramaschi 2003). The skin is predominantly dark brown with dorsal yellow spots and ventral yellow and red spots (Weiler et al. 2003). Additionally, its hands and palms are bright red, a coloration pattern that has been associated with the unken reflex (Cruz and Caramaschi 2003; Weiler et al. 2003). Its aposematic coloration and behavior suggest the presence of chemical defenses such as alkaloids, as occurs in several species of Anura, including *Melanoprhyniscus* (Wells 2007; Daly et al. 2008). This pattern is repeated all over the world, including species of Myobatrachidae (Australia; Sague et al. 2023), Mantellidae (Madagascar; Clark 2006), Dendrobatidae, and Bufonidae (Central and South America; Drast et al. 2005; Daly et al. 2007). Additionally, anurans with this kind of defense usually sequester their alkaloids from the diet, making necessary specialist habits and diets that are mostly composed of ants and mites (Darst et al. 2005; Saporito et al. 2007; Saporito et al. 2009a; Walters et al. 2023).

Other variables that affect the quantity of chemical defense acquired by the diet are those related to body size, like snout-vent length and wet skin mass (Jeckle et al. 2015), factors that defer between sexes, such as the fact that usually in anurans females are larger than males (Shine 1975), a common pattern in *Melanophryniscus* (Bidau et al. 2011).

Despite the current panorama of *M. fulvoguttatus* and its potential for the discovery of unknown chemical defenses, multiple natural historical features such as diet, tadpole morphology, advertisement call, reproductive mode, and reproductive strategies remain unknown. Therefore, it is paramount to unveil the multiple aspects of this species natural history in order to assist with future conservation assessments. In order to better understand the natural history aspects of this species, we described the diet, tested the sexual dimorphism, and calculated the fertility index of this conspicuous toad from South America.

Material and methods

A total of 29 individuals (eight males and 21 females) of *Melanophryniscus fulvoguttatus* were collected in a Cerrado area from Bela Vista municipality, Mato Grosso do Sul state, Central-East Brazil (21º59'53.0"S, 56º43'25.0"W; SISBIO license 49080-5). Organisms were euthanized with lidocaine 5%, fixed in formaldehyde 10%, and preserved in alcohol 70%. All samples were housed at the Coleção Zoológica da Universidade Federal de Mato Grosso do Sul (ZUFMS-AMP 15777–15805).

The stomachs of 29 collected specimens were removed by ventral dissection, and they were subsequently preserved in 70% alcohol at ZUFMS-AMP along with the specimens. Samples with empty stomachs (six females and two males) were not considered in the analyses. Diet items were classified by order level, and prey belonging to the Formicidae (Hymenoptera) were identified by genera and species level whenever possible. From there, we calculated an index of relative importance (IRI) per prey category present in the diet following Pinkas et al. (1971): IRI = (N%+V%)*F%, where F% is the relative frequency of an item, N% is the relative number of an item, and V% is the relative volume of an item. To calculate the relative volume, we used a chamber made with microscopy slides nearest to 1 mm³ (Camera et al. 2014; Carrillo et al. 2022). The comparison for possible differences between male and female diets was made through a PERMANOVA analysis using the volume of consumed prey. All statistical analyses were conducted using the vegan package for the R platform (Oksanen et al. 2022; R-Core-Team-D 2022).

All organisms collected were characterized as sexually mature by direct inspection of gonads. We used the 15 morphometric measurements following Heyer et al. (1990) and Cruz and Caramaschi (2003) to assess the sexual dimorphism in size and shape: snout vent length (SVL), head length (HL), head width (HW), inter orbital distance (IOD), inter nostril distance (IND), eye diameter (ED), eye nostril distance (END), nostril snout distance (NSD), upper eyelid width (UEW), thigh length (THL), tibia length (TL), foot length (FL), foot and tarsus length (FTL), hand length (HAL), and forearm length (FAL). Measurements were taken using a caliper with a minimal accuracy of 0.01 mm or under a dissecting microscope when needed. We tested for differences in body size between sexes using a principal component analysis (PCA) and a multivariate analysis of variance (MANOVA) with the two first components of the PCA.

Considering the females collected, we also tested whether larger females are capable of investing more in oocytes. This was done by assessing the relationship between body size and the number of eggs. Eggs were removed using a ventral dissection and preserved in 70% alcohol at ZUFMS-AMP along with the specimens. We compared the female size (SVL) and the number of oocytes using a linear model. The reproductive investment was calculated following Pereira and Maneyro (2018): RI = number of Oocytes/SVL. Oocyte size was considered as the mean of the oocyte diameter per female, using 10 oocytes per female.

Results

The most consumed order was Hymenoptera (Table 1), over Arachnida, Coleoptera, and Diptera. Within Hymenoptera, most prey items belonged to the Formicidae. *Pheidole radoszkowskii* (Formicidae) was the most common species in frequency, volume, and total number, thus being the most important species consumed (IRI = 1088.49), followed by *Crematogaster* sp. (IRI = 233.52) and *Cyphomyrmex transversus* (IRI = 190.11). There were no significant differences in diet composition or in the volume of prey between males and females (F = 0.60, p = 0.55).

We observed a significant difference in mean body size between males and females of *Melanophryniscus fulvoguttatus* (F = 3.62, p = 0.04; Fig. 1). Despite overlap in the size ranges of several morphometric measurements (Table 2), on average, females are larger than males (SVL: 22.87 ± 1.31 and 20.91 ± 0.39 mm, respectively).

Females of *Melanophryniscus fulvoguttatus* presented an average of 110.9 ± 79.94 eggs (from 12 to 246 mm). Egg polarization was always well defined. There was a positive relation between the size (SVL) of females and the number of eggs (F = 14.95; p = 0.001), in which larger females presented more eggs than the small ones (Fig. 2). The mean egg size was 1.10 ± 0.06 mm. The reproductive investment was estimated at 4.89 ± 3.47 (from 0.56 to 10; Fig. 3).

Table 1. Diet of *Melanophryniscus fulvoguttatus* from Bela Vista, Mato Grosso do Sul (Brazil). Number of prey items (N), Frequency (F), Volume in mm³ (V), and index of relative importance (IRI).

	N	N%	F	F%	V	V%	IRI
Arachnida							
Araneae	1	0.24	1	4.76	1	0.24	1.19
Insecta							
Coleoptera	2	0.48	2	9.52	8	1.91	19.1
Diptera	1	0.24	1	4.76	2	0.48	2.39
Hymenoptera							
Formicidae							
Pheidole radoszkowskii	176	42	12	57.14	46	10.98	1088.49
Cyphomyrmex transversus	36	8.59	7	33.33	19	4.53	190.11
Wasmannia auropunctata	6	1.43	1	4.76	1	0.24	1.48
Crematogaster sp.	128	30.55	5	23.81	18	4.3	233.52
Non Formicidae	24	5.73	4	19.05	12	2.86	70.96
Vegetation	2	0.48	2	9.52	5	1.19	11.93
Undetermined	43	10.26	11	52.38	37	8.83	553.18

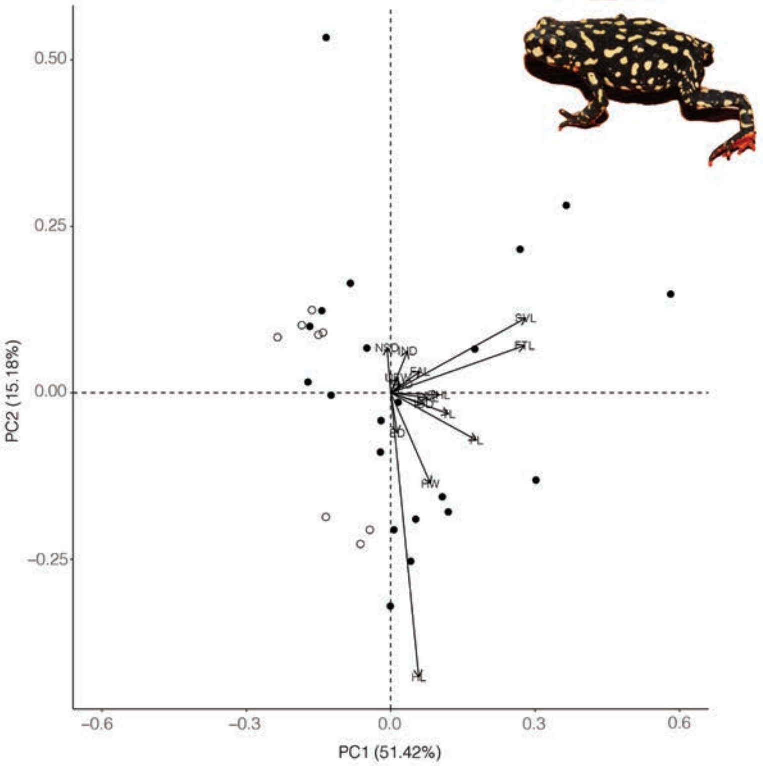

Figure 1. Principal components analysis for male (white) and female (black) body measures. Snout vent length (SVL), head length (HL), head width (HW), inter orbital distance (IOD), inter nostril distance (IND), eye diameter (ED), eye nostril distance (END), nostril snout distance (NSD), upper eyelid width (UEW), thigh length (THL), tibia length (TL), foot length (FL), foot and tarsus length (FTL), hand length (HAL) and forearm length (FAL).

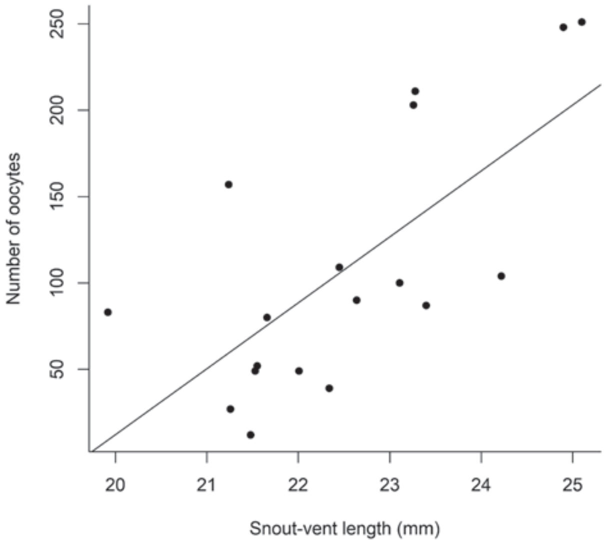

Figure 2. Relationship between female snout vent length and number of eggs of *Melanophryniscus fulvoguttatus*.

Table 2. Mean, standard deviation, and range (mm) of 15 morphometric features of *Melanophryniscus fulvoguttatus* from Bela Vista, Mato Grosso do Sul (Brazil).

	Female (N= 21)	Male (N= 8)
Snout vent length	22.87 ± 1.31 (20.55–25.63)	20.91 ± 0.39 (20.29–21.40)
Head length	5.96 ± 1.15 (3.05–7.90)	5.65 ± 0.92 (4.66–6.95)
Head width	7.07 ± 0.67 (5.52–8.57)	6.67 ± 0.33 (6.14–7.20)
Inter orbital distance	2.70 ± 0.81 (1.56–4.55)	2.99 ± 0.83 (1.67–4.05)
Inter nostril distance	1.68 ± 0.37 (0.90–2.40)	1.45 ± 0.55 (0.66–2.15)
Eye diameter	1.68 ± 0.33 (1.06–2.23)	1.67 ± 0.51 (1.07–2.67)
Eye nostril distance	1.74 ± 0.19 (1.15–2.19)	1.72 ± 0.31 (1.46–2.41)
Nostril Snout distance	1.26 ± 0.33 (0.69–1.80)	1.13 ± 0.18 (0.82–1.33)
Upper eyelid width	2.31 ± 0.46 (0.84–2.87)	2.39 ± 0.34 (1.81–2.92)
Thigh length	7.73 ± 0.69 (6.78–9.44)	6.99 ± 0.55 (6.31–7.65)
Tibia length	7.23 ± 0.53 (6.62–8.40)	6.45 ± 0.41 (5.94–7.17)
Foot length	6.57 ± 1.03 (4.56–9.10)	6.44 ± 0.87 (5.60–8.31)
Foot and tarsus length	10.64 ± 1.42 (8.85–14.67)	9.96 ± 0.81 (8.90–10.94)
Hand Length	5.13 ± 0.41 (4.73–6.25)	4.77 ± 0.31 (4.40–5.40)
Forearm Length	5.49 ± 0.40 (4.98–6.50)	5.24 ± 0.30 (4.86–5.77)

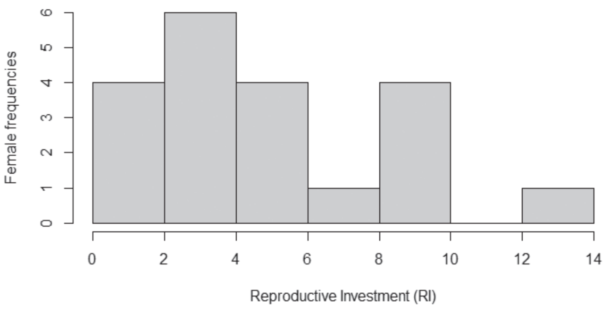

Figure 3. Frequency of the reproductive investment in females of *Melanophryniscus fulvoguttatus*.

Discussion

All the most important items in the diet of *Melanophryniscus fulvoguttatus* belonged to the Order Hymenoptera (Formicidae), followed by Coleoptera and Diptera. Although species of *Melanophryniscus* typically have a very restricted diet, the most important prey are usually Hymenoptera and Aracnida (Acari), as in *M. devincenzii*, *M. rubriventris*, *M. stelzneri*, *M. klappenbachi* and *M. cupreuscapularis* (Filipello and Crespo 1994; Bustos-Singer and Rodriguez 1997; Bonansea and Vaira 2007; Daly et al. 2008; Quiroga et al. 2011; Bortolini et al. 2013); hence, the results presented herein indicate that *M. fulvoguttatus* may have the most restricted diet within *Melanophryniscus* (Bonansea and Vaira 2007; Bortolini et al. 2013). Although we did not account for prey availability in this study, we highlight that the ant species *Pheidole radoszkowskii*, *Crematogaster* sp., and *Cyphomyrmex transversus* (Formicidae) were the most relevant for the diet of *M. fulvoguttatus*. Similarly to the ants found in *M. klappenbachi*, *M. rubriventris*, and *M. stelzneri*, the ant species reported herein all present chemical defenses and alkaloids (Filipello and Crespo 1994; Bonansea and Vaira 2007; Quiroga et al. 2011; Arias et al. 2021). Additionally, it has been reported that the diet of *M. rubriventris* can change between populations, which may also be the case with *M. fulvoguttatus* (Bonansea and Vaira 2007; Quiroga et al. 2011).

Multiple species of anurans present chemical defense complemented with aposematic colorations, e.g., Bufonidae, Dendrobatidae, and Mantellidae (Saporito et al. 2009a). Some of its congenerics have alkaloids described, i.e., *M. stelzneri*, *M. rubriventris*, and *M. klappenbachi* (Daly et al. 2007, 2008; Mebs et al. 2018; Arias et al. 2021). These species also presented a similar diet to *M. fulvoguttatus*. In fact, diet is a main factor in frogs's chemical defenses; species with alkaloids-rich skins usually consume a very specific diet to sequester those substances from ants and mites; otherwise, toxicity decreases (Mebs et al. 2018). In this context, the diet and coloration of *Melanophryniscus fulvoguttatus* are typical of frog species that use alkaloids as chemical defense, and they need to be confirmed experimentally.

The redescription of *Melanophryniscus fulvoguttatus* (Cruz and Caramaschi 2003) presented a clear difference in size between males and females, with descriptive statistics indicating females are larger than males, which is in agreement with the results presented herein. Moreover, it is worth mentioning that our sample included males and females smaller than the redescription. This may be because we only analyze organisms from one locality, while the redescription included samples from six localities (Cruz and Caramaschi 2003). Nevertheless, sexual dimorphism has been demonstrated in numerous amphibian species (Shine 1975; Wells 2007), including the congeneric *M. montevidensis* (Pereira and Maneyro 2018). Furthermore, these differences may be a pattern that changes between populations or geographically (Yu et al. 2010). Sexual size dimorphism within bufonids is well known and well-established in most of the toads' genera (e.g., Mueses-Cisneros et al. 2012; Peloso et al. 2012; Rojas et al. 2022). Additionally, species from other families such as Ranidae, Hylidae, and Leptodactylidae have shown variation in size and sexual dimorphism due to geographic variation (Yu et al. 2010; Boaratti and Da Silva 2015). Size also may affect the quantity of acquired chemical defenses that can be sex-related, as proven in *Oophaga pumilio* and *Melanophryniscus moreirae* (Saporito et al. 2009b; Jeckel et al. 2015); however, this relation has not been tested in *M. fulvoguttatus*.

Bufonidae species mainly invest in large clutches of small eggs, which, in theory, depend on the availability of environmental energy (Vitt and Caldwell 2014). For instance, *Melanophryniscus krauczuki* has clutches of up to 401 eggs (Baldo and Basso 2004). The females of *Melanophryniscus fulvoguttatus* here analyzed presented similar clutch sizes to other congeneric species of *Melanophryniscus*, such as *M. stelzneri* and *M. montevidensis* (Bustos-Singer and Rodriguez 1997; Cairo et al. 2008; Pereira and Maneyro 2018). Species of *Melanophryniscus* are usually explosive breeders, and reproduction relies on temporary ponds formed after heavy rainfalls (Cairo et al. 2008). Eggs can be laid under water or above the water surface, depending on the species (Bustos-Singer and Rodriguez 1997; Vaira 2005; Goldberg et al. 2006; Cairo et al. 2008). On the other hand, a phytotelmata reproductive mode has also been described for the genus (Langone et al. 2008; Steinbach-Padilha 2008; Bornschein et al. 2015). In this case, female investment is in small clutches like *M. alipioi* with 16 eggs (Bornschein et al. 2015). Now that we know that phytotelmata present smaller clutches, it would be expected to find bigger eggs than those from species from temporary ponds like *M. fulvoguttatus*, but it needs to be tested.

Still, there is no specific information on the reproduction of *M. fulvoguttatus*. Although it belongs to the *M. stelzneri* species group and reproduction may be similar, we do not know if there are variations for *M. fulvoguttatus* in reproductive period, reproductive mode, egg laying, amplexus, advertisement call, or call locations.

Further analyses of the diet should account for prey availability to determine the selectivity of *Melanophryniscus fulvoguttatus* for ant-related diets for chemical defenses such as alkaloid sequestering. It is necessary to describe the skin-acquired chemical defenses to compare with the diet, establish a direct relationship with the chemical defenses presented in the community of arthropods that compose the *M. fulvoguttatus* diet, and study if there are additional factors involved, such as body size or sex. Additionally, the rate of acquisition of chemical defenses is unknown, and the efficiency of chemical defense acquisition may be an interesting ecological feature to explore between the genus.

Reproductive investment from species that reproduce in phytotelmata needs to be described and compared with water egg-laying species. Besides, several reproductive features mentioned above remain unknown, and they need to be described for a better understanding of the ecology and reproduction of the species, thus helping future conservation assessments to enhance species preservation.

Acknowledgements

Mariáh Tibcherani helped with the taxonomic determination of the ant species. Lukas Landler and two anonymous reviewers provided valuable suggestions that greatly improved our work. This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) - Finance Code 001, and by Universidade Federal de Mato Grosso do Sul. Carrillo JFC thanks Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) - Funding code 001. DJS thanks Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq process 404239/2021-8) and Fundação de Apoio ao Desenvolvimento do Ensino, Ciência e Tecnologia do Estado de Mato Grosso do Sul (FUNDECT process 054/2017) for financial support. DJS also thanks CNPq for his research fellowship (CNPq 309420/2020-2).

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ÖGH Herpetozoa
Austrian Herpetological Society

Herpetozoa 37: 149–156 (2024)
DOI 10.3897/herpetozoa.37.e121610

First record of *Amolops truongi* Pham, Pham, Ngo, Sung, Ziegler & Le, 2023 (Anura, Ranidae) from China

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Academic editor: Peter Mikulíček ♦ **Received** 25 February 2024 ♦ **Accepted** 16 April 2024 ♦ **Published** 29 May 2024

Abstract

We report the first record of *Amolops truongi* Pham, Pham, Ngo, Sung, Ziegler & Le, 2023 from China, based on four specimens collected in Yunnan Huanglianshan National Nature Reserve. This species was previously known only from the type locality in north-western Vietnam. Morphologically, the specimens from China correspond to the original description of *A. truongi* with a few variations and, phylogenetically, they are clustered with the type specimens of *A. truongi* from Vietnam with strong support. In this study, we also provide an updated diagnosis of this species combining the original description and new data.

Key Words

distribution, morphology, ND2, updated diagnosis, Yunnan Huanglianshan National Nature Reserve

Introduction

Amolops Cope, 1865 is a genus with a rapid increase in the number of species recently, making it the most speciose genus within the family Ranidae and having a wide distribution range from southern and eastern Himalayas to the Peninsular Malaysia (Dever et al. 2012; Pham et al. 2019; Wu et al. 2020; Mahony et al. 2022; Tang et al. 2023; Frost 2024; Li et al. 2024). Currently, 85 species are included in the genus (Frost 2024) and 52 have been recorded in China (AmphibiaChina 2024). However, it is important to note that the taxonomic systems of Frost (2024) and AmphibiaChina (2024) have different views on the validity of some species, thus not allowing a direct comparison of the total number of species in the genus *Amolops* and the number of species recorded in China.

Amolops truongi Pham, Pham, Ngo, Sung, Ziegler & Le, 2023 is a species which was originally discovered in Muong La District, Son La Province, north-western Vietnam (Pham et al. 2023). Currently, *A. truongi* is known only from the type locality (Pham et al. 2023).

During our herpetological expedition in Yunnan Huanglianshan National Nature Reserve, Yunnan Province, China, in 2023, we collected some specimens of *Amolops*. Morphological and molecular data show that four of them should be assigned to *A. truongi*. Herein, we report the distribution of this species in China for the first time, describe the specimens collected from China and provide an updated diagnosis of this species.

PENSOFT.

Material and methods

The fieldwork was carried out under the permission of Yunnan Huanglianshan National Nature Reserve Management and Protection Bureau. Specimens were collected by hand at night. Liver tissues were stored in analytical pure ethanol and specimens were preserved in 75% ethanol. The newly-collected specimens were deposited at Kunming Natural History Museum of Zoology, Kunming Institute of Zoology, Chinese Academy of Sciences (KIZ).

Measurements were taken with a digital caliper to the nearest 0.1 mm. The methodology of measurements followed Pham et al. (2023) and the terminology describing the webbing formula followed Glaw and Vences (2007). The following morphological characteristics were recorded: snout-vent length (SVL), from the tip of the snout to the cloacal; head length (HL), from the back of the mandible to the tip of the snout; maximum head width (HW), across the angles of the jaws; snout length (SE), distance from the tip of the snout to the anterior corner of the eye; nostril to snout distance (SND), from the nostril to the tip of the snout; eye to nostril distance (END), from the anterior corner of the eye to the nostril; internarial distance (IND), the distance between nostrils; upper eyelid width (UEW), the maximum width of the upper eyelid; interorbital distance (IOD), the minimum distance between the upper eyelids; horizontal eye diameter (ED), from the anterior corner to the posterior corner of the eye; tympanum diameter (TD), the maximum diameter of the tympanum; tympanum-eye distance (TED), from the anterior margin of the tympanum to the posterior corner of the eye; fore-limb length (FLL), from the tip of the disc of the third finger to the axilla; finger III disc width (FTD), the maximum width of the disc of the third finger; hindlimb length (HLL), from the tip of the disc of the fourth toe to the groin; femur length (FL), from the vent to the knee; tibia length (TL), from the knee to the tarsus; foot and tarsus length (FOT), from the tarsus to the tip of the fourth toe; toe IV disc width (HTD), the maximum width of the disc of the fourth toe; inner metatarsal tubercle length (MTTi), the maximum length of the inner metatarsal tubercle.

A fragment of the mitochondrial NADH dehydrogenase subunit 2 gene (ND2) was amplified via the polymerase chain reaction (PCR) using the primers Met-LND2: 5′–CAATGTTGGTTAAAATCCTTCC–3′ and Trp–HND2: 5′–AGGCTTTGAAGGCCTTTGGTC–3′ (Stuart et al. 2006). The experimental protocols of amplification and sequencing followed Pham et al. (2023). Sequences were assembled using SeqMan in Lasergene 7.1 (Burland 2000). All new sequences have been deposited in GenBank and additional ND2 sequences used in this study were also obtained from GenBank (Table 1).

The sequences were aligned using ClustalW (Thompson et al. 1994) integrated in MEGA X and the genetic divergences (uncorrected p-distance) were calculated in MEGA X (Kumar et al. 2018). The best substitution models GTR+F+I+G4 for Bayesian Inference and TIM+F+I+G4 for Maximum Likelihood phylogenetic analysis were selected using the Akaike Information Criterion (AIC) in ModelFinder (Kalyaanamoorthy et al. 2017). Maximum Likelihood phylogenetic analysis was performed in IQ-TREE 1.6.12 (Nguyen et al. 2015) with the nodal support estimated by 1,000 ultrafast bootstrap replicates. Bayesian Inference was performed in MrBayes 3.2.7 (Ronquist et al. 2012) and the Markov chains run for 1,000,000 generations and sampled every 100 generations. The first 25% of the sampled trees were discarded as burn-in and the remaining trees were used to estimate Bayesian posterior probabilities.

Results

The morphological measurements of the specimens from China are presented in Table 2. There is no significant difference in morphological characteristics between the specimens from China and the type specimens of *Amolops truongi* from Vietnam, except for minor variations. Genetically, Bayesian Inference and Maximum Likelihood phylogeny obtained the same topology, with slightly different supports for some nodes. The sequences of the specimens from China clustered with the type specimens of *A. truongi* from Vietnam with strong support (Fig. 1). The genetic distance (uncorrected p-distance) between the specimens from China and the type specimens of *A. truongi* was only 0.4% (Table 3).

Taxonomic account

Amolops truongi Pham, Pham, Ngo, Sung, Ziegler & Le, 2023

Figs 2, 3

Specimen examined. KIZ2023080–KIZ2023083, four adult males, all collected on 22 July 2023 by Shuo Liu from Qimaba Township, Luchun County, Honghe Prefecture, Yunnan Province, China (22°56'29"N, 102°6'52"E, elevation 1430 m a.s.l.).

Description of the specimens from China. Male body size relatively small, SVL 39.3–39.9 mm in adult males; head moderate long (HL/SVL 0.36–0.38), longer than wide (HL/HW 1.09–1.15); snout relatively long (SE/SVL 0.16), projecting beyond lower jaw; canthus rostralis distinct; loreal region concave; distance from nostril to snout tip equal to or slightly greater than distance from eye to nostril (SND/END 1.00–1.03); internarial distance greater than interorbital distance (IND/IOD 1.22–1.39); upper eyelid width narrower than interorbital distance (UEW/IOD 0.78–0.94); pupil oval, horizontal; tympanum distinct (TD/ED 0.35–0.40); tympanum-eye distance smaller than tympanum diameter (TED/TD 0.77–0.90); vomerine teeth present; choanae rounded; tongue cordiform, notched posteriorly; vocal sac opening on floor of mouth at corner, sac-like gular pouch, front margin positioned near to level of centre of orbit.

Table 1. Samples used for the phylogenetic analyses in this study.

Species	Voucher	Locality	Accession
Amolops adicola	BNHS 6121	Arunachal Pradesh, India	MZ231116
Amolops akhaorum	FMNH 271355	Luang Namtha, Laos	FJ417207
Amolops akhaorum	FMNH 271406	Luang Namtha, Laos	FJ417208
Amolops aniqiaoensis	SYNU04ll6016	Tibet, China	MN958715
Amolops aniqiaoensis	KIZ011136	Tibet, China	MN958717
Amolops archotaphus	CUMZ A 2000.62	Chiang Mai, Thailand	FJ417173
Amolops archotaphus	KIZ030888	Chiang Mai, Thailand	MN958719
Amolops cf. *bellulus*	KIZYPX9037	Yunnan, China	MN958723
Amolops cucae	AMNH 168727	Lao Cai, Vietnam	FJ417193
Amolops cucae	AMNH 168729	Lao Cai, Vietnam	FJ417194
Amolops compotrix	FMNH 256499	Khammouane, Laos	FJ417185
Amolops compotrix	FMNH 256500	Khammouane, Laos	FJ417190
Amolops chunganensis	KIZ03756	Hubei, China	MN958729
Amolops daorum	ROM 38501	Lao Cai, Vietnam	FJ417199
Amolops deng	KIZ014115	Tibet, China	MW111443
Amolops iriodes	AMNH 163926	Ha Giang, Vietnam	FJ417201
Amolops iriodes	AMNH 163928	Ha Giang, Vietnam	FJ417202
Amolops kohimaensis	WIIADA 751	Nagaland, India	MZ231118
Amolops mengdingensis	KIZ20160265	Yunnan, China	MK501814
Amolops mengdingensis	KIZ20160266	Yunnan, China	MK501815
Amolops monticola	WIIADA 544	Sikkim, India	MZ231117
Amolops nyingchiensis	KIZ012629	Tibet, China	MN958773
Amolops nyingchiensis	KIZ016416	Tibet, China	MW133377
Amolops putaoensis	GXNU W011	Kachin, Myanmar	MT901213
Amolops putaoensis	GXNU W005	Kachin, Myanmar	MT901214
Amolops truongi	IEBR 4995	Son La, Vietnam	OP157199
Amolops truongi	ZVNU.2022.01	Son La, Vietnam	OP157200
Amolops truongi	KIZ2023080	Yunnan, China	PP663261
Amolops truongi	KIZ2023081	Yunnan, China	PP663262
Amolops truongi	KIZ2023082	Yunnan, China	PP663263
Amolops truongi	KIZ2023083	Yunnan, China	PP663264
Amolops tuanjieensis	GXNU YU110003	Yunnan, China	MN832756
Amolops tuanjieensis	GXNU YU110006	Yunnan, China	MN832757
Amolops tuberodepressus	SCUM050433CHX	Yunnan, China	MN958786
Amolops viridimaculatus	KIZ048488	Yunnan, China	MN958789
Amolops vitreus	FMNH 258183	Phongsaly, Laos	FJ417212
Amolops vitreus	FMNH 258187	Phongsaly, Laos	FJ417213
Amolops wenshanensis	KU 292045	Guangxi, China	FJ417178
Amolops wenshanensis	KIZ021425	Yunnan, China	MG996763

Fore-limb moderate long (FLL/SVL 0.66–0.68); relative length of fingers III > IV > II > I; tips of outer three fingers expanded into discs with circum-marginal grooves; webbing between fingers absent; subarticular tubercles present, oval, formula 1, 1, 2, 2; supernumerary tubercles absent; inner metacarpal (thenar) tubercle large, oval; outer metacarpal tubercle indistinct; glandular nuptial pad on finger I.

Hind-limb relatively long (HLL/SVL 1.78–1.84); tibia longer than thigh length (TL/FL 1.08–1.12); relative length of toes IV > V > III > II > I; all toe tips expanded into discs; webbing between toes deeply incurved, webbing formula I0–1/2II0–1III0–1IV1–0V; subarticular tubercles distinct, oval, formula 1, 1, 2, 3, 2; inner metatarsal tubercle elongated; outer metatarsal tubercle absent.

Dorsal and lateral surface of head and body smooth with few very small tubercles present on temporal sides of head, above tibiae and vent; supratympanic fold indistinct; dorsolateral fold distinct; ventral surface smooth with flat tubercles on basal ventral thigh.

Colouration in life. Dorsal sides of head and body green or olive brown with some black dots; lateral side of head and tympanum dark brown or black; a white stripe extending from tip of snout to shoulder on each side; iris pale gold; flanks light brown or brown; dorsal surface of fore-limbs and hind-limbs light brown or brown with dark bands; throat, chest and belly cream with some brown dots; vocal sac orange or light yellow; ventral surface of fore-limbs light red or flesh-coloured; ventral surface of hind-limbs red flesh-coloured or flesh-coloured with some dark brown dots; toe webbing dark brown.

Updated diagnosis. SVL 37.5–41.3 mm in adult males, 61.5–62.5 mm in adult females; head moderate long (HL/SVL 0.35–0.38 in males, 0.35–0.36 in females), longer than wide; snout relatively long (SE/SVL 0.16 in males, 0.15 in females); vomerine teeth present; tympanum distinct, round (TD/ED 0.35–0.40 in males, 0.36–0.37 in females); skin smooth; supratympanic fold indistinct; dorsolateral fold present; fore-limb moderate long (FLL/SVL 0.65–0.72 in males, 0.64–0.66 in fe-

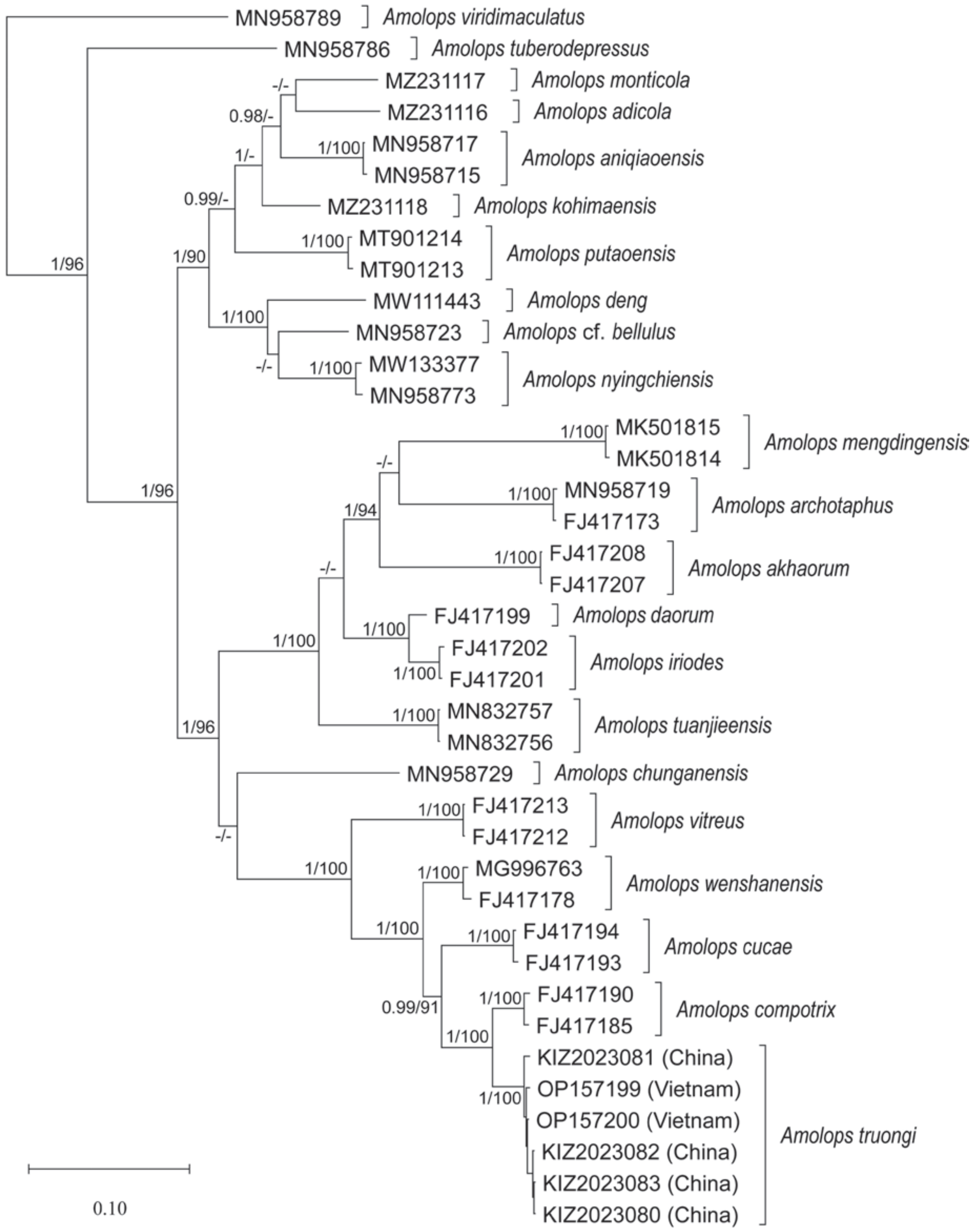

Figure 1. The Bayesian phylogenetic tree, based on the ND2 sequences. The numbers after and behind the "/" are the Bayesian posterior probabilities and the Maximum Likelihood ultrafast bootstrap values (> 0.90/90), respectively.

males); hind-limb relatively long (HLL/SVL 1.78–1.92 in males, 1.77–1.79 in females); webbing formula I0–1/2II0–1III0–1IV1–0V; external vocal sac present and finger I with nuptial pad in adult males.

Distribution. *Amolops truongi* is currently known from the type locality in Son La Province, north-western Vietnam and Lvchun County, Honghe Prefecture, southern Yunnan Province, China (Fig. 4).

Recommended common name. We suggest 山罗湍蛙 (Pinyin: shān luó tuān wā) as the Chinese name, deriving from the type locality Son La Province, Vietnam.

Figure 2. Dorsal view (top) and ventral view (bottom) of the specimens of *Amolops truongi* from China in preservative.

Table 2. Measurements (in mm) and proportions of the specimens of *Amolops truongi* from China (for abbreviations, see Material and methods).

	KIZ2023080	**KIZ2023081**	**KIZ2023082**	**KIZ2023083**
	Male	**Male**	**Male**	**Male**
SVL	39.4	39.7	39.3	39.9
HL	15.0	14.3	14.8	14.4
HW	13.3	13.1	13.4	12.5
SE	6.5	6.2	6.4	6.3
SND	3.4	3.4	3.2	3.2
END	3.4	3.3	3.2	3.2
IND	5.0	5.0	5.0	5.0
UEW	3.4	3.4	3.2	3.0
IOD	3.6	4.1	4.1	3.8
ED	5.6	5.4	5.7	5.3
TD	2.2	2.0	2.0	2.1
TED	1.7	1.8	1.7	1.7
FLL	26.1	27.0	26.2	26.5
FTD	1.9	2.0	1.8	1.9
HLL	70.8	72.9	70.0	71.6
FL	21.5	22.9	21.4	21.4
TL	23.6	24.7	23.9	23.8
FOT	32.2	32.4	31.9	32.4
HTD	1.6	1.6	1.6	1.5
MTTi	1.8	1.7	1.7	1.6
HL/SVL	0.38	0.36	0.38	0.36
HW/SVL	0.34	0.33	0.34	0.31
HL/HW	1.13	1.09	1.10	1.15
SE/SVL	0.16	0.16	0.16	0.16
SND/END	1.00	1.03	1.00	1.00
IND/IOD	1.39	1.22	1.22	1.32
UEW/IOD	0.94	0.83	0.78	0.79
ED/HL	0.37	0.38	0.39	0.37
ED/SE	0.86	0.87	0.89	0.84
TD/ED	0.39	0.37	0.35	0.40
TED/TD	0.77	0.90	0.85	0.81
FLL/SVL	0.66	0.68	0.67	0.66
HLL/SVL	1.80	1.84	1.78	1.79
TL/FL	1.10	1.08	1.12	1.11

Discussion

The morphological characteristics of the specimens of *Amolops truongi* from China mostly agree with the original description by Pham et al. (2023), but there are also a few differences between them. The distance from the nostril to the snout tip is equal to or slightly greater than the distance from the eye to the nostril in the specimens from China, while the distance from the nostril to the snout tip is smaller than the distance from the eye to the nostril in the original description. The upper eyelid width is narrower than the interorbital distance in the specimens from China. while the upper eyelid width is wider than the interorbital distance in the original description. In addition, according to the original description of this species, the colour of the dorsum is light grey, while some specimens from China have a green dorsum. Therefore, based on these variants, we revised the diagnosis of this species.

Yunnan Huanglianshan National Nature Reserve is located in the south of Yunnan Province, it has a variety of terrain and vegetation types, which has long established its rich species diversity. In recent years, many new species have been discovered in this region, such as frogs *Leptobrachella aspera* Wang, Lyu, Qi & Wang, 2020 (Wang et al. 2020) and *Raorchestes huanglianshan* Jiang, Wang, Ren & Li, 2020 (Jiang et al. 2020) and plants *Agapetes heana* Y.H. Tong & J.D. Ya (Tong et al. 2021) and *Primula zhengyii* Bin Yang & Y.H. Tan (Yang et al. 2023). In addition, Yunnan Huanglianshan National Nature Reserve borders Vietnam in the south, so it is not surprising to find species that were previously considered to be distributed only in Vietnam. Previously, 52 species of the genus *Amolops* were recorded in China, 20 of which were distributed in Yunnan (Tang et al. 2023; Li et al. 2024). Our discovery of *A. truongi* in Yunnan increased the number of *Amolops* species in China to 53 and the number of *Amolops* species in Yunnan to 21.

Figure 3. Dorsal, lateral and ventral views of the male specimen KIZ2023081 (**A–C**) and dorsal, lateral and ventral views of the male specimen KIZ2023082 (**D–F**) of *Amolops truongi* from China in life.

Table 3. Uncorrected pairwise genetic distance (%) matrix, based on ND2 sequences.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
1 *Amolops adicola*																				
2 *Amolops akhaorum*	17.4																			
3 *Amolops aniqiaoensis*	8.1	14.8																		
4 *Amolops archotaphus*	17.7	13.3	17.0																	
5 *Amolops* cf. *bellulus*	10.7	15.8	11.7	16.3																
6 *Amolops chunganensis*	13.5	14.6	13.5	15.8	11.8															
7 *Amolops compotrix*	15.1	17.3	15.3	18.8	15.7	15.3														
8 *Amolops cucae*	15.8	16.8	15.3	17.2	15.4	15.1	7.5													
9 *Amolops daorum*	16.8	12.1	15.9	12.3	15.1	13.7	17.0	17.6												
10 *Amolops deng*	12.9	15.9	13.3	17.0	8.4	13.9	16.8	15.3	14.1											
11 *Amolops iriodes*	17.1	12.4	15.7	13.1	15.1	13.6	17.0	17.3	2.8	14.3										
12 *Amolops kohimaensis*	7.0	15.6	6.6	16.3	9.4	13.3	14.9	14.8	14.6	10.4	14.9									
13 *Amolops mengdingensis*	18.6	14.1	16.2	13.4	17.0	16.1	18.3	17.9	13.7	17.2	14.1	17.1								
14 *Amolops monticola*	8.3	16.3	8.3	17.5	11.2	14.2	16.0	15.7	16.1	12.0	16.0	8.2	17.5							
15 *Amolops nyingchiensis*	13.1	16.5	12.0	16.8	7.7	13.8	16.2	16.2	14.7	8.6	14.4	10.5	17.5	11.5						
16 *Amolops putaoensis*	11.4	15.8	10.3	16.9	11.0	15.1	16.2	15.7	15.8	12.6	15.3	9.7	17.9	9.3	12.0					
17 *Amolops truongi* (China)	15.5	16.9	14.9	18.5	15.4	15.0	4.0	7.8	16.8	16.6	16.6	14.7	18.2	16.2	16.1	16.4				
18 *Amolops truongi* (Vietnam)	15.2	16.6	14.7	18.4	15.3	15.0	3.6	7.5	16.4	16.6	16.2	14.6	17.9	15.8	16.1	16.1	0.4			
19 *Amolops tuanjieensis*	17.2	13.1	15.1	13.0	15.4	14.2	16.5	16.5	9.5	15.0	10.3	13.9	15.0	14.5	14.5	14.8	16.9	16.8		
20 *Amolops vitreus*	14.4	15.6	14.6	16.9	14.7	13.9	11.1	12.1	16.0	14.8	16.1	13.4	18.6	14.0	15.7	14.6	12.0	11.5	14.8	
21 *Amolops wenshanensis*	15.0	15.5	14.3	16.6	15.1	13.6	6.8	6.7	15.8	15.7	15.3	14.0	17.1	13.9	14.8	14.7	7.1	6.8	14.5	10.2

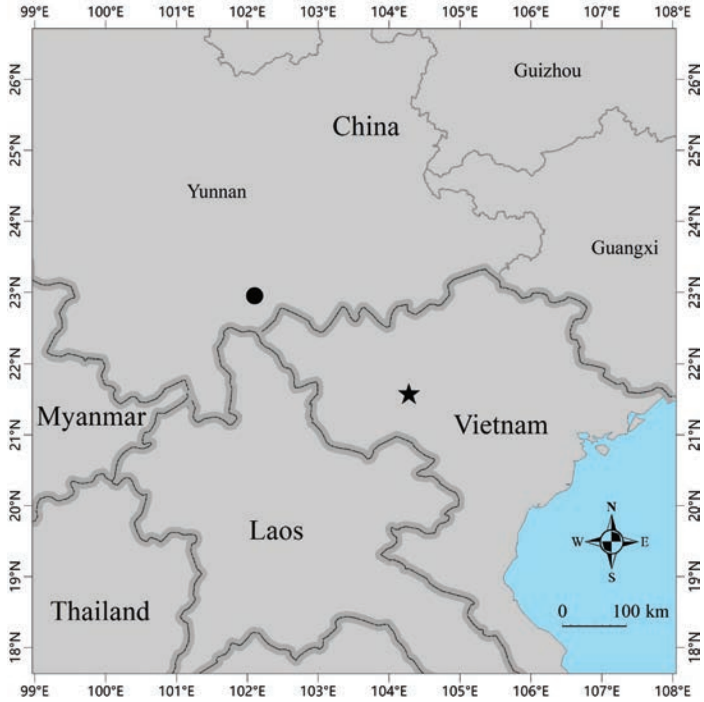

Figure 4. Map showing the type locality of *Amolops truongi* in north-western Vietnam (black star) and the new collection site from Lvchun County, Yunnan Province, China (black dot).

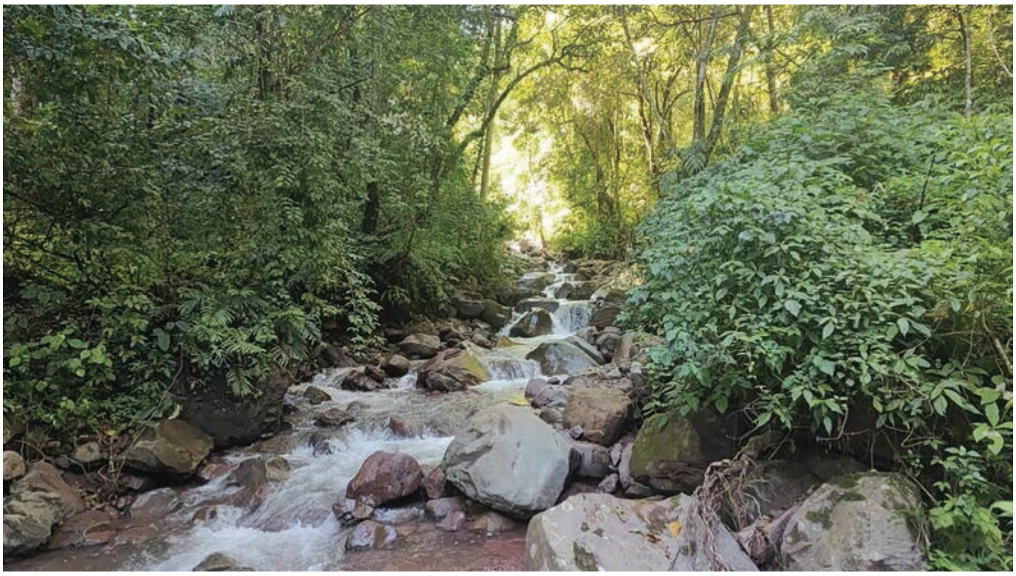

Figure 5. Habitat of the specimens of *Amolops truongi* collected in China.

Amolops truongi was previously known only from the type locality in north-western Vietnam (Pham et al. 2023). The new collection site in China extends the distribution range of this species to the northwest by approximately 290 km. The altitude of the type locality is 1360 m a.s.l., while the altitude of the new collection site in China is 1430 m a.s.l., the altitude difference between the two localities not being significant. According to Pham et al. (2023), the type specimens of this species were found on trees or limestone cliffs nearby a stream; however, the newly-collected specimens from China were all found on herbaceous plants nearby a river (Fig. 5), this being slightly different from those at the type locality. In addition, Pham et al. (2023) did not mention the courtship calls and breeding season of the species. During the surveys, we only collected males and did not find females or tadpoles and did not hear the courtship calls of males. Therefore, we are also unable to determine the breeding habits of this species. More field observations are needed to understand the habits and ecological information of *A. truongi*.

Acknowledgements

We thank the forest rangers of Yunnan Huanglianshan National Nature Reserve for their assistance in the fieldwork. We would like to thank the editors and reviewers for their valuable comments on the manuscript. This work was supported by the Project of Huanglian Mountains National Nature Reserve Animal Diversity Expedition (Grant No. E2023HLS001), National Natural Science Foundation Project: Classificatory and Phylogenetic Studies on the *Amolops* frogs of China (Grant No. NSFC-31772424) and the project of the Ministry of Ecology and Environment of China: Investigation and assessment of amphibians and reptiles in southern Yunnan.

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ÖGH Herpetozoa
Austrian Herpetological Society

Herpetozoa 37: 157–179 (2024)
DOI 10.3897/herpetozoa.37.e122213

A new species of *Thamnophis* (Serpentes, Colubridae) from Jalisco, Mexico, with a discussion on the phylogeny, taxonomy, and distribution of snakes related to *Thamnophis scalaris*

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Academic editor: Günter Gollmann ♦ **Received** 4 March 2024 ♦ **Accepted** 11 June 2024 ♦ **Published** 26 June 2024

Abstract

Garter snakes in the genus *Thamnophis* from Mexico have a long and convoluted taxonomic history. From 2015 to 2022, we conducted a comprehensive sampling of Mexican *Thamnophis* species, aiming to link molecular phylogenies with the recognized species related to *T. scalaris* in the highlands of Mexico. Here, we present an analysis of mitochondrial DNA to resolve the status of two enigmatic highland *Thamnophis* populations. Our research resulted in the identification and morphological characterization of a previously undescribed *Thamnophis* species from the state of Jalisco in western Mexico. We also clarify the identity and relationships of several previously enigmatic populations of *Thamnophis*. This work presents new data for *Thamnophis* phylogenetics from the Mexican highlands and offers a framework for future conservation efforts.

Resumen

Las culebras del género *Thamnophis* de México tienen una historia taxonómica larga y complicada. De 2015 a 2022, realizamos un muestreo integral de las especies de *Thamnophis* de México, con el objetivo de conciliar la filogenética molecular con las delimitaciones de especies establecidas en el complejo relacionado con *T. scalaris* en las tierras altas de México. Aquí presentamos un análisis del ADN mitocondrial para resolver las trayectorias evolutivas y el estado de dos enigmáticas poblaciones de *Thamnophis* de las tierras altas. Nuestra investigación resultó en la identificación y caracterización morfológica de una especie de *Thamnophis* no descrita previamente del estado de Jalisco en el oeste de México. También aclaramos la identidad y las relaciones de varias poblaciones de *Thamnophis* previamente enigmáticas. Este trabajo presenta nuevos datos para nuestra comprensión de la filogenética de *Thamnophis* del altiplano mexicano y ofrece un marco para futuros esfuerzos de conservación.

Kurzfassung

Strumpfbandnattern der Gattung *Thamnophis* aus Mexiko haben eine lange und verworrene taxonomische Geschichte. Von 2015 bis 2022 führten wir eine umfassende Probenahme der *Thamnophis*-Arten Mexikos durch, mit dem Ziel die Phylogenetik mit etablierten Artenabgrenzungen im *T. scalaris* Komplex im Hochland Mexikos in Einklang zu bringen. Hier präsentieren wir eine Analyse der mitochondrialen DNA, um die Evolutionsverläufe und den Status zweier rätselhafter *Thamnophis*-Populationen aufzuklären. Unsere Forschung führte zur Identifizierung und Charakterisierung einer bisher unbeschriebenen *Thamnophis*-Art aus dem Bundesstaat Jalisco im Westen Mexikos. Wir klären auch die Identität und Beziehungen mehrerer bisher rätselhafter *Thamnophis*-Populationen. Diese Arbeit stellt neue Daten für unser Verständnis der Phylogenetik von *Thamnophis* aus dem mexikanischen Hochland vor und bietet einen Rahmen für zukünftige Schutzbemühungen.

Key Words

Adelophis, conservation, *errans*, *godmani*, pine-oak woodland, Mexican Transverse Ranges, *scaliger*

Palabras clave

Adelophis, bosque pino-encino, conservación, Eje Neovólcanico, *errans*, *godmani*, *scaliger*

Schlüsselwörter

Adelophis, Eje Neovólcanico, *errans*, *godmani*, Kiefern-Eichen Wald, Naturschutz, *scaliger*

Introduction

The taxonomy of the Longtail Alpine Garter Snake, *Thamnophis scalaris* Cope, 1861, has been a subject of confusion since its description. Historically, *Thamnophis scalaris* was confused with both *Thamnophis scaliger* (Jan, 1863) and *Thamnophis godmani* (Günther, 1894), and these two species were considered subspecies of *T. scalaris* at one time (Smith 1942). The type specimen of *T. scalaris* was collected near Xalapa, Veracruz (Cope, 1861). Later, Cope (1887) referred additional specimens from Orizaba, Veracruz, and nearby localities in Puebla to *T. scalaris*. Jan (1863) described *T. scaliger* but did not specify a type locality, leaving the association of this name with certain *Thamnophis* populations unclear. However, it should be noted that Jan had access to material collected by François Sumichrast in Xalapa and other areas of Veracruz at the time he described *T. scaliger* (Grünwald et al. 2015, p. 402).

The first accounts of *Thamnophis scalaris* from Jalisco were derived from material collected by A. C. Buller in 1892, which Boulenger (1893) reported as "*Tropidonotus scalaris*." His concept of *T. scalaris* was based on seven specimens from Jalisco (which we now recognize in this study as a distinct species) and one specimen from Oaxaca, which was likely mislabeled. Later, Smith (1942) reviewed the *Thamnophis* of Mexico and considered *T. scaliger* and *T. godmani* to be subspecies of *T. scalaris.* Smith et al. (1950) also examined specimens of *Thamnophis* at the Natural History Museum, London, and categorized the Jalisco specimens reported by Boulenger (1893) within their concept of *T. scalaris scaliger.* Subsequently, Rossman and Lara-Gongora (1997) removed *Thamnophis scaliger* from the synonymy of *T. scalaris* and proposed a neotype for *T. scaliger* from Mexico City. This was done in order to preserve the name "*T. scaliger*" for the population that Smith (1943) and Smith et al. (1950), among others, had associated with it. Jan's (1863) description was extremely vague, and Smith (1942) recognized this and ignored the issue regarding the lost "*T. scaliger*" type and decided to apply the name to one of the morphologically distinct populations that he had at hand. At the time Rossman and Lara-Gongora (1997) were investigating the group, most authors were following Smith's (1942) concept of *T. scaliger*, so Rossman and Lara-Gongora (1997) wanted to stabilize the name *T. scaliger*, and thus they neotypified the name for populations in central Mexico.

Rossman and Lara (1997) helped clarify the relationships between *T. scalaris* and *T. scaliger*, and they considered *T. scalaris* to be distributed along the Mexican Transverse Ranges from western Jalisco east to central Veracruz and eastern Puebla. These authors partially recognized the distinct nature of the Jalisco *T.* "*scalaris*" from typical *T. scalaris* in their review and grouped these individuals as part of their "western *T. scalaris.*" However, they continued to confuse these populations with true "*T. scalaris*" from eastern Michoacán and the high mountains around Mexico City. Unfortunately, these authors did not publish more specific data that would facilitate comparisons between the different geographical populations of *T. scalaris*.

Against this historical backdrop, our research was motivated by the discovery of garter snakes in Jalisco that were morphologically distinct and could not be assigned to any known species of *Thamnophis*. To resolve the taxonomic status of these populations, we undertook a comprehensive molecular and morphological analysis of Mexican highland *Thamnophis*. Morphologically, the Jalisco population appeared to be intermediate between *T. scalaris* and *Thamnophis errans* Smith, 1942, and molecular data were needed to determine their specific identity. This study presents these molecular findings and provides a morphological description of the Jalisco population, which we recognize as a new species. In addition, we resolve some longstanding taxonomic conundrums around *T. scalaris*. This work underlines the urgent need for conservation measures since the habitats of these populations are increasingly threatened by human activities. By clarifying the taxonomy of these garter snakes, we aim to establish a foundation for future research and conservation efforts in the highlands of Jalisco.

Materials and methods

Taxonomic sampling

We collected multiple specimens of garter snakes of the genus *Thamnophis* from the highlands of Mexico between 2015 and 2022. We photographed all live snakes, including dorsal, lateral, and ventral profiles, and euthanized them with pentobarbital. We took tissue samples from muscle or liver upon death and preserved them in 96% ethanol. We fixed specimens in 10% formalin and transferred them to 70% ethanol for permanent storage.

All collected materials were deposited at the Instituto de Investigaciones sobre los Recursos Naturales (INIRENA) of the Universidad Michoacana de San Nicolás de Hidalgo (UMSNH) in Morelia, Mexico; the Museo de Zoología, Facultad de Ciencias (MZFC) of the Universidad Nacional Autónoma de México (UNAM) in Mexico City; and the Facultad de Estudios Superiores, Zaragoza (MZFZ) of the Universidad Nacional Autónoma de México (UNAM), also in Mexico City. Museum acronyms throughout the text follow Sabaj (2020). Specimen numbers for all material examined are provided in Appendices 1, 2. We were not able to measure type specimens of previously described taxa, so we used measurements of the type specimens provided in original descriptions and other published literature (Cope 1861, 1866; Jan 1863; Boulenger 1893; Smith 1942; Walker 1955; Rossman 1969; Rossman and Burbrink 2005). Measurements for *T. errans* were taken from Webb (1976), whereas measurements from *Thamnophis sumichrasti* Cope, 1866, and *Thamnophis mendax* Walker, 1955, were taken from their original descriptions.

Distribution maps were generated based on the GBIF database (www.gbif.org), which includes both museum records and distribution records from the citizen scientist platform iNaturalist (inaturalist.org). iNaturalist records were curated by us to assure that no misidentifications were present before generating the maps and are current up through January 2024. Observations that could not be positively identified were removed.

The mountains of central Jalisco have numerous other species of *Thamnophis* that occur in sympatry or near sympatry with the species described herein. These include *Thamnophis copei* (Dugés, 1879), *Thamnophis cyrtopsis* (Kennicott, 1860), *Thamnophis eques* (Reuss, 1834), *Thamnophis melanogaster* (Peters, 1864), and *Thamnophis pulchrilatus* (Cope, 1885). While these species are not closely related to the species described herein, we include them in our comparatives within the species description to aid in the identification of individuals in the field.

Measurements and character states

Abbreviations used in the text and tables are as follows: snout–vent length (**SVL**), tail length (**TL**), total length (**TotL**), head length (**HL**), head width (**HW**), eye diameter (**ED**), rostral height (**RH**), rostral width (**RW**), internasal length (**INL**), internasal width (**INW**), prefrontal length (**PFL**), prefrontal width (**PFW**), frontal length (**FL**), maximum anterior frontal width (**MAFW**), maximum posterior frontal width (**MPFW**), parietal width (**PW**), parietal length (**PL**), loreal length (**LL**), loreal height (**LH**), mental length (**ML**) mental width (**MW**), anterior chin shield length (**ACSL**), posterior chin shield length (**PCSL**), internasal suture length (**INK**), prefrontal suture length (**PFK**), internasal rostral contact (**INR**), rostral nasal contact (**NR**), distance from frontal to snout (**DFS**), muzzle length (**MZL**), number of labials in contact with anterior chin shields (**LCAC**), ventral scales (**VS**) (Dowling (1951) method and explained in Rossman et al. (1996), subcaudal scales (**SC**), dorsal scale rows at one head length behind parietal (**DSRA**), dorsal scale rows at midbody (**DSRM**), dorsal scale rows at one head length prior to anal scale (**DSRT**), supralabials (**SL**), infralabials (**IL**), preoculars (**PRO**), postoculars (**POO**), maxilliary teeth (**MT**).

Scale measurements were taken in the following manner: **HL**, distance from the tip of the snout to the posterior border of the parietal scales; **HW**, distance taken at the posterior edge of the jaw; **RH**, the distance of the rostral scale from the median point of the mouth to the vertex formed by the internasal suture; **RW**, distance of the rostral scale measured between each suture formed by the 1st supralabial and prenasal; **INL**, distance of the internasal scale from its anterior border with the prenasal and rostral to its posterior border with the prefrontal; **INW**, distance from the prenasal to the medial suture between each internasal; **PFL**, distance from the postnasal, nasal, and internasal borders back to the border between the frontal and supraocular; **PFW**, distance from the postnasal to the median suture between the prefrontals; **FL** maximum length of the frontal shield; distance from the posterior part of the prefrontals to the medial union between the parietals; **MAFW**, the maximum width of the anterior portion of the frontal scale measured between each vertex formed by the prefrontal and supraocular scales; **MPFW**, the maximum width of the posterior portion of the frontal scale measured between each vertex formed by the parietal and supraocular scales, **PW**, distance from the union of the postocular and anterior temporal scales to the median suture between the parietals; **PL**, distance from the border between the supraocular and frontal to the posteriormost point of each parietal scale; **LL**, maximum length from the upper anterior border of the nasal to the lower posterior border with the preocular and supralabial; **LH**, distance from the supralabial to the union with the prefrontal and preocular; **ML**, taken from the medial point of the mouth to the posterior end of the mental scale, where the first pair of infralabials meet each other; **MW**, measured along the border of the mouth from one infralabial border to the other; **ACSL**, distance from the suture between the first and second infralabial posteriorly to the median suture between posterior chinshields; **PCSL**, maximum length from the union with the anterior chinshield and infralabial to the posterior border of the posterior chinshield; **SC**, counted on the left and right sides, with the first subcaudal scale interpreted as the first scale posterior to the cloaca that was not counted by the anal scale; Dorsal scales were counted at one head length behind the posterior edge of the parietals, at midbody, and at one head length before the anterior border of the anal scale. Muzzle length was defined as the combined length of the internasal suture (**INK**) and pre-frontal suture (**PFK**); muzzle shape was calculated by dividing INR by NR.

DNA extraction, amplification, and molecular analyses

All laboratory procedures were carried out at the UNAM FES-Zaragoza in Mexico City. We used a standard ammonium acetate protocol (Fetzner 1999) to extract genomic DNA from liver or muscle tissue. We then sequenced two mitochondrial loci: Cytochrome b (Cytb) and NADH dehydrogenase subunit 4 (ND4). For Cytb, we used Gludg-L (Palumbi 1996) as the forward primer and ATRCB3 (Harvey et al. 2000) as the reverse primer. For ND4, we employed ND4 and ND4_Leu (Arevalo et al. 1994) as the forward and reverse primers, respectively. Both loci were amplified using a standard polymerase chain reaction (PCR) protocol: an initial denaturation at 95 °C for 3 minutes and 30 seconds, followed by 35 cycles of denaturation at 95 °C for 30 seconds, annealing at 53 °C for 30 seconds, extension at 72 °C for 1 minute, and a final extension at 72 °C for 15 minutes, with a terminal hold at 10 °C. The PCR products were purified using a polyethylene glycol method (Lis 1980) and sequenced by Macrogen Korea (Standard-Seq of Macrogen Inc.).

Raw chromatograms were trimmed and edited using Geneious v. 2023.1 (Biomatters Ltd., Auckland, NZ). To infer phylogenetic relationships from the new samples, we included additional sequences of the genus *Thamnophis* as well as two outgroups obtained from GenBank. All new sequences were deposited in GenBank (Appendix 2).

Each gene was aligned separately in MAFFT version 7 (Katoh et al. 2017) using the Q-INS-I option. The alignments were then concatenated using FASconCAT v.1.04 (Kück and Longo 2014). The final alignment comprised 1828 base pairs (1104 bp for Cytb and 724 bp for ND4), including sequences from 81 representatives of the genus *Thamnophis* and individuals each of "*Adelophis*" *foxi* Rossman & Blaney, 1968 and "*Adelophis*" *copei* (Dugès, 1879), as well as one individual of *Nerodia erythrogaster* (Forster, 1771) and *Tropidoclonion lineatum* (Hallowell, 1856) as outgroups. We calculated pairwise genetic distances in the mitochondrial, Cytb, and ND4 genes using MEGA X software (Kumar et al. 2018).

Maximum likelihood (ML) analysis of the concatenated dataset was performed using IQ-TREE (Nguyen et al. 2015) using the IQ-TREE web server (Trifinopoulos et al. 2016). We employed an auto-substitution model and conducted 1,000 bootstrap replicates for support assessment. Separate ML analyses were also performed for each mitochondrial gene. The resulting topologies are presented in Suppl. materials 1, 2.

For Bayesian phylogenetic inference (BI), we initially used PartitionFinder v1.1.1 (Lanfear et al. 2012) to determine the most suitable model of partitions and nucleotide evolution for each locus using the Bayesian information criterion (BIC). The identified optimal partitions were: GTR + I + gamma for the first codon position of both Cytb & ND4, HKY + I + gamma for the second codon position of Cytb, HKY + gamma for the second codon position of ND4, and GTR + gamma for the third codon position of both Cytb & ND4. Our dataset was organized accordingly by locus and codon position. We then conducted BI using Mr. Bayes v3.2.2 (Ronquist et al. 2012) on the CIPRES science gateway server (Miller et al. 2011). This analysis involved four runs, each with 10 million generations and a sampling interval of 1,000 generations, incorporating three heated chains and one cold chain. Convergence was assessed using Tracer v1.6 (Rambaut et al. 2015), focusing on likelihood and parameter estimate overlaps, effective sample sizes, and the potential scale reduction factor (PSRF). Convergence was achieved within 200,000 generations, allowing us to discard the initial 25% of each run as burn-in. The results of these runs were combined using TreeAnnotator 2.7.4. (Bouckaert et al. 2019) to create a concatenated tree and visualized with FigTree v1.4.2 (Rambaut 2014).

Considering that the topologies from both ML and BI analyses were almost identical, we have included only the maximum likelihood phylogeny in this paper, with the Bayesian Inference phylogeny provided as Suppl. material 3.

Results

Molecular phylogenetic results

In our ML phylogeny (Fig. 1), we find concordance with prior evolutionary hypotheses, including those derived from mitochondrial DNA (mtDNA) studies (de Queiroz et al. 2002), those combining mtDNA and nuclear DNA in their analyses (McVay et al. 2015, Deepak et al. 2022), as well as analyses by Hallas et al. (2022), which were based on ddRADseq data. The robustness of our phylogenetic nodes is largely high, with most nodes garnering greater than 95% bootstrap support. However, several internal nodes exhibited lower support values, so we collapsed nodes below the 50% bootstrap support threshold.

Our analysis corroborates the delineation of three primary clades within *Thamnophis*, aligning with previous studies. The first clade, "Ribbon Snakes," is composed of *Thamnophis sirtalis* (Linnaeus, 1758), *Thamnophis proximus* (Say, 1823), and *Thamnophis saurita* (Linnaeus, 1766), collectively forming a lineage sister to the remaining members of *Thamnophis*. The other sampled *Thamnophis* segregate into two major clades. The first of these, referenced as the "Widespread Clade" by de Queiroz et al. (2002), predominantly comprises species from the USA and northern Mexico. Our data support the close phylogenetic relationship between *Thamnophis fulvus* (Bocourt, 1893) and *Thamnophis chrysocephalus* (Cope, 1885), albeit with notable genetic divergence among *T. chrysocephalus* populations from Guerrero compared to those from Oaxaca and Veracruz. Further, the clade encompassing *T. fulvus* and *T. chrysocephalus* formed a sister lineage to a cluster of species endemic to the USA and northern Mexico, with *Thamnophis. cyrtopsis* forming a sister relationship to a clade comprising species from the USA and Baja California, inclusive of "*T.* aff. *pulchrilatus*" and an individual matching the original description of *Thamnophis vicinus* Smith, 1942 (currently a junior synonym of *T. cyrtopsis*), which is notable as this population has never been studied phylogenetically.

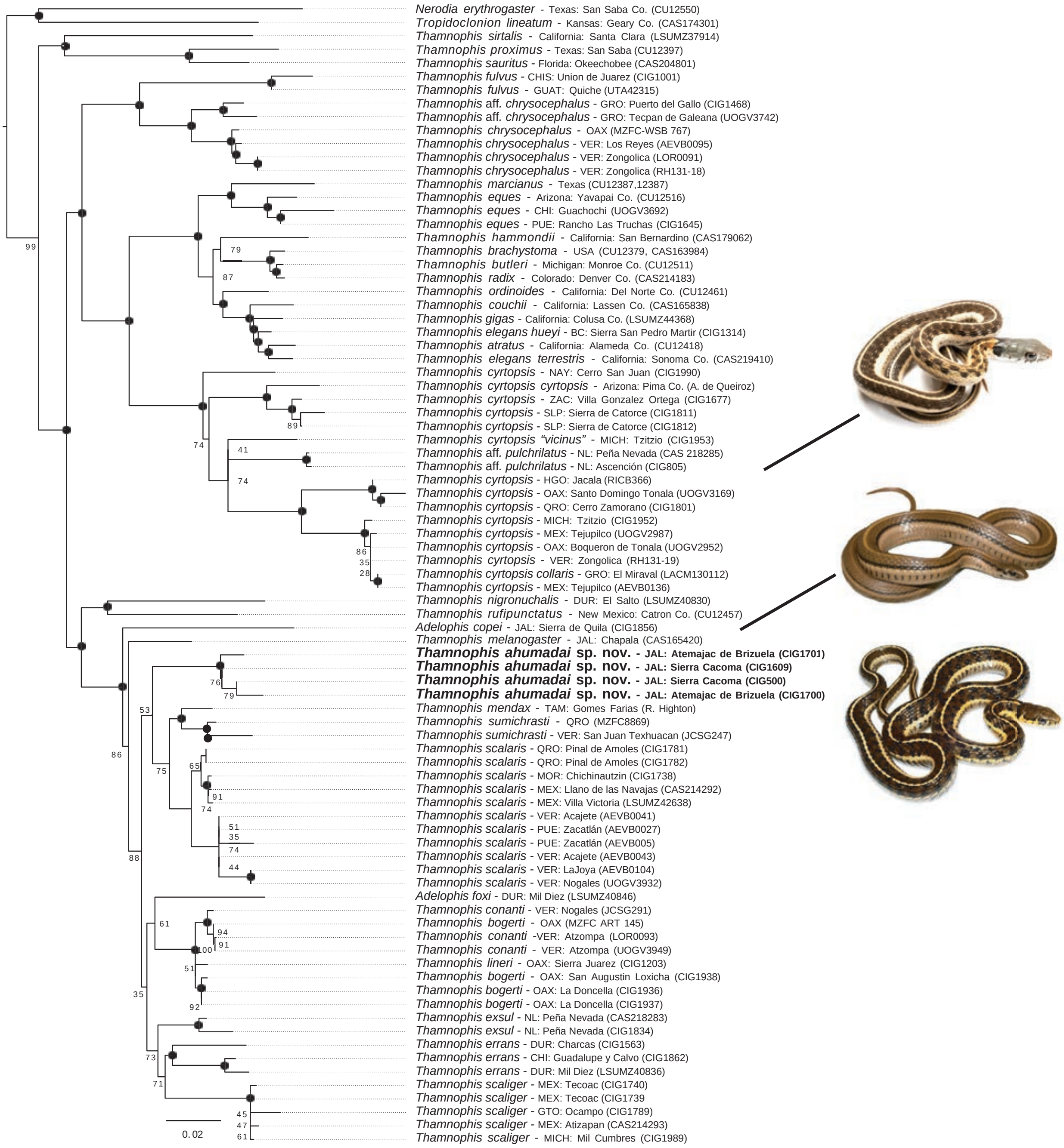

Figure 1. Concatenated maximum likelihood inference of the phylogenetic relationships of *Thamnophis* and closely related Natricines based on the mitochondrial genes Cytb and ND4.

In the other major clade, referenced as the "Mexican Clade," our results recover an early split between *T. nigronuchalis* Thompson, 1957 + *T. rufipunctatus* (Cope, 1875), sister to all other lineages. This split is followed by the divergence of *T. copei* and *T. melanogaster*. Remarkably, this study represents the first inclusion of *T. copei* in a molecular phylogeny, revealing an unexpected non-sister relationship to *T. foxi*, which was its sole congener in the now invalid genus *Adelophis* (see below). The new species reported herein formed a polytomy with *T. mendax*, *T. sumichrasti*, and *T. scalaris*. That group was recovered as the sister clade to a group that includes *Thamnophis exsul* Rossman, 1969; *T. errans* + *T. scaliger*; and a group comprising *T. foxi* alongside *T. bogerti* Rossman & Burbrink, 2005; *T. conanti* Rossman & Burbrink, 2005; and *T. lineri* Rossman & Burbrink, 2005. However, it is noteworthy that the support for this latter grouping is very low, and *T. bogerti*, *T. conanti*, and *T. lineri* do not form monophyletic groups.

Finally, our BI analysis exhibited a high degree of similarity to those relationships obtained through maximum likelihood, with an almost identical topology. The key distinction lies in the variation of support values for certain groups. In the BI analysis, *T. ahumadai* was recovered as a sister to *T. mendax* + *T. sumichrasti* + *T. scalaris*, but with low support (posterior probability = 0.61). The BI tree, elucidating these differences, is presented in Suppl. material 3.

Our molecular results support the hypothesis that populations formerly assigned to *Thamnophis scalaris* from Jalisco, Mexico, belong to an undescribed species. We analyzed molecular samples from two isolated highland populations (Sierra Cacoma, Sierra de Tapalpa; Appendix 2) of "*T. scalaris*" from Jalisco. Both are each other's closest relatives, with 100% bootstrap support. These populations fall within the "Mexican Clade" of our phylogenetic tree (Fig. 1). Together, these two populations are sister to a clade comprising *T. scalaris*, *T. sumichrasti*, and *T. mendax*, albeit with low bootstrap support (67%). The two Jalisco populations have similar genetic distances to their closest relatives. These genetic distances are 0.04–0.05 (ND4) and 0.03 (Cytb) to *Thamnophis bogerti* (as understood herein, see below), which appears to be their closest relative according to genetic distances. The two Jalisco populations also have genetic distances of 0.04–0.06 (ND4) and 0.03–0.04 (Cytb) from the superficially similar *T. scalaris*; 0.05–0.07 (ND4) and 0.04–0.05 (Cytb) from geographically proximate populations of *T. errans*; and 0.04–0.05 (ND4) and 0.04 (Cytb) from *T. exsul*. In comparison, the *T. scalaris* populations analyzed herein (Morelos, Estado de México, Querétaro, Puebla, and Veracruz) have intraspecific genetic distances of 0.00–0.02 (ND4) and 0.00–0.01 (Cytb). Within the *T. scalaris* populations analyzed, it should be noted that one specimen (AEVB 0104) from La Joya, Acajete, Veracruz had a ND4 genetic distance of 0.02–0.04 from other non-Veracuz specimens of *T. scalaris*. Similarly, another specimen (UOGV 3932) from nearby Nogales, Veracruz, had a higher Cytb genetic distance (0.02) from all other specimens of *T. scalaris*. These genetic distances are still low when compared to the interspecific genetic distances that we recovered between other species, but they are nonetheless interesting and may show evidence of hybridization with nearby populations of *T. bogerti* (see below).

Systematic account

***Thamnophis ahumadai* sp. nov.**

https://zoobank.org/3D2B6CF0-C0D6-443C-B7A4-3AF2DC32AF77

Figs 2, 4, 5, 7a–e

Proposed standard English name. Ahumada's Alpine Garter Snake.

Proposed standard Spanish name. Culebra Listonada de Montaña de Ahumada.

Type materials. ***Holotype*** (Fig. 2). INIRENA 2933 (original field number CIG-1612). Adult male collected under a log in a marshy meadow near a pond, 2.8 km E of Cumbre de Guadalupe, Municipio de Cuautla, Jalisco, Mexico (20.168991, -104.684925, 2,353 m asl; datum = WGS84; Fig. 4a), by Christoph I. Grünwald, María del Carmen G. Mendoza-Portilla, Iván Ahumada-Carrillo, and Ginny N. Weatherman on 23 August 2019.

Paratypes (Fig. 3). MZFZ 4593 (original field number CIG-0500) adult male collected under a rock on a hillside above a stream at Cumbre de Guadalupe, Municipio de Talpa de Allende, Jalisco, Mexico (20.168902, -104.711252, 2,137 m asl; datum = WGS84) by Christoph I. Grünwald, Héctor Franz-Chávez, André J. Grünwald, Ámbar Lanomy Grünwald, Janelle Morales-Flores, and Karen I. Morales-Flores on 11 July 2015. MZFZ 4594 (original field number CIG-1609) adult female collected under trash in a grassy meadow amongst pine forest, 1.5 km N of Cumbre de Guadalupe, Municipio de Talpa de Allende, Jalisco, Mexico (20.186581, -104.716188, 2,179 m asl; datum = WGS84), by Christoph I. Grünwald, María del Carmen G. Mendoza-Portilla, Iván Ahumada-Carrillo, and Ginny N. Weatherman on 23 August 2019. INIRENA 2932 and 2934 (original field numbers CIG-1611 and CIG-1613) adult male and adult female, respectively, with the same collection data as Holotype. MZFZ 4595 (original field number CIG-1610) adult male collected under rock in meadow, 4.2 km airline ESE of Cumbre de Guadalupe, Municipio de Tomatlán, Jalisco, Mexico (20.157217, -104.675059, 2,319 m asl; datum = WGS84), by Christoph I. Grünwald, María del Carmen G. Mendoza-Portilla, Iván Ahumada-Carrillo, and Ginny N. Weatherman on 23 August 2019. INIRENA 2935–36 (original field number CIG-1700–01) 2 adult males, collected under logs at a large pond at 2.5 km SE of Atemajac de Brizuela, Municipio de Atemajac de Brizuela, Jalisco, Mexico (20.1151, -103.7036, 2,422 m asl; datum = WGS84; Fig. 4b), by Christoph I. Grünwald, André J. Grünwald, and Ámbar Lanomy Grünwald on 21 June 2020.

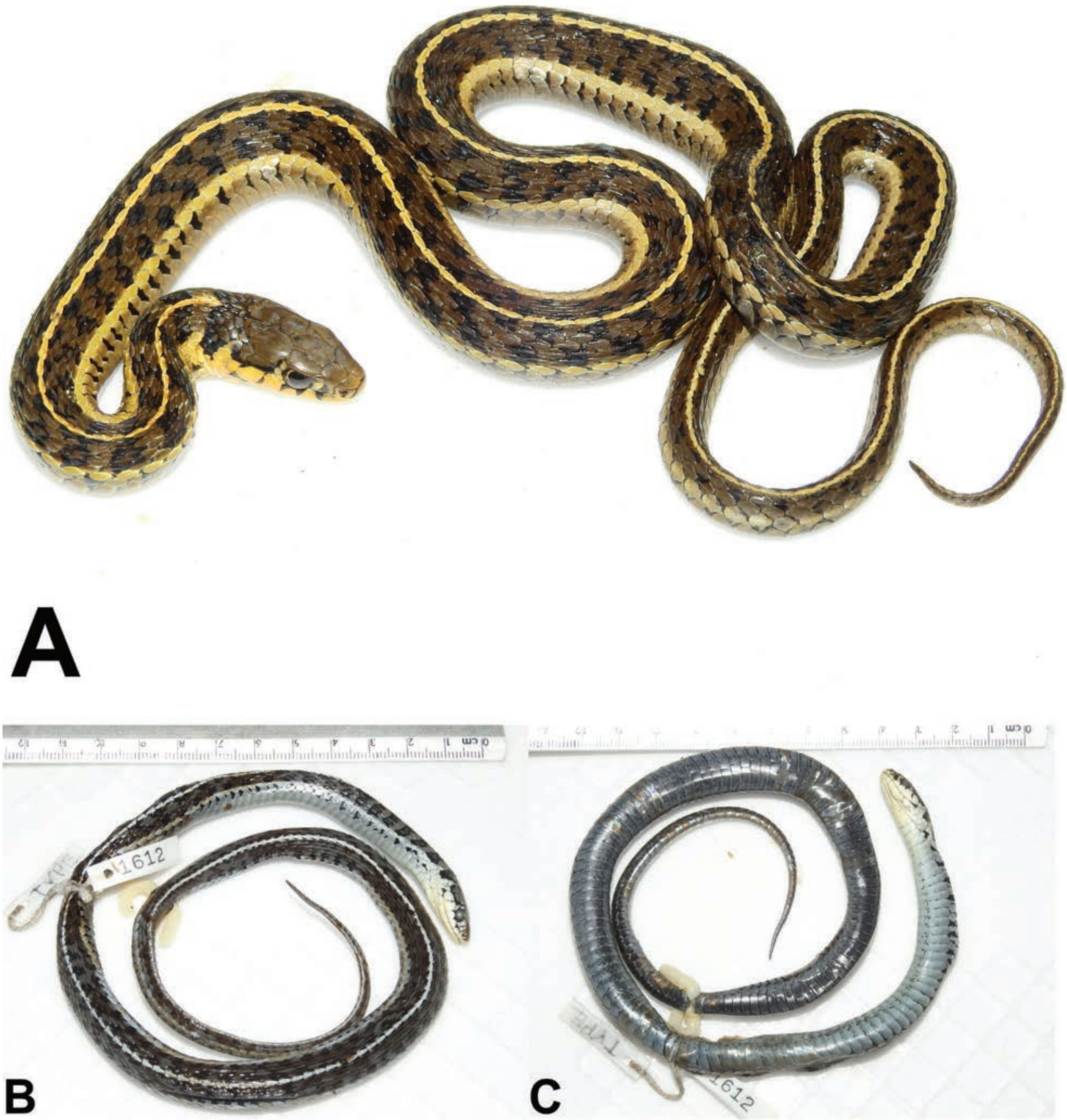

Figure 2. *Thamnophis ahumadai* sp. nov. holotype (INIRENA 2933) from the vicinity of Cumbre de Guadalupe, in the Municipio de Cuautla, Jalisco, Mexico.

Diagnosis. A relatively small Garter Snake, with a maximum of 565 mm SVL and 705 TotL; head narrow, scarcely wider than neck, with a short muzzle (INK + PFK = 3.0 mm); tail long in length, 31–36% of SVL and 23–27% of TotLin males, 25–26% of SVL and 20–21% of TotLin females; internasals wider than long; frontal 2–2.25 times longer than wide; loreal slightly longer than wide; one preocular; 2–4 postoculars; temporals 1 + 2; 7 supralabials, third and fourth entering orbit; 9–10 infralabials, first four in contact with anterior chinshields; anterior chinshields shorter than posterior. Dorsal scales always in 19-17-17 rows, strongly keeled except for the outermost row, which is smooth; ventral scales in males 134–142, in females 139–141; subcaudals in males 61–69, in females 54–55; anal scale undivided. Dorsal pattern consists of a pale mid-vertebral stripe restricted to the mid-dorsal row and a pale lateral stripe on second scale row. One or two rows of dark brown or black dorso-lateral spots, usually in one row on the anterior third of the dorsum, then divided into two rows along the remaining posterior two thirds. Ventral coloration dark, pale anteriorly, becoming progressively darker until dark gray or black posteriorly.

Figure 3. *Thamnophis ahumadai* sp. nov. paratypes. **A.** Male (INIRENA 2932) from same locality as holotype; **B.** Male (INIRENA 2936) from 2.5 km SE of Atemajac de Brizuela, Municipio de Atemajac de Brizuela, Jalisco, Mexico; **C.** Male (MZFZ 4595) from 4.2 km airline ESE of Cumbre de Guadalupe, Municipio de Tomatlán, Jalisco, Mexico; **D.** Male (INIRENA 2935) from 2.5 km SE of Atemajac de Brizuela, Municipio de Atemajac de Brizuela, Jalisco, Mexico; **E.** Male (MZFZ 4593) from Cumbre de Guadalupe, Municipio de Talpa de Allende, Jalisco, Mexico.

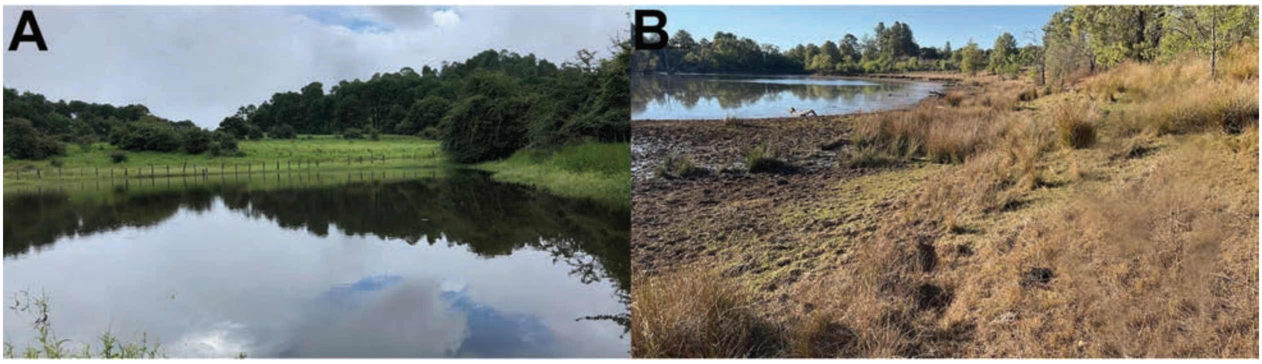

Figure 4. Collection localities for *Thamnophis ahumadai* sp. nov., including the type locality at 2.8 km E of Cumbre de Guadalupe, Municipio de Cuautla, Jalisco, Mexico (**A**) and 2.5 km SE of Atemajac de Brizuela, Municipio de Atemajac de Brizuela, Jalisco, Mexico (**B**).

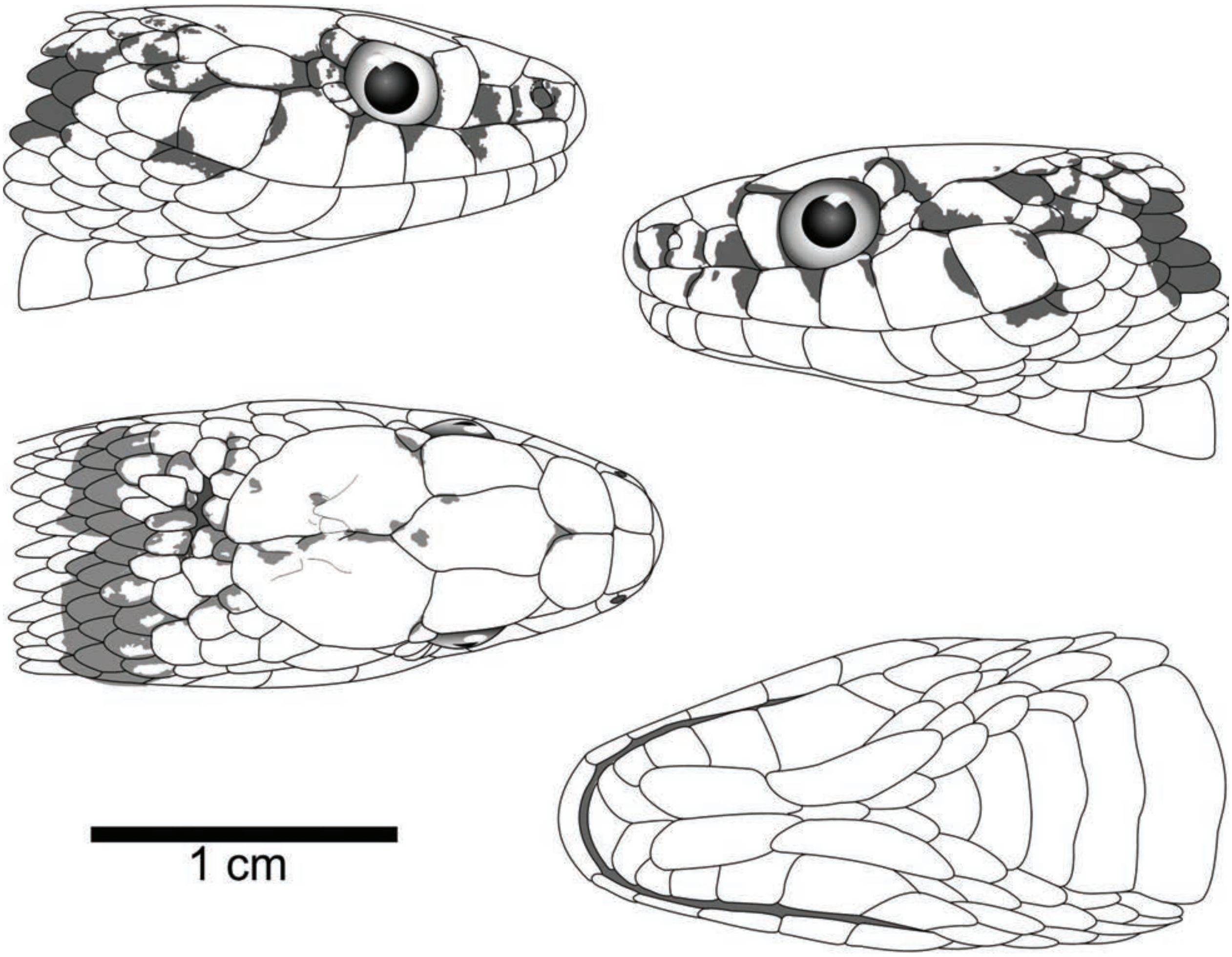

Figure 5. Head drawings of the holotype of *Thamnophis ahumadai* sp. nov. (INIRENA 2933). A solid line depicts 1 cm. Drawings by Iván T. Ahumada-Carrillo.

Comparisons. *Thamnophis ahumadai* can be distinguished from *T. scalaris* (comparison character traits in parenthesis) by possessing 19-17-17 dorsal scale rows (vs. 17-17-17); 61–69 subcaudals in males (vs. 69–77, but see below for Querétaro population); a shorter tail length in males, 23–27% TL/Totl ratio (vs. 26–33%) and also in females, 20–21% (vs. 20–25%); a black nuchal blotch (vs. brown); two rows of dark brown or black dorsal blotches between mid-dorsal and dorso-lateral pale stripes (vs. one row of large brown dorsal blotches); and a dark gray to black ventral coloration on latter two thirds of body (vs. ventral coloration same as darker portions of lateral coloration).

Thamnnophis ahumadai can be distinguished from *T. errans* (*T. errans* character traits in parenthesis) by possessing 134–142 ventral scales in males (vs. 150–166) and 139–141 ventral scales in females (vs. 146–160); 61–

69 subcaudals in males (vs. 78–94) and 54–55 subcaudals in females (vs. 67–83); a shorter tail length in females, 20–21% TL/TotL ratio (vs. 23–25%); a dorsal pattern with at least some larger dorsal spots fusing together towards the anterior portion of the body (vs. all dorsolateral spots in two alternating rows); and by possessing small black spots on the lateral pale stripe (vs. black spotting absent on lateral pale stripe).

Thamnophis ahumadai can be distinguished from *T. godmani* (*T. godmani* character traits in parenthesis) by possessing 19-17-17 dorsal scale rows (vs. 17-17-17); 54–69 subcaudals in males (vs. 74–88) and 54–55 subcaudals in females (vs. 61–77); a shorter tail length in males, 23–27% TL/TotL (vs. 25–29%), and also in females, 20–21% (vs. 25–27%). *Thamnophis ahumadai* can be distinguished from *T. bogerti* (*T. bogerti* character traits in parenthesis) by possessing 19-17-17 dorsal scale rows (vs. 17-17-15), 134–142 ventral scales in males (vs. 135–157); 54–55 subcaudal scales in females (vs. 56–68). Moreover, *Thamnophis ahumadai* is distinguished from both species by possessing two rows of alternating dark brown or black blotches between the mid-dorsal stripe and lateral stripes on dorsum (vs. two rows of dark spots between mid-dorsal stripe and lateral stripe may be absent, limited to randomly occurring black scale outlines, or consist of small black spots).

Thamnophis ahumadai can be distinguished from *T. scaliger* (*T. scaliger* character traits in parenthesis) by possessing supralabials that are all entirely pale-colored, with black pigment along each scale suture (vs. several posterior supralabials darkly pigmented by the encroachment of an occipital blotch, anterior ground color of anterior supralabials pale); 61–69 subcaudal scales in males (vs. 49–58), and 54–55 subcaudal scales in females (vs. 40–49); a longer tail length in males, 23–27% TL/Totl ratio (vs. 17–21%) and in females 20–21% TL/TotL (vs. 16–19%); no pale coloration along head scale sutures (vs. pale coloration present along head scale sutures); a black nuchal blotch (vs. brown); and two rows of dark brown or black dorsal blotches between the mid-dorsal and dorso-lateral pale stripes (vs. one row of large brown dorsal blotches).

Thamnophis ahumadai can be distinguished from *T. exsul* (*T. exsul* character traits in parenthesis) by possessing 19-17-17 dorsal scale rows (vs. 17-17-17); 134–142 ventral scales in males (vs. 142–150) and 139–141 ventral scales in females (vs. 152–156); and a pale mid-dorsal stripe always present (vs. absent or only present anteriorly).

Thamnophis ahumadai can be distinguished from both *T. mendax* and *T. sumichrasti* by possessing 134–142 ventral scales in males (vs. 145–149 in *T. mendax* and 151–161 in *T. sumichrasti*); from *T. sumichrasti* by possessing 139–141 ventral scales in females (vs. 147–159) and by possessing 61–69 subcaudals in males (vs. 72–78); from *T. mendax* by possessing 54–55 subcaudal scales in females (vs. 56–60) and by shorter tail in females 20–21% TL/TotL (vs. 21–23%); from both *T. mendax* and *T. sumichrasti* by possessing a lateral pale stripe (vs. absent); furthermore *T. ahumadai* does not present two pattern classes, and can readily distinguished from the blotched pattern class of *T. mendax* and both pattern classes of *T. sumichrasti* by possessing a pale mid-dorsal stripe (vs. lacking in both species).

The mountains of central Jalisco have numerous other species of *Thamnophis* that occur in sympatry or near sympatry with *T. ahumadai*. These species (except *T. copei*) are all distantly related to *T. ahumadai* and can be readily distinguished by their appearance. *Thamnophis ahumadai* differs from *T. cyrtopsis*, *T. eques*, and *T. pulchrilatus* by having 17 dorsal scale rows at mid-body (vs. 19 or more) and by having a tongue that is black (vs. red with black tips). From the apparently closely related *T. copei* (see below), *T. ahumadai* differs by possessing a loreal (vs. fused with prefrontal), 17 dorsal scale rows (vs. 15), and a longer head with seven supralabials (vs. 5).

For comparative purposes, we include photographs of *T. ahumadai* and closely related species in Fig. 6 and at a higher resolution in Suppl. material 5.

Description of holotype (Fig. 2). All measurements in mm. An adult male, 357 SVL, 122 TL, 479 TotL, tail relatively long, with TL/ SVL ratio 0.34 and TL/TotL ratio 0.25. Head short, twice as long as wide, and HL/SVL 0.04. All dorsal scales keeled except in first lateral row, dorsal scales in 19 rows anteriorly, reducing to 17 at the 21st ventral scale row and then 17 throughout rest of body. Ventral scales 135, anal scale undivided, 62/63 divided subcaudal scales. Rostral wider (3.2) than high (2.3); internasals wider (2.2/2.1) than long (1.7); prefrontal subequal, slightly longer (2.7/2.6) than wide (2.5/2.6); frontal 5.4 long, anterior edge 4.0 from snout; frontal 2.7 wide anteriorly and 2.5 wide posteriorly; parietal longer (6.3/6.1) than wide (3.4/3.5); loreal slightly longer (1.7/1.6) than wide (1.3); seven supralabials on each side and nine infralabials on each side. One preocular on each side and ¾ postoculars. Eye relatively small, ED 3.2, and ED/HL ratio 0.22. Mental wider (2.4) than long (1.25); anterior chin shields (4.8/4.3), in contact with 4 infralabials on each side; posterior chin shields longer than anterior chin shields (5.1/5.0). For a detailed drawing of the head scalation, see Fig. 5.

Everted hemipenes are the length of seven subcaudals; they are long and narrow with no noticeable widening in the apical region.

Coloration in preservative (Fig. 2b, c). Head scales brown, but frontal, supraoculars, and parietals pale gray, apparently damaged as the snake was pre-ecdysis. Supralabials cream-white, with black lines along sutures. Dark nuchal blotch 1–3 scales long, black in coloration, complete dorsally from below the labial region on both sides. Pale mid-dorsal stripe white, starting on the fourth dorsal scale posterior to the parietals and running to the tip of the tail. One row of large dorso-lateral blotches, nine on the left, six on the right; large blotches divided into two rows of alternating dorso-lateral blotches. Lateral pale stripe bluish-gray, one second and third scale rows on the neck, and then limited to the second scale row on the rest of body and tail, slowing fading in intensity on tail. A single, lateral row of black spots present on the first dorsal scale row,

Figure 6. Photographs of species related to *Thamnophis ahumadai* sp. nov. in life, all from Mexico. *Thamnophis ahumadai* sp. nov. (**A**) from Cumbre de Guadalupe, Municipio de Talpa de Allende, Jalisco; *Thamnophis ahumadai* sp. nov. (**B**) from 2.8 km E of Cumbre de Guadalupe, Municipio de Cuautla, Jalisco; *Thamnophis ahumadai* sp. nov. (**C**) 4.2 km airline ESE of Cumbre de Guadalupe, Municipio de Tomatlán, Jalisco; *Thamnophis ahumadai* sp. nov. (**D**) from 2.5 km SE of Atemajac de Brizuela, Municipio de Atemajac de Brizuela, Jalisco; *Thamnophis ahumadai* sp. nov. (**E**) from Tapalpa, Municipio de Tapalpa, Jalisco; *Thamnophis ahumadai* sp. nov. (**F**) from 2.5 km SE of Atemajac de Brizuela, Municipio de Atemajac de Brizuela, Jalisco; *Thamnophis errans* (**G**) from vicinity of Los Amoles, Sierra Huichol, Jalisco; *Thamnophis errans* (**H**) from Los Charcos, Municipio de Mezquital, Durango; *Thamnophis errans* (**I**) from Los Charcos, Municipio de Mezquital, Durango; *Thamnophis errans* (**J**) from 28 km SSW of Tepehuanes, Municipio de Tepehuanes, Durango; *Thamnophis errans* (**K**) from La Catedral, Municipio de Guadalupe y Calvo, Chihuhua *Thamnophis exsul* (**L**) from Peña Nevada, Nuevo León (photo by Robert Hansen) *Thamnophis scalaris* (**M**) from Volcán Chichinautzin, Municipio de Huitzilac, Morelos; *Thamnophis scalaris* (**N**) from Villa Victoria, Municipio de Villa Victoria, Estado de México (photo by Hugo Plata-Tinoco); *Thamnophis scalaris* (**O**) from Huamantla, Municipio de Huamantla, Tlaxcala (photo by Anibal Díaz de la Vega Pérez); *Thamnophis scalaris* (**P**) from Pico de Orizaba, Veracruz (photo by Alfredo Gutiérrez); *Thamnophis scalaris* (**Q**) from 1 km N of Pinal de Amoles, Municipio de Pinal de Amoles, Quéretaro; *Thamnophis scalaris* (**R**) from 1 km N of Pinal de Amoles, Municipio de Pinal de Amoles, Quéretaro; *Thamnophis godmani* (**S**) from San Vicente, Municipio de Chilpancigo de los Bravo, Guerrero; *Thamnophis godmani* (**T**) from San Vicente, Municipio de Chilpancigo de los Bravo, Guerrero; *Thamnophis bogerti* (**U**) from La Doncella, Municipio de San Mateo Río Hondo, Oaxaca; *Thamnophis bogerti* (**V**) from Sierra Miahuatlán, Oaxaca; *Thamnophis bogerti* (**W**) from Llano de las Flores, Sierra Juárez, Oaxaca; *Thamnophis bogerti* (**X**) from Puerto del Aire, Municipio de Acultzingo, Veracruz.

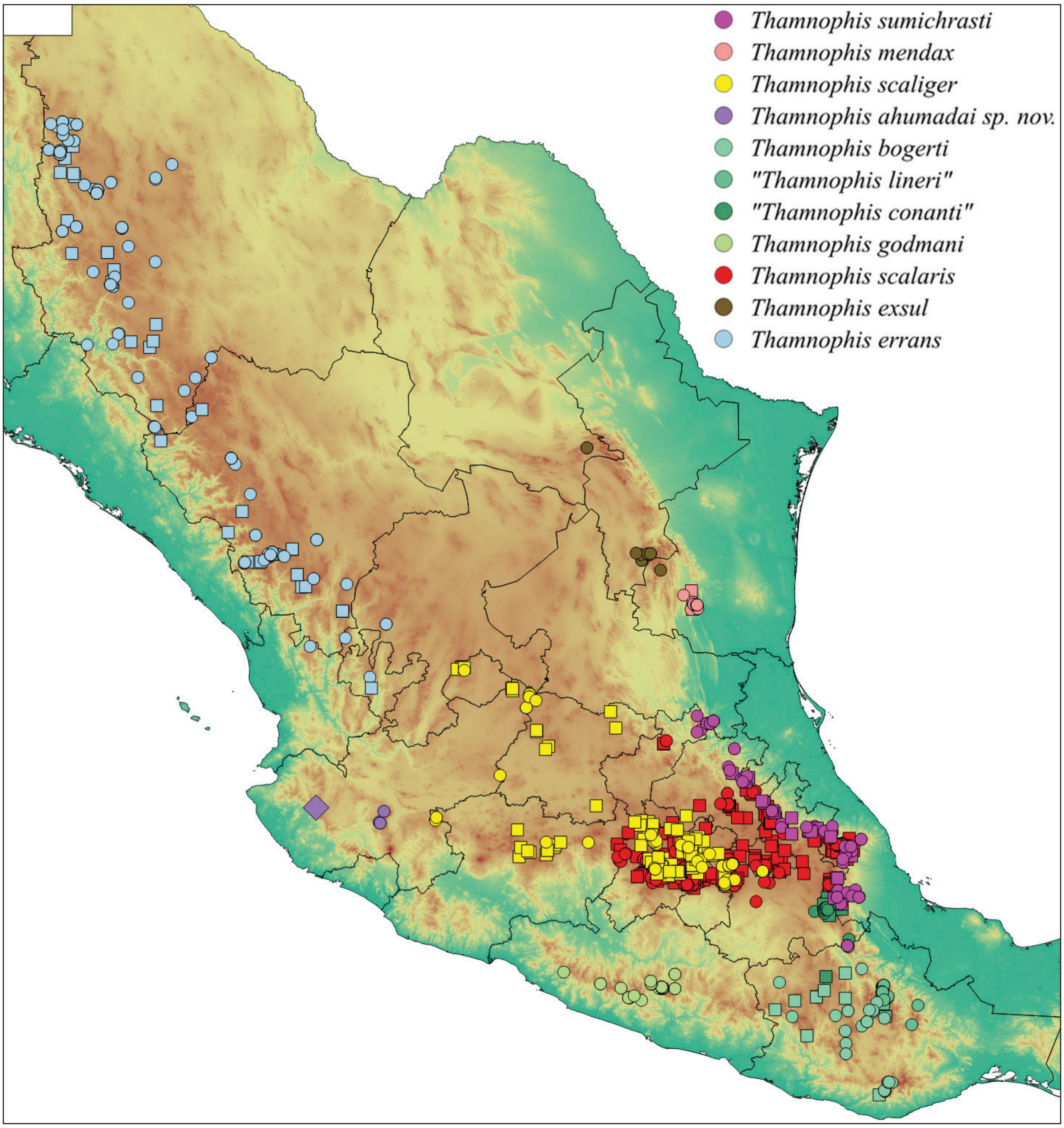

Figure 7. Distribution map of snakes similar to *Thamnophis scalaris* in Mexico. Circles represent museum records; squares represent verified iNaturalist observations, or field observations made by us but without a specimen deposited in a collection. The diamond represents the type locality of *Thamnophis ahumadai* sp. nov. We have included the different populations of *Thamnophis bogerti* in the key with their former names in quotation marks, and we used different shades of green to depict their individual ranges.

spots about half a scale in size on the anterior quarter of the body, then reducing to about a third a scale in size on the posterior three quarters of the body. Ventral surfaces of head and throat creamy-white until the fourth ventral scale, becoming pale bluish gray until approximately the 50th ventral scale, then progressively turning darker until the 78th ventral scale, where the venter takes on a dark gray-black coloration that continues posteriorly to the tip of the tail.

Coloration in life (Fig. 2a). Dorsal coloration chocolate brown, with 80 pairs of black dorso-lateral blotches present on dorsum. Pale mid-dorsal stripe present, pale yellow on anterior half of body, and then fading to a cream pigment posteriorly until the tail tip. Pale stripe involving only the vertebral row of scales. Top of head brown, including parietals, frontal, supraoculars, prefrontals and internasals, with rostral and front part of internasal pale cream, similar to supralabials. Supralabials pale cream on first three supralabials, then pale yellow on the latter four supralabials. Supralabials 2–6 have a black outline along the upper posterior suture. Nasal cream below, brown above, with a black outline posteriorly. Loreal anterior half cream, posterior half black. Single preocular cream;

four postoculars also cream; upper one with black outline on posterior border. Anterior temporal brown above and black below; posterior temporals brown and black. A bilobed dark nuchal spot present behind head, dark brown anteriorly, and black posteriorly. Nuchal spot three scales long at mid-dorsal line, extending ventrally to the height of the jawline. This nuchal blotch partially divides the pale-yellow coloration on the posterior supralabials and the pale lateral stripe on the lower portion of the dorsum. Pale lateral stripe on first three scale rows anteriorly, pale yellow, then restricted to second scale row on the anterior four-fifths of the dorsum, only slightly involving the first and third scale rows. After the anterior fifth of the dorsum, the first scale row is cream with dark black markings on the posterior edge of each scale, giving the appearance of small black vertical blotches. Tail same color as posterior body, black above, cream below, with a pale cream mid-dorsal stripe and small black spots on the cream colored first scale row. Iris copper.

Measurements (mm). SVL 357; TL 122 mm; TotL; 479; HL 14.; HW 7.3; ED 3.2 mm; RH 2.3; RW 3.2; INL 1.7; INW 2.1; PFL 2.7/2.6; PFW2.5/2.6; FL 5.4; MAFW 2.7; MPFW 2.4; PL 6.2/6.1; PW 3.4/3.5; LL 1.6; LH 1.3; ML 1.25; MW 2.4; ACSL 4.8/4.3; PCSL 5.1/5.0.

Variation. Meristic variation is minimal amongst the paratypes. Specimen ranged in size from 255 mm (INIRENA 2935) to 565 mm (INIRENA 2934). The shortest relative tail length was 0.20 TL/TotL in INIRENA 2934, whereas the longest relative tail length was 0.27 in INIRENA 2932. Interestingly, both were males from the same locality. Most all specimens had more ventral scales than the holotype, with MZFZ 4595 possessing the highest count (142). The ventral scale + subcaudal scale combinations amongst specimens ranged from 194 (INIRENA 2934) to 210/211 (MZFZ 4595). No variation in number of dorsal scale rows or supralabials was documented. Two specimens (INIRENA 2934, 2936) had 10 infralabials on both sides. Postoculars usually three on at least one side, but MZFZ 4593 has 4/4 and MZFZ 4594 has 4/6. The two females (INIRENA 2934, MZFZ 4594) have a head HL/HW ratio of 1.1–1.4, whereas the males have a HL/HW ratio of 1.5–2.0. Color pattern variation exists. The vertebral stripe ranges from orange (INIRENA 2932), yellow or yellowish (INIRENA 2936, MZFZ 4595), to cream (MZFZ 4593, INIRENA 2935). MZFZ 4593 is unique in that it presents dorsolateral blotches that are fused, similar to *T. scalaris* and *T. scaliger*. INIRENA 2934 has a dark dorsal coloration, which makes the dorsal pattern barely visible and gives the snake a dark, unpatterned appearance. Morphological and meristic variation of the all available specimens, including the holotype and all paratypes is given in Table 1.

Distribution and habitat. This species appears to be restricted to grasslands and meadows in pine-oak woodland and pine forest above 2100 m asl. Only known from two mountain ranges in Jalisco, the Cumbre de Guadalupe region of the Sierra Cacoma, and in the vicinities of the towns of Atemajac de Brizuela and Juanacatlán in the Sierra de Tapalpa. This species has been collected at elevations ranging from 2140 to 2450 m asl. We have included a range map with known localities of this species and closely related species in Fig. 7.

Etymology. A patronym honoring Iván Trinidad Ahumada-Carrillo (1984–), who has made many contributions to diverse areas in herpetology, including extensive studies of the herpetofauna of Jalisco and Zacatecas. Iván collected the first specimen of this new species in the Sierra Cacoma (MZFZ 4593) and pointed out its distinctiveness from typical *T. scalaris* and *T. errans*.

Conservation. This species of garter snake is only known from two relatively small high-elevation areas in the highlands of Jalisco, which fall within the "Jaliscan Transverse Range Pine-Oak Woodland (42)" and "Jaliscan Sierra Madre del Sur Mixed Temperate Woodland (46)" biogeographical formations as mapped by Grünwald et al. (2015). Due to its small distribution, we recommend that this species be rewarded with the highest level of protection possible from the Mexican government. As habitat destruction such as logging and farming is ongoing in both localities where it occurs, this species may qualify as Endangered under the IUCN criterion. However, it can also be considered to be in the DD (data deficient) category due to uncertainties about whether or not this species occurs in Michoacán (see below). More fieldwork should be done to determine the full extent of the distribution of *T. ahumadai* and whether or not it is present in other highland regions of Jalisco or adjacent Michoacán.

Discussion

With the description of *T. ahumadai*, we remove *T. scalaris* from the herpetofauna of Jalisco. We also suggest that all specimens of "*T. scalaris*" collected from Jalisco (NHMUK 92.9.5.39, 92.10.31.20–24; UTA 4040, 4932–47, 4949, 5991–93; KU 87472–73) are assignable to *T. ahumadai*. A detailed morphological examination of these specimens will undoubtedly expand the known variation in this newly described species. Boulenger (1893) gave a detailed description of specimens of "*T. scalaris*" from Jalisco. The combination of 7 supralabial scales and low ventral and subcaudal scale counts that he reported precludes their assignment to any other species of *Thamnophis* known from Jalisco except for *T. scaliger*. While we cannot definitely exclude *T. scaliger* from the description given by Boulenger, it is important to note that despite intensive recent collecting at both localities (Cumbre de Guadalupe and Atemajac de Brizuela), no *T. scaliger* has been collected from either of these mountain ranges. We tentatively suggest that these specimens are referrable to *T. ahumadai*. Smith et al. (1950) reviewed these same specimens, but they confused them with their concept of "*T. scalaris scaliger.*"

Apparently, *T. ahumadai* and *T. scalaris* are widely isolated along the Mexican Transverse Ranges, with no known records between the Sierra Tapalpa in central Jalisco and the closest populations occurring near

Table 1. Morphometric and meristic variation of *Thamnophis ahumadai* sp. nov. Measurements of the holotype are shaded gray.

	MZFZ 4593	MZFZ 4594	MZFZ 4595	INIRENA 2932	INIRENA 2933	INIRENA 2934	INIRENA 2935	INIRENA 2936
Sex	Male	Female	Male	Male	Male	Female	Male	Male
SVL	262	426	297	335	357	565	255	327
TL	86	112	103	122	122	140	78	114
TotL	348	538	400	457	479	705	333	441
TL/SVL	0.33	0.26	0.35	0.36	0.34	0.25	0.31	0.35
TL/TotL	0.25	0.21	0.26	0.27	0.25	0.20	0.23	0.26
VS	140	141	142	135	135	139	134	139
SC	68/68	54/54	68/69	67/67	62/63	55/55	61/62	64/65
VS+SC	208	195	210.5	203	197.5	194	195.5	203.5
DSRA	19	19	19	19	19	19	19	19
DSRM	17	17	17	17	17	17	17	17
DSRT	17	17	17	17	17	17	17	17
SL	7/7	7/7	7/7	7/7	7/7	7/7	7/ 7	7/7
IL	9/9	9/9	9/9	9/10	9/9	10/10	9/9	10/10
PRO	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1
POO	4/4	4/6	3/3	3/3	3/4	3/3	3/3	3/3
ED	2.61	3.96	3.11	3.15	3.19	4.50	3.01	3.54
ED/HL	0.21	0.21	0.24	0.21	0.22	0.20	0.24	0.26
HL	12.44	18.59	12.70	15.08	14.43	22.90	12.56	13.65
HW	8.24	13.34	8.59	8.96	7.34	20.54	7.70	8.74
HL/HW	1.51	1.39	1.48	1.68	1.97	1.11	1.63	1.56
HL/SVL	0.05	0.04	0.04	0.05	0.04	0.04	0.05	0.04
MAFW	2.65	3.41	2.39	3.63	2.71	4.58	2.53	2.92
MPFW	2.17	2.91	2.25	2.62	2.46	3.54	1.84	2.24
PFK	1.6	2.39	1.82	2.12	1.67	2.15	1.69	1.74
INK	1.21	2.07	1.42	1.44	1.31	*	1.29	1.52
RH	2.41	2.93	2.12	2.48	2.27	3.46	1.58	2.59
RW	3.4	4.41	3.17	3.53	3.23	5.87	3.03	3.47
INL	1.61 / 1.48	2.69/2.82	1.62/ 1.61	1.67/ 1.66	1.71/ 1.66	*	1.52/ 1.75	1.66/ 1.43
INW	1.86 / 1.73	2.55/ 2.58	1.88/ 1.81	2.21/ 2.08	2.08/ 2.11	*	1.68/ 1.79	1.89/ 2.21
PFL	2.31 / 2.36	3.49/ 3.52	2.38/ 2.30	2.73/ 2.82	2.71/ 2.64	3.49/ 3.39	2.31/ 2.38	2.54/ 2.66
PFW	2.43 / 2.39	3.35/ 3.23	2.46/ 2.56	2.66/ 2.68	2.51/ 2.59	4.25/ 4.46	2.12/ 2.18	2.58/ 2.36
FL	4.92	6.57	4.76	5.44	5.41	8.34	4.58	4.93
PL	5.41 / 5.15	7.51/7.22	5.20/ 5.33	5.74/ 5.88	6.27/ 6.11	9.30/ 9.55	4.95/ 5.28	5.72/ 5.95
PW	3.43 / 3.69	2.67/2.68	3.57/ 3.63	3.79/ 3.94	3.43/ 3.49	5.38/ 5.24	3.22/ 3.35	3.87/ 3.55
DFS	3.89	5.32	4.04	4.56	3.95	7.27	4.14	4.12
LL	1.47/ 1.46	2.49/ 2.56	1.48/ 1.39	1.69/ 1.61	1.68/ 1.62	2.61/ 2.53	1.20/ 1.38	1.34/ 1.43
LH	1.27/ 1.23	1.83/ 1.76	1.64/ 1.68	1.26/ 1.29	1.28/ 1.29	2.30/ 2.23	1.15/ 1.10	1.26/ 1.21
ML	1.22	1.81	1.21	1.38	1.25	2.43	0.88	1.35
MW	2.08	2.84	2.34	2.73	2.36	3.83	2.01	2.45
LCAC	4/4	4/4	4/4	4/5	4/4	5/5	4/4	5/5
ACSL	4.0 / 4.05	5.89/ 5.62	3.85/ 4.10	4.49/ 4.43	4.77/ 4.34	7.98/ 8.50	3.50/ 3.57	4.21/ 4.0
PCSL	4.10 / 3.99	6.62/ 6.32	5.02/ 4.57	4.88/ 4.74	5.11/ 5.03	10.02/ 9.24	4.51/ 4.71	5.65/ 6.10

the vicinity of Nahuatzen in central Michoacán (Rossman et al. 1996; Rossman and Lara-Gongora 1997). The Nahuatzen population is of special interest itself. The Nahuatzen area is a particularly high elevation (ca. 3000 m) upland situated in the middle of a continuous swath of moderate mountains (ca. 2000 m) in central Michoacán. While this region is not particularly isolated, the nearest collecting localities of highland *Thamnophis* are *T. ahumadai*, 190 km to the west at Atemajac de Brizuela, Jalisco, and *T. scalaris*, 170 km to the east near Zitacuaro, Michoacán. Based on color pattern alone, these snakes seem very similar to and may be conspecific with *T. ahumadai*. We were unable to acquire molecular data from this population as all specimens in Mexican collections are fixed in formalin. Field work should be done around Nahuatzen to determine whether this population represents an isolated population of *T. ahumadai*, an isolated population of *T. scalaris*, or another yet undescribed species of *Thamnophis*.

Monophyly of *Thamnophis scaliger* and *Thamnophis scalaris*

Based on eleven genetic samples of *Thamnophis scalaris* collected across its range and an additional five samples of *Thamnophis scaliger* from several localities in central Mexico, we found that both *T. scalaris* and *T. scaliger* are monophyletic and do not represent sister species (Fig. 1). De Queiroz et al. (2002) had confusing results for the relationship between *T. scalaris* and *T. scaliger* based on the placement of their "*T. scaliger* 2" within *T. scalaris*. Their "*T. scaliger* 2" was based on LSUMZ 42638 (Accession Number: AF420189), which is supposedly a *T. scaliger* from the vicinity of Villa Victoria, Estado de México. We compared this sample to five *T. scaliger* samples and nine *T. scalaris* samples, all identified in the field by us. This sample was consistently recovered in a clade containing morphologically verified *T. scalaris* in all of our analyses. While we were not able to examine the specimen, we hypothesize that this genetic sample was misidentified as a *T. scaliger* by de Quieroz et al. (2002) and consequently re-identify it as *T. scalaris*.

The positioning of the former genus *Adelophis* within *Thamnophis*

The genus *Adelophis* Dugès *in* Cope, 1879, was originally described as a close relative of the North American genus *Tropidoclonion* Cope, 1860. The first authors to suggest a close relationship between *Adelophis* and *Thamnophis* were Dunn (1931) and Rossman and Blaney (1968), who suggested that *Adelophis* was derived from *Thamnophis.* Later, de Queiroz et al. (2002) obtained molecular sequences from a specimen of *Adelophis foxi* (LSUMZ 40846) and found that it grouped within *Thamnophis*, with its closest relatives being *T. melanogaster* and *T. validus*. De Queiroz et al. (2002) suggested that their sample of *Adelophis* may have been mixed up with another sample; however, they assessed that the genetic distances of the analyzed sequences were too far removed from any of the other analyzed species to represent any of them. These authors had samples from all valid Mexican species of *Thamnophis* at that time except for two (*Thamnophis postremus* Smith, 1942, and *Thamnophis rossmani* Conant, 2000) and cautioned against making any taxonomical changes pending further sampling of "*A. foxi*" and the similar "*A. copei.*" McVay et al. (2015) obtained additional sequences from the same specimen of "*A. foxi*" and confirmed the results obtained by de Queiroz et al. (2002), thus dispelling the possibility of PCR contamination. Nonetheless, these authors also refrained from making any taxonomical changes because the sequenced specimen was the same individual used earlier by de Queiroz et al. (2002) and was thus subject to the same caveats as the prior study. Later, Hallas et al. (2022) found *Adelophis* to be placed within *Thamnophis* across three molecular datasets (mtDNA, nDNA, and ddRADseq) and formally sank the genus *Adelophis* into *Thamnophis*. They cautioned that "some might refrain from formal changes to *A. copei* until that taxon can be suitably evaluated in a phylogenetic analysis." However, Hallas et al. (2022) considered the putative sister relationship between "*A. copei*" and "*A. foxi*" (Rossman and Blaney 1968; Rossman and Wallach 1987) to be sufficient evidence refuting the argument that "*A. copei*" could be nested outside of *Thamnophis*. In this study, we sequenced samples from a specimen of *Thamnophis copei* for the first time and confirmed that *T. copei* falls within the "southern clade" of *Thamnophis* like *T. foxi* (de Queiroz et al. 2002; McVay et al. 2015; Hallas et al. 2022). This confirms that the placement of *Thamnophis copei* is correct and that *Adelophis* should be subsumed into *Thamnophis*. Surprisingly, however, our results suggest that the morphologically similar *T. copei* and *T. foxi* are not sisters to one another, though both are consistently nested within the "Mexican clade" of *Thamnophis*. More extensive sequence data is needed to determine the exact phylogenetic relationship between these two species and closely related species. It is noteworthy that *T. foxi* has not been collected since the 1970s, despite several recent collection attempts.

The validity of the species related to *Thamnophis godmani* in the Sierra Madre del Sur

Thamnophis godmani was described from Omiltemi and "Amula" in Guerrero (Günther 1886) and was later considered to be distributed in the Sierra Madre del Sur and Sierra Madre Oriental from central Guerrero and central Veracruz (respectively) to the Isthmus of Tehuantepec (Rossman et al. 1996). *Thamnophis godmani* has historically been recognized as a close relative of *T. scalaris*, and Smith (1942) even considered *T. godmani* to be a subspecies of the former taxon. The specific identity of *T. godmani* was reaffirmed when more specimens and morphological data became available (Rossman in Varkey 1979). Later, Rossman and Burbrink (2005) conducted a multivariate study of morphological characters in various populations of *T. godmani* and described three species: *T. bogerti*, *T. conanti*, and *T. lineri*.

Rossman and Burbrink (2005) provided subtle morphological and mensural characters to justify the description of *T. bogerti*, *T. conanti*, and *T. lineri* as species distinct from *T. godmani*. While the morphological data provided does suggest that *T. godmani* may be specifically distinct from the other three, the morphological differences are trivial once data from *T. bogerti*, *T. conanti*, and *T. lineri* are compared to one another. When compared to the intraspecies variation of other widespread highland *Thamnophis* such as *T. scaliger*, *T. scalaris*, *T. errans*, *T. chrysocephalus*, and *T. sumichrasti*, the subtle geographic variation found across *T. bogerti* and its relatives over a relatively broad range suggests that these taxa likely represent a single evolutionary species unit. Morphological and mensural differences for the three species were presented in the description only in text; however, here we compare them in Table 2.

We evaluated the status of these species (*T. bogerti*, *T. conanti*, and *T. lineri*) in our phylogeny, and our results indicate that *T. bogerti* is paraphyletic with respect to *T. conanti* and *T. lineri* (Fig. 1, Suppl. materials 1–3). Additionally, we found very low levels of genetic divergence between these species. Genetic distances in the mitochondrial gene ND4 were as low as 0.002 between *T. bogerti* and *T. conanti*, whereas both of these species show a genetic distance of 0.04–0.05 with their closest relative, *T. scalaris*. Genetic distances in the Cytb between *T. bogerti*, *T. conanti*, and *T. lineri* ranged from 0.00–0.01, whereas all three of these "species" had distances between 0.03–0.04 from their closest relative, *T. ahumadai*. (Suppl. material 4). These results support the tree topologies of McVay et al. (2015) and

Table 2. Comparisons of *Thamnophis godmani*, *T. bogerti*, *T. conanti*, and *T. lineri* taken from the descriptions of the latter three (Rossman and Burbrink 2005).

	T. godmani	*T. bogerti*	*T. conanti*	*T. lineri*
DSR	maximum DSR 17	maximum DSR 17	maximum DSR 17	maximum DSR 17
Maxillary Teeth	Maxillary Teeth 17–21	Maxillary Teeth 17–20	Maxillary Teeth 16–18	Maxillary Teeth 18–20
Dorsal Head Coloration	top of head unpatterned	top of head unpatterned	top of head unpatterned	top of head unpatterned
Dorsal Pattern	two rows of small black spots between light lines	two rows of small black spots between light lines	two rows of small black spots between light lines	two rows of small black spots between light lines
Nuchal Blotch Coloration	nuchal blotch coloration black	nuchal blotch coloration variable, only 15% brown	nuchal blotches brown	nuchal blotches predominately brown
Suture coloration on SL	prominence of black bar along posterior suture of SL 5 equal to or greater than bar along SL 6 and SL 7 suture	prominence of black bar along posterior suture of SL 5 equal to or less than bar along SL 6 and SL 7 suture	prominence of black bar along posterior suture of SL 5 equal to or less than bar along SL 6 and SL 7 suture	black bar along posterior suture of SL 5 reduced or absent
VS	V 144 males, 138 in females	V 145 males, 140 females	V 150 in males, 144 females	140 in males, 136 in females
SC	SC average 79 in males, 71 females	SC average 70 males, 62 females	SC average 72 males, 62 females	SC average 62 males, 55 females
TL/TotL	TL/TotL 27% males, 26% females	TL/TotL 25% males, 23% females	TL/TotL 25% males, 23% females	TL/TotL 23.5% males, 21.5% females
Prefrontal Length	prefrontal suture usually slightly shorter than internasal suture PFK/INK 94%	prefrontal suture usually slightly longer than internasal suture PFK/INK 106%	prefrontal suture usually slightly longer than internasal suture PFK/INK 105%	prefrontal suture usually slightly longer than internasal suture PFK/INK 106%
Muzzle Shape	muzzle tip very broad, INR / NR 134%	muzzle tip usually broad, INR / NR 115%	muzzle tip broad INR / NR 107%	muzzle tip usually broad, INR / NR 116%
Nasal Condition	anterior and posterior nasal subequal	anterior nasal usually shorter than posterior nasal	anterior nasal usually shorter than posterior nasal	anterior nasal usually shorter than posterior nasal
Parietal Length	parietal short FL/PL 88%	FL/PL 77%	FL/PL 77%	FL/PL 80%
Frontal Condition	Frontal Broad Posteriorly / MPFW/ MAFW 72%	Frontal Broad Posteriorly / MPFW/ MAFW 79%	Frontal Broad Posteriorly / MPFW/ MAFW 75%	Frontal Broad Posteriorly / MPFW/ MAFW 85%

Hallas et al. (2022). Unfortunately, we did not have any genetic material of *T. godmani* to compare with, and recent analyses that did include *T. godmani* (Hallas et al. 2022) are based on a sample of *T. bogerti* from Oaxaca (J. Campbell, pers. comm). *Thamnophis godmani* is not known to occur in Oaxaca, and all samples of "*T. godmani*" found on Genbank are based on specimens that were collected before *T. bogerti* was described (Rossman and Burbrink 2005). Consequently, our phylogenetic results indicate that *T. bogerti*, *T. conanti*, and *T. lineri* belong to the same evolutionary unit. As these three species were described in the same paper, we invoke Article 24.2 of the International Code of Zoological Nomenclature (ICZN 1999) and suggest that *T. lineri* and *T. conanti* are junior synonyms of *T. bogerti*, the latter of which was alphabetically and sequentially described first in Rossman and Burbrink (2005). The specific relationship between *T. godmani* and *T. bogerti* remains to be tested; however, the isolated nature of the Guerrero populations of this species may indeed prove to be of a specific nature.

Rossman and Burbrink (2005), in their review of the populations formerly assigned to *T. godmani*, stated that "*T. godmani* occurs in at least four discrete geographic areas that are effectively separated at the present time by habitat disjunctions unsuitable for these residents of montane pine-oak forests (1768–3048 m)." However, these sky islands inhabited by the snakes formerly assigned to *T. godmani* have not been proven to act as biogeographic barriers for other reptiles (Bryson et al. 2011; Palacios-Aguilar et al. 2021). Furthermore, *T. bogerti* is frequently collected as low as 1100–1200 m in humid environs in Oaxaca, and oak woodland in the Sierra Juárez and Sierra Zongolica ranges down to at least 1500 m. Thus, these perceived "isolated mountain ranges" are not barrier-isolated for an elevation-adapted snake like *T. bogerti*.

Identity of *Thamnophis* similar to *T. scalaris* in the Sierra de Pinal de Amoles, Querétaro

An unidentified population of *Thamnophis* occurs in the vicinity of Pinal de Amoles in the Sierra Gorda region of Querétaro (Rossman and Lara-Gongora, 1997). These specimens (UMMZ 105415–416) are cataloged as *T. scalaris* but originate from well outside of the known range of this species. Rossman and Lara-Gongora (1997) discussed these specimens in detail, comparing them to *T. scalaris*, *T. scaliger*, and *T. exsul*, and concluded that "until fresh material becomes available, it would seem prudent to defer judgment of the identity of these Querétaro specimens." We collected two specimens (INIRENA 2937–38, Fig. 8) of this population in 2020 and included them in our analyses.

Morphologically, the two specimens seem to match *T. scalaris* relatively well. INIRENA 2937 is an adult female and has 17-17-17 dorsal scale rows, whereas INIRENA 2938 is a subadult male and has 19-17-17 dorsal scales. Supralabials are seven on both specimens; infralabials are 9/10 on INIRENA 2937 and 10/10 on INIRENA 2938. Ventrals 141 on both specimens, subcaudals 52 (INIRENA 2937) and 57 (INIRENA 2938), respectively. The subcaudal count is lower than the ranges given for eastern *T. scalaris* by Rossman and Lara-Gongora (1997), which were 53–69 (females) and 64–85 (males). The tail lengths are short for *T. scalaris*, with TL/TotL 19.8% on INIRENA 2937 and 21.4% on INIRENA 2938. Rossman and Lara (1997) gave TL/TotL ranges of 20.2–25.0% (females) and 25.9–33.2% (males). Rossman and Lara (1997) noted that the hemipenes on UMMZ 104415 were nine subcaudals long. The hemipenes on INIRENA 2938 are six subcaudals long, although they may not have been everted to their fullest extent. While the tail lengths

Figure 8. Photographs of *Thamnophis scalaris* from Querétaro. Female (INIRENA 2937) (**A, B**) and male (INIRENA 2938) (**C, D**) both from 1 km N of Pinal de Amoles, Municipio de Pinal de Amoles, Querétaro.

and subcaudal counts are very low for eastern *T. scalaris* and the hemipenes are short, in all other aspects, these specimens seem to match *T. scalaris* reasonably well.

Our phylogenetic analysis suggests that these specimens are nested with *T. scalaris*. Genetic distances for ND4 between this population and nearby Veracruz *T. scalaris* range from 0.00–0.01. In comparison, the genetic distances of ND4 between the Queretaro specimens and *T. scaliger* range from 0.02–0.06, between *T. exsul* 0.04 and *T. errans* 0.05. The genetic distance of Cytb in these specimens to nearby *T. scalaris* in Puebla and northern Veracruz ranges from 0.00–0.01. Comparatively, the genetic distances of Cytb in the Queretaro specimen to specimens of *T. scaliger* are 0.04–0.07, and for *T. exsul*, they are 0.04. Based on these results, we formally assign the Pinal de Amoles (Sierra Gorda) population to *T. scalaris* and confirm the presence of *T. scalaris* in Querétaro.

Acknowledgements

We thank our field crew for their courageous enthusiasm to go out in the field in Guerrero, including but not limited to Iván Ahumada-Carrillo, Brandon T. La Forest, Jason M. Jones, Ricardo Ramírez-Chaparro, Jorge A. Bañuelos-Alamillo, Ginny N. Weatherman, Karen I. Morales-Flores, Ámbar Lanomy Grünwald, Janelle Morales-Flores, and Alejandro Lara. We thank Adrian Nieto Montes de Oca, Oscar Flores-Villela and Leticia Ochoa (MZFC), Ireri Suazo-Ortuño and Jonathan Torres-Pérez-Coeto (INIRENA), and Gregory Pandelis (UTA) for letting us examine preserved specimens under their care; the latter two also took photographs of specimens for our review. For providing photographs of museum specimens, we are indebted to Joshua Mata and Rachunliu G. Kamei (FMNH), Gregory Schneider (UMMZ), Teresa Hsu (USNM), and Jonathan Campbell (UTA). We thank Jason M. Jones, Iván Ahumada-Carrillo, Robert Hansen, Hugo Plata-Tinoc, and Anibal Díaz de la Vega-Pérez for providing photographs of live specimens in the field. We thank Justin Lee and four anonymous reviewers for their detailed review and feedback on an earlier version of the manuscript. Funding for molecular data generation was provided by the Consejo Nacional de Ciencia y Tecnología (CONACyT A1-S-37838) and the Dirección General de Asuntos del Personal Académico, Universidad Nacional Autónoma de México (DGAPA, UNAM) (PAPIIT IN218022 y PAPIME PE208422) to U.O. García-Vázquez (UOG-V). We thank María de los Ángeles Palma Irizarry of the Secretaría de Medio Ambiente y Recursos Naturales (SEMARNAT) for providing and collecting permits.

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Appendix 1

Table A1. Specimens examined. Two samples are from shed skins where specimens were not collected, but genetic material was included in the analysis and these are included in bold print.

Field number	Museum number	Date	Species	Locality	State	Coordinates	Elevation
CIG-1936	pending	12-Jun-21	*Thamnophis bogerti*	Municipio de San Mateo Río Hondo: La Doncella	Oaxaca	16.121632, -96.505225	2736 m
CIG-1937	pending	12-Jun-21	*Thamnophis bogerti*	Municipio de San Mateo Río Hondo: La Doncella	Oaxaca	16.121632, -96.505225	2736 m
CIG-1938	pending	13-Jun-21	*Thamnophis bogerti*	Municipio de San Augustín Loxicha: La Paz Obispo	Oaxaca	16.069370, -96.577196	2160 m
CIG-0626	pending	10-Oct-15	*Thamnophis cyrtopsis*	Municipio de Santiago: La Camotera, near Laguna de Sánchez	Nuevo León	25.318675, -100.212369	1583 m
CIG-0805	pending	28-May-16	*Thamnophis cyrtopsis*	Municipio de Aramberri: S of La Ascención	Nuevo León	22.22677, -99.87677	2010 m
CIG-1677	pending	NA	*Thamnophis cyrtopsis*	Municipio de Noria de Angeles: Villa de Gonzalez Ortega	Zacatecas	22.407739, -101.875726	2300 m
CIG-1801	pending	02-Aug-20	*Thamnophis cyrtopsis*	Municipio de Colón: Los Trigos, Cerro Zamorano	Querétaro	20.906709, -100.206763	2606 m
CIG-1811	pending	08-Aug-20	*Thamnophis cyrtopsis*	Municipio de Catorce: 11.3 km airline ESE of Estación Wadley	San Luis Potosí	23.577618, -100.878592	2794 m
CIG-1812	pending	08-Aug-20	*Thamnophis cyrtopsis*	Municipio de Catorce: 11.3 km airline ESE of Estación Wadley	San Luis Potosí	23.577618, -100.878592	2794 m
CIG-1990	pending	11-Jul-21	*Thamnophis cyrtopsis*	Municipio de Tepic: NE side of Rancho La Noria	Nayarit	21.4860, -104.9921	1572 m
CIG-1991	pending	11-Jul-21	*Thamnophis cyrtopsis*	Municipio de Tepic: NE side of Rancho La Noria	Nayarit	21.4860, -104.9922	1572 m
CIG-1992	pending	11-Jul-21	*Thamnophis cyrtopsis*	Municipio de Tepic: NE side of Rancho La Noria	Nayarit	21.4860, -104.9923	1572 m
CIG-1993	pending	11-Jul-21	*Thamnophis cyrtopsis*	Municipio de Tepic: NE side of Rancho La Noria	Nayarit	21.4860, -104.9924	1572 m
CIG-1994	pending	11-Jul-21	*Thamnophis cyrtopsis*	Municipio de Tepic: NE side of Rancho La Noria	Nayarit	21.4860, -104.9925	1572 m
CIG-1995	pending	11-Jul-21	*Thamnophis cyrtopsis*	Municipio de Tepic: NE side of Rancho La Noria	Nayarit	21.4860, -104.9926	1572 m
CIG-1996	pending	11-Jul-21	*Thamnophis cyrtopsis*	Municipio de Tepic: NE side of Rancho La Noria	Nayarit	21.4860, -104.9927	1572 m
CIG-1997	pending	11-Jul-21	*Thamnophis cyrtopsis*	Municipio de Tepic: NE side of Rancho La Noria	Nayarit	21.4860, -104.9928	1572 m
CIG-1998	pending	11-Jul-21	*Thamnophis cyrtopsis*	Municipio de Tepic: NE side of Rancho La Noria	Nayarit	21.4860, -104.9929	1572 m
CIG-1999	pending	11-Jul-21	*Thamnophis cyrtopsis*	Municipio de Tepic: NE side of Rancho La Noria	Nayarit	21.4860, -104.9930	1572 m
CIG-2000	pending	11-Jul-21	*Thamnophis cyrtopsis*	Municipio de Tepic: NE side of Rancho La Noria	Nayarit	21.4860, -104.9931	1572 m
CIG-2001	pending	11-Jul-21	*Thamnophis cyrtopsis*	Municipio de Tepic: NE side of Rancho La Noria	Nayarit	21.4860, -104.9932	1572 m
CIG-2002	pending	11-Jul-21	*Thamnophis cyrtopsis*	Municipio de Tepic: NE side of Rancho La Noria	Nayarit	21.4860, -104.9933	1572 m
CIG-2003	pending	11-Jul-21	*Thamnophis cyrtopsis*	Municipio de Tepic: NE side of Rancho La Noria	Nayarit	21.4860, -104.9934	1572 m
CIG-2004	pending	11-Jul-21	*Thamnophis cyrtopsis*	Municipio de Tepic: NE side of Rancho La Noria	Nayarit	21.4860, -104.9935	1572 m

Field number	Museum number	Date	Species	Locality	State	Coordinates	Elevation
CIG-2006	pending	06-Sep-21	*Thamnophis cyrtopsis*	Municipio de Yécora: 3.8 km W of Yécora on Hwy. 16	Sonora	28.3619, -108.96497	1691 m
CIG-2183	pending	18-Aug-22	*Thamnophis cyrtopsis*	Municipio de Tecpán de Galeana: La Laguna	Guerrero	17.577641, -100.767848	1055 m
CIG-2005	pending	06-Sep-21	*Thamnophis eques*	Municipio de Yécora: west of Yécora	Sonora	28.374691, -109.028245	1942 m
CIG-2138	pending	30-Jun-22	*Thamnophis eques*	Municipio de Aguascalientes: Hwy. 36, 1.2 km NW of Jalisco stale line.	Aguascalientes	21.720405, -102.155511	2007 m
CIG-1645	pending	06-Mar-19	*Thamnophis eques*	Municipio de Huachinango: Rancho Las Truchas	Puebla	20.114709, -98.107585	2041 m
CIG-1646	pending	06-Mar-19	*Thamnophis eques*	Municipio de Huachinango: Rancho Las Truchas	Puebla	20.114709, -98.107585	2041 m
CIG-1562	pending	08-Aug-19	*Thamnophis errans*	Municipio de Mezquital: Los Charcos	Durango	23.012354, -104.299608	2693 m
CIG-1563	pending	08-Aug-19	*Thamnophis errans*	Municipio de Mezquital: Los Charcos	Durango	23.012354, -104.299608	2693 m
CIG-1564	pending	08-Aug-19	*Thamnophis errans*	Municipio de Mezquital: Los Charcos	Durango	23.012354, -104.299608	2693 m
CIG-1862	pending	26-Oct-20	*Thamnophis errans*	Municipio de Guadalupe y Calvo: Terreros	Chihuahua	26.194814, -106.588628	2590 m
CIG-2239	pending	30-Jun-22	*Thamnophis errans*	Municipio de Aguascalientes: Hwy. 36, 1.2 km NW of Jalisco stale line.	Aguascalientes	25.066337, -106.319703	2537 m
CIG-1833	**NA**	**10-Aug-20**	***Thamnophis exsul***	**Cerro Peña Nevada, Puerto Peña Nevada**	**Nuevo León**	**23.823812, -99.878490**	**2657 m**
CIG-1834	**NA**	**10-Aug-20**	***Thamnophis exsul***	**Cerro Peña Nevada, salamander spot 2**	**Nuevo León**	**23.836308, -99.856751**	**2658 m**
CIG-1001	pending	06-Sep-16	*Thamnophis fulvus*	Puente Malá, El Chiquihuite, Unión Juárez	Chiapas	15.093985, -92.091741	1790 m
CIG-1468	pending	29-Jun-19	*Thamnophis* aff. *chrysocephalus*	just below Puerto del Gallo	Guerrero	17.48739, -100.19932	2457 m
CIG-1315	pending	23-Apr-18	*Thamnophis hammondii*	Cienega La Grulla, Sierra San Pedro Martir	Baja California	30.892369, -115.481361	2069 m
CIG-1314	pending	23-Apr-18	*Thamnophis hueyi*	Cienega La Grulla, Sierra San Pedro Martir	Baja California	30.892369, -115.481361	2069 m
CIG-1448	pending	19-May-19	*Thamnophis hueyi*	Cienega La Grulla, Sierra San Pedro Martír	Baja California	30.889159, -115.463460	2068 m
CIG-1449	pending	19-May-19	*Thamnophis hueyi*	Cienega La Grulla, Sierra San Pedro Martír	Baja California	30.889159, -115.463460	2068 m
CIG-1494	pending	19-May-19	*Thamnophis hueyi x hammondii*	Cienega La Grulla, Sierra San Pedro Martír	Baja California	30.894320, -115.481086	2066 m
CIG-0810	pending	29-May-16	*Thamnophis* aff. *pulchrilatus*	Near Valle Hermoso, Sierra de Miquihuana	Tamaulipas	22.672603, -99.7941210	2472 m
CIG-0811	pending	29-May-16	*Thamnophis* aff. *pulchrilatus*	Near Valle Hermoso, Sierra de Miquihuana	Tamaulipas	22.672603, -99.7941210	2472 m
CIG-0812	pending	29-May-16	*Thamnophis* aff. *pulchrilatus*	La Marcela	Tamaulipas	23.744825, -99.816433	2490 m
CIG-1848	pending	10-Sep-20	*Thamnophis rossmani*	Municipio de Tepic: between El Armadillo and San Cayetano	Nayarit	21.4392664, -104.842978	924 m
CIG-0500	MZFZ-4593	11-Jul-15	*Thamnophis ahumadai* sp. nov.	Municipio de Talpa de Allende: nr. Cumbre de Guadalupe,	Jalisco	20.169099, -104.711496	2129 m
CIG-1609	MZFZ-4594	23-Aug-19	*Thamnophis ahumadai* sp. nov.	Municipio de Talpa de Allende: nr. Cumbre de Guadalupe,	Jalisco	20.186581, -104.716188	2178 m
CIG-1610	MZFZ-4595	23-Aug-19	*Thamnophis ahumadai* sp. nov.	Municipio de Tomatlán: Cumbre de Guadalupe, Sierra Cacoma	Jalisco	20.157217, -104.675059	2319 m
CIG-1611	INIRENA-2932	23-Aug-19	*Thamnophis ahumadai* sp. nov.	Municipio de Cuautla: Cumbre de Guadalupe, Sierra Cacoma	Jalisco	20.168991, -104.684925	2353 m
CIG-1612	INIRENA-2933	23-Aug-19	*Thamnophis ahumadai* sp. nov.	Municipio de Cuautla: Cumbre de Guadalupe, Sierra Cacoma	Jalisco	20.168991, -104.684925	2353 m
CIG-1613	INIRENA-2934	23-Aug-19	*Thamnophis ahumadai* sp. nov.	Municipio de Cuautla: Cumbre de Guadalupe, Sierra Cacoma	Jalisco	20.168991, -104.684925	2353 m
CIG-1700	INIRENA-2935	21-Jun-20	*Thamnophis ahumadai* sp. nov.	Municipio de Atemajac de Brizuela: Presa near Atemajac	Jalisco	20.1151731, -103.7036033	2422 m
CIG-1701	INIRENA-2936	21-Jun-20	*Thamnophis ahumadai* sp. nov.	Municipio de Atemajac de Brizuela: Presa near Atemajac	Jalisco	20.1151731, -103.7036033	2422 m
CIG-1738	INIRENA 2939	29-Jun-20	*Thamnophis scalaris*	Municipio de Huitzilac: Volcán Chichinautzín	Morelos	19.08675, -99.148971	3278 m
CIG-1781	INIRENA 2937	12-Jul-20	*Thamnophis scalaris*	Municipio de Pinal de Amoles: 1.0 km N of Pinal de Amoles	Querétaro	21.1431, -99.6222	2241 m
CIG-1782	INIRENA 29378	13-Jul-20	*Thamnophis scalaris*	Municipio de Pinal de Amoles: 1.0 km N of Pinal de Amoles	Querétaro	21.1431, -99.6222	2241 m
CIG-1739	pending	29-Jun-20	*Thamnophis scaliger*	Municipio de Atlacomulco: Tecoac	México	19.77559, -99.845494	2525 m
CIG-1740	pending	29-Jun-20	*Thamnophis scaliger*	Municipio de Atlacomulco: Tecoac	México	19.77559, -99.845494	2525 m
CIG-1789	pending	01-Aug-20	*Thamnophis scaliger*	Municipio de Ocampo: 8 km NW of Ocampo, on Hwy. 51	Guanajuato	21.718780, -101.507760	2273 m
CIG-1961	pending	04-Jul-21	*Thamnophis scaliger*	Municipio de Hidalgo: 6.4 km NNE of Mil Cumbres	Michoacán	19.664948, -100.750429	2308 m
CIG-1989	pending	04-Jul-21	*Thamnophis scaliger*	Municipio de Hidalgo: 6.4 km NNE of Mil Cumbres	Michoacán	19.664948, -100.750429	2308 m
CIG-1203	pending	01-Jul-17	*Thamnophis bogerti*	Municipio: San Juan Atepec: Llano de las Flores, Sierra Juarez	Oaxaca	17.440299, -96.508401	2912 m
CIG-1913	pending	19-Jun-21	*Thamnophis validus*	Municipio de Coahuayana: Hwy. 200 at Coahuayana Rd.	Michoacán	18.67476, -103.67911	15 m

Field number	Museum number	Date	Species	Locality	State	Coordinates	Elevation
CIG-1952	pending	05-Jul-21	*Thamnophis vicinus*	Municipio de Charo: pond near Pontezuelas	Michoacán	19.641433, -100.995025	2233 m
CIG-1953	pending	05-Jul-21	*Thamnophis vicinus*	Municipio de Charo: pond near Pontezuelas	Michoacán	19.641433, -100.995025	2233 m
AEVB-0095	pending	03-Nov-17	*Thamnophis chrysocephalus*	Municipio de Los Reyes: Finca Santa Martha	Veracruz	18.652957, -97.009057	1421 m
AEVB-0136	pending	26-Mar-18	*Thamnophis cyrtopsis*	Municipio de Tejupilco: El Tule	México	19.0161, -100.11149	1562 m
AEVB-0005	pending	05-Feb-17	*Thamnophis scalaris*	Municipio de Zacatlán: Valle de piedras encimadas	Puebla	20.024296, -98.050986	2535 m
AEVB-0027	pending	05-Feb-17	*Thamnophis scalaris*	Municipio de Zacatlán: Valle de piedras encimadas	Puebla	20.024296, -98.050986	2535 m
AEVB-0041	pending	19-Nov-16	*Thamnophis scalaris*	Municipio de Acajete: La Joya	Veracruz	19.618235, -97.023109	2170 m
AEVB-0043	pending	19-Nov-16	*Thamnophis scalaris*	Municipio de Acajete: La Joya	Veracruz	19.618235, -97.023109	2170 m
JCSG-0291	pending	28-Apr-19	*Thamnophis conanti*	Municipio de Nogales: Sierra de Agua	Veracruz	18.874395, -97.209868	2249 m
JCSG-0247	pending	21-Sep-18	*Thamnophis sumichrasti*	Municipio de San Juan Tehuacán: El Pedregal	Veracruz	18.618352, -97.047550	2130 m
LOR-0091	pending	28-Jul-18	*Thamnophis chrysocephalus*	Municipio de Zongolica: Tlaquilpa	Veracruz	18.608160, -97.113641	2325 m
LOR-0093	pending	28-Jul-18	*Thamnophis conanti*	Municipio de Soledad Atzompa: Acultzinapa	Veracruz	18.689601, -97.191876	2660 m
RICB-0366	pending	NA	*Thamnophis cyrtopsis*	Municipio de Jacala: La Placita	Hidalgo	20.976902, -99.211789	1425 m
UOGV-3949	pending	15-Aug-20	*Thamnophis conanti*	Municipio de Soledad Atzompa: Acultzinapan	Veracruz	18.689601, -97.191876	2660 m
UOGV-2952	pending	25-Jun-17	*Thamnophis cyrtopsis*	Municipio de Santo Domingo Tonalá: Boquerón de Tonala	Oaxaca	17.63895, -97.94565	1945 m
UOGV-2987	pending	28-Jul-17	*Thamnophis cyrtopsis*	Municipio de Tejupilco: El Tule	México	19.0149, -100.1017	1540 m
UOGV-3932	pending	27-Jun-20	*Thamnophis scalaris*	Municipio de Nogales: Santa Cruz	Veracruz	18.8741683, -97.2063083	2211 m
UOGV-3692	pending	16-Sep-19	*Thamnophis eques*	Municipio de El Tule: Carretera 23 en dirección a Guachochi	Chihuahua	27.0644, -106.26837	1552 m
UOGV-3742	pending	NA	*Thamnophis* aff. *chrysocephalus*	Municipio de Tecpan de Galeana: El Pinito	Guerrero	17.5730, -100.5660	1986 m

Appendix 2

Table A2. Genbank accession numbers used in this study. New sequences generated by us are indicated in bold.

Species	Locality	Catalogue #	Cytb	ND4
Nerodia erythrogaster	USA: Texas, San Saba Co.	CU12550	AF420081	AF420084
Thamnophis ahumadai sp. nov.	Mexico: Jalisco, Atemajac de Brizuela	CIG1700 / INIRENA2935	-	**PP273357**
Thamnophis ahumadai sp. nov.	Mexico: Jalisco, Sierra Cacoma	CIG0500 / MZFC4593	-	**PP273368**
Thamnophis ahumadai sp. nov.	Mexico: Jalisco, Atemajac de Brizuela	CIG1701 / INIRENA2936	**PP273342**	**PP273358**
Thamnophis ahumadai sp. nov.	Mexico: Jalisco, Sierra Cacoma	CIG1609 / MZFZ 4595	**PP273343**	**PP273354**
Thamnophis atratus	USA: California	CU12418	AF420085	AF420088
Thamnophis bogerti	Mexico: Oaxaca	MZFC ART 145	AF420135	AF420138
Thamnophis bogerti	Mexico: Oaxaca, La Doncella	CIG1936	**PP273312**	-
Thamnophis bogerti	Mexico: Oaxaca, La Doncella	CIG1937	**PP273313**	-
Thamnophis bogerti	Mexico: Oaxaca, San Augustin Loxicha	CIG1938	**PP273314**	-
Thamnophis brachystoma	USA: Pennsylvania	CU12379, CAS163984	AF420089	AF420092
Thamnophis butleri	USA: Michigan, Monroe Co.	CU12511	AF420107	AF420095
Thamnophis chrysocephalus	Mexico: Guerrero, Puerto del Gallo	CIG1468	**PP273310**	**PP273352**
Thamnophis chrysocephalus	Mexico: Guerrero, Tecpan de Galeana	UOGV3742	**PP273311**	**PP273375**
Thamnophis chrysocephalus	Mexico: Oaxaca	MZFC-WSB 767	AF420108	AF420098
Thamnophis chrysocephalus	Mexico: Veracruz, Los Reyes	AEVB095	**PP273315**	**PP273350**
Thamnophis chrysocephalus	Mexico: Veracruz, Zongolica	LOR0091	**PP273316**	**PP273370**
Thamnophis chrysocephalus	Mexico: Veracruz, Zongolica	RH13118	**PP273317**	-
Thamnophis "conanti"	Mexico: Veracruz, Atzompa	LOR0093	**PP273318**	**PP273371**
Thamnophis "conanti"	Mexico: Veracruz, Atzompa	UOGV3949	**PP273319**	-
Thamnophis "conanti"	Mexico: Veracruz, Nogales	JCSG291	**PP273320**	-
Thamnophis copei	Mexico: Jalisco, Sierra Quila	CIG1856	**MZ287373**	**MZ287399**
Thamnophis couchii	USA: California, Lassen Co.	CAS165838	AF420103	AF420106
Thamnophis cyrtopsis	Mexico: Hidalgo, Jacala	RICB366	-	**PP273372**
Thamnophis cyrtopsis	Mexico: Mexico State	AEVB136	-	**PP273349**
Thamnophis cyrtopsis	Mexico: Mexico, Tejupilco	UOGV2987	**PP273321**	-
Thamnophis cyrtopsis	Mexico: Michoacan, Tzitzio	CIG1952	**PP273322**	-
Thamnophis cyrtopsis	Mexico: Michoacan, Tzitzio	CIG1953	**PP273345**	-
Thamnophis cyrtopsis	Mexico: Nayarit, Cerro San Juan	CIG1990	**PP273323**	-
Thamnophis cyrtopsis	Mexico: Oaxaca, Santo Domingo Tonala	UOGV3169	-	**PP273373**
Thamnophis cyrtopsis	Mexico: Oaxaca, Boqueron de Tonala	UOGV2952	**PP273324**	-
Thamnophis cyrtopsis	Mexico: Querétaro, Cerro Zamorano	CIG1801	-	**PP273362**
Thamnophis cyrtopsis	Mexico: San Luis Potosí, Sierra de Catorce	CIG1811	-	**PP273363**
Thamnophis cyrtopsis	Mexico: San Luis Potosí, Sierra de Catorce	CIG1812	-	**PP273364**

Species	Locality	Catalogue #	Cytb	ND4
Thamnophis cyrtopsis	Mexico: Sonora, Yecora	CIG2006	**PP273325**	-
Thamnophis cyrtopsis	Mexico: Veracruz, Zongolica	RH13119	**PP273326**	-
Thamnophis cyrtopsis	Mexico: Zacatecas, Villa Gonzalez Ortega	CIG1677	-	**PP273356**
Thamnophis cyrtopsis	Mexico: San Luis Potosí, Real de Catorce	CIG0805	-	**PP273369**
Thamnophis cyrtopsis collaris	Mexico: Guerrero, El Miraval	LACM130112	AF420099	AF420102
Thamnophis cyrtopsis cyrtopsis	USA: Arizona, Pima Co.	ADQ194A	AF420109	AF420112
Thamnophis elegans hueyi	Mexico: Baja California, Sierra San Pedro Martir	CIG1314	**PP273327**	-
Thamnophis elegans terrestris	USA: California, Sonoma Co.	CAS219410	AF420113	AF420116
Thamnophis eques	Mexico: Chihuahua, Guachochi	UOGV3692	-	**PP273374**
Thamnophis eques	Mexico: Puebla, Rancho Las Truchas	CIG1645	-	**PP273355**
Thamnophis eques	USA: Arizona, Yavapai Co.	CU12516	AF420117	AF420120
Thamnophis errans	Mexico: Chihuahua, Guadalupe y Calvo	CIG1862	-	**PP273366**
Thamnophis errans	Mexico: Durango, Charcas	CIG1563	-	**PP273353**
Thamnophis errans	Mexico: Durango, Mil Diez	LSUMZ40836	AF420121	AF420124
Thamnophis exsul	Mexico: Nuevo León, Peña Nevada	CIG1834	-	**PP273365**
Thamnophis exsul	Mexico: Nuevo Leon, Peña Nevada	CAS218283	AF420125	AF420128
Thamnophis foxi	Mexico: Durango, Mil Diez	LSUMZ40846	AF420069	AF420072
Thamnophis fulvus	Mexico: Chiapas, Union de Juarez	CIG1001	-	**PP273351**
Thamnophis fulvus	Guatemala: Quiche	UTA42315	AF420129	AF420132
Thamnophis gigas	USA: California, Colousa Co.	LSUMZ44368	AF420133	AF420134
Thamnophis hammondii	USA: California, San Bernardino Co.	CAS179062	AF420139	AF420142
Thamnophis "*lineri*"	Mexico: Oaxaca, Sierra Juarez	CIG1203	**PP273329**	-
Thamnophis marcianus	USA: Texas	CU12387	AF420143	AF420146
Thamnophis melanogaster	Mexico: Jalisco, Chapala	CAS165420	AF420147	AF420150
Thamnophis mendax	Mexico: Tamaulipas, Gomes Farías	R. Highton	AF420151	-
Thamnophis nigronuchalis	Mexico: Durango, El Salto	LSUMZ40830	AF420153	AF420156
Thamnophis ordinoides	USA: California, Del Norte Co.	CU12461	AF420157	AF420160
Thamnophis proximus	USA: Texas, San Saba Co.	CU12397	AF420161	AF420164
Thamnophis pulchrilatus	Mexico: Nuevo León, Peña Nevada	CAS 218285	AF420165	AF420168
Thamnophis radix	USA: Colorado, Denver Co.	CAS214183	AF420169	AF420172
Thamnophis rufipunctatus	USA: New Mexico, Catrom Co.	CU12457	AF420173	AF420176
Thamnophis saurita	USA: Florida, Okeechobee	CAS204801	AF420177	AF420180
Thamnophis scalaris	Mexico: Estado de Mexico, Navajas	CAS214292	AF420181	AF420184
Thamnophis scalaris	Mexico: Estado de Mexico, Villa Victoria	LSUMZ42638	AF420185	AF420188
Thamnophis scalaris	Mexico: Morelos, Chichinautzin	CIG1738 / INIERNA2939	**PP273330**	**PP273359**
Thamnophis scalaris	Mexico: Puebla, Zacatlan	AEVB005	**PP273336**	**PP273347**
Thamnophis scalaris	Mexico: Puebla, Zacatlan	AEVB027	**PP273331**	-
Thamnophis scalaris	Mexico: Queretaro, Pinal de Amoles	CIG1781 / INIRENA2937	**PP273332**	-
Thamnophis scalaris	Mexico: Queretaro, Pinal de Amoles	CIG1782 / INIRENA2938	**PP273333**	**PP273360**
Thamnophis scalaris	Mexico: Veracruz, La Joya	AEVB104	-	**PP273348**
Thamnophis scalaris	Mexico: Veracruz, Acajete	AEVB041	**PP273334**	-
Thamnophis scalaris	Mexico: Veracruz, Acajete	AEVB043	**PP273335**	**PP273346**
Thamnophis scalaris	Mexico: Veracruz, Nogales	UOGV3932	**PP273337**	**PP273376**
Thamnophis scaliger	Mexico: Estado de Mexico, Atizapán	CAS214293	AF420189	AF420192
Thamnophis scaliger	Mexico: Guanajuato, Ocampo	CIG1789	**PP273338**	**PP273361**
Thamnophis scaliger	Mexico: Mexico, Tecoac	CIG1739	**PP273339**	-
Thamnophis scaliger	Mexico: Mexico, Tecoac	CIG1740	**PP273340**	-
Thamnophis scaliger	Mexico: Michoacan, Mil Cumbres	CIG1989	**PP273341**	**PP273367**
Thamnophis sirtalis infernalis	USA: California, Santa Clara	LSUMZ37914	AF420193	AF420196
Thamnophis sumichrasti	Mexico: Queretaro	MZFC8869	AF420197	AF420200
Thamnophis sumichrasti	Mexico: Veracruz, San Juan Texhuacan	JCSG247	**PP273344**	-
Tropidoclonion lineatum	USA: Kansas, Geary Co.	CAS174301	AF420205	AF420208

Supplementary material 1

Cytb phylogenetic tree

Author: Jacobo Reyes-Velasco

Data type: pdf

Explanation note: Maximum Likelihood phylogenetic inference of members of the genus *Thamnophis* and closely related genera, based on the mitochondrial gene Cytb.

Link: https://doi.org/10.3897/herpetozoa.37.e122213.suppl1

Supplementary material 2

ND4 phylogenetic tree

Author: Jacobo Reyes-Velasco

Data type: pdf

Explanation note: Maximum Likelihood phylogenetic inference of members of the genus *Thamnophis* and closely related genera, based on the mitochondrial gene ND4.

Link: https://doi.org/10.3897/herpetozoa.37.e122213.suppl2

Supplementary material 3

BI phylogenetic tree

Author: Jacobo Reyes-Velasco

Data type: pdf

Explanation note: Concatenated Bayesian Inference phylogenetic tree based on the mitochondrial genes Cytb and ND4 for members of the genus *Thamnophis* and closely related genera.

Link: https://doi.org/10.3897/herpetozoa.37.e122213.suppl3

Supplementary material 4

Genetic distance

Author: Jacobo Reyes-Velasco

Data type: xlsx

Explanation note: : Genetic distance table of the genetic distances of Cytb and ND4 of numerous samples *Thamnophis* and related Natricinae.

Link: https://doi.org/10.3897/herpetozoa.37.e122213.suppl4

Supplementary material 5

Large comparable image spread of live photographs of *Thamnophis* related to *Thamnophis. ahumadai* sp. nov.

Author: Christoph I. Grünwald

Data type: jpg

Explanation note: Large spread of high resolution photographs of species related to *Thamnophis ahumadai* sp. nov.

Link: https://doi.org/10.3897/herpetozoa.37.e122213.suppl5

ÖGH Herpetozoa
Austrian Herpetological Society

Herpetozoa 37: 181–187 (2024)
DOI 10.3897/herpetozoa.37.e118699

Origins and taxonomic status of *Hemidactylus* geckos on the Îles Éparses of the Western Indian Ocean

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https://zoobank.org/C8A61420-5F60-49F3-AE09-8BEA4877B0E0

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Academic editor: Günter Gollmann ♦ **Received** 12 January 2024 ♦ **Accepted** 19 March 2024 ♦ **Published** 3 July 2024

Abstract

Distinguishing between anthropogenic introductions and natural colonizations can be complex, especially in groups that are evidently both capable natural colonizers and anthropophilic, such as some geckos of the genus *Hemidactylus*. However, such information is fundamental both for constructing appropriate conservation approaches, as well as to identify biogeographical patterns. Here we assessed the origins and taxonomic status of the *Hemidactylus* geckos found on the Îles Éparses, disjunct small islands of the western Indian Ocean located around Madagascar, using partial mitochondrial DNA sequences. *Hemidactylus platycephalus* was confirmed on Juan de Nova, presumed introduced from continental Africa. The *H. mabouia-mercatorius* complex was identified on Juan de Nova, Europa, Tromelin, and Grande Glorieuse, with *H. mabouia* (*sensu stricto*) on Juan de Nova and *H. mercatorius* on Europa, from where it is likely to have been introduced to Tromelin. An unnamed lineage within *H. mercatorius*, previously known only from the Aldabra Islands, was identified on Grande Glorieuse, and further demonstrated the unexpectedly high genetic diversity within this lineage.

Key Words

16S rRNA, colonization, Europa, Glorioso, *Hemidactylus mabouia*, *Hemidactylus mercatorius*, introduction, Juan de Nova, Tromelin

Introduction

Extensive evidence indicates that invasive alien species are significant drivers of population declines and species extinctions in island ecosystems worldwide (Reaser et al. 2007). However, determining the alien status of many species is complex, since natural long-distance colonizations have been demonstrated for many different taxa. Geckos are an evident example of this, with numerous overwater dispersals across both ancient and recent timeframes indicating both a natural ability for island colonization as well as extensive, well documented anthropogenic introductions (Agarwal et al. 2021). Species of the genus *Hemidactylus* are an exemplar of this, with seven species found across the remote islands of the Indian Ocean (Rocha et al. 2022). Since neither the volcanic Mascarene islands nor the atolls of the western Indian Ocean have been connected to continental source populations, either natural or human-mediated transmarine colonizations must be invoked to explain their distribution. Separating these two phenomenona is further complicated by the convoluted taxonomic history of some of these species. In particular *Hemidactylus platycephalus* and the *Hemidactylus mabouia-mercatorius* complex were historically amalgamated as "*H. mabouia*" due to overall similar morphological aspects, so that early historical records cannot be reliably used to infer exact species presences (reviewed in Rocha et al. 2022). Disentangling these species, primarily through the use of genetic sequences, has helped elucidate colonization patterns across the Indian Ocean islands (Rocha et al. 2010a). However, most studies have still focused on larger islands, such as those of the Comoros group (Rocha et al. 2005), Madagascar (Vences et al. 2004) and Réunion (Sanchez et al. 2012). Assessment of genetic lineages, as well as confirmation of species level taxonomy, on the smaller islands are often still lacking, although these may be crucial to disentangle natural from anthropogenic introductions, and thus the native or alien status of these taxa.

Genetic data has also only partially resolved the taxonomic situation within the *Hemidactylus mabouia-mercatorius* complex. Vences et al. (2004) identified two highly divergent lineages of *H. mercatorius* within Madagascar. Rocha et al. (2005) demonstrated that individuals of *H. mabouia* from the Gulf of Guinea islands clearly grouped within their *H. mercatorius* group from northern Madagascar and Comoros. Boumans et al. (2007) identified three lineages within Madagascar, all of which were considered *H. mercatorius*. Similarly, Rocha et al. (2010a) proposed that the insular populations, including Comoros, Madagascar and the Seychelles, be treated as *H. mercatorius*, and identified three divergent groups within a paraphyletic *H. mabouia*. This would indicate that *H. mercatorius* was then also introduced to other regions, such as the Gulf of Guinea islands. More recently, comparing the data from this study with that from the comprehensive study of Agarwal et al. (2021), which found *H. mabouia* to harbor more than 20 (still unnamed) species-level lineages within which *H. mercatorius* is also embedded, led Pinho et al. (2023) to consider that one of the three groups (Clade A, group I) of Rocha et al. (2010a) actually corresponds to *H. mabouia sensu stricto*, a widespread clade both in West Africa and in the Americas, with a distribution clearly driven by anthropogenic movements. It also includes populations found in Mayotte, Mahé (Seychelles), Réunion, Zanzibar and Pemba. This means that both *H. mabouia* (*sensu stricto*) and *H. mercatorius* are found on various Indian Ocean islands, and that there have been multiple introductions in some areas, highlighting the need to assess the smaller islands.

In this study we include specimens of *Hemidactylus* from the “Îles Éparses”, disjunct small islands off the western Indian Ocean, including Europa, Juan de Nova, Tromelin and Grande Glorieuse of the Glorioso islands. We sequenced part of the 16S rRNA mitochondrial gene and compared this to previously published exemplars from the larger islands to infer colonization patterns and the possible native status of these geckos on the individual islands.

Methods

The sampled islands form part of the 5th district of the French Southern and Antarctic lands, a French overseas territory. They include the main Glorioso island (Grande Glorieuse), Juan de Nova, Europa and Tromelin, with fieldwork carried out between 2013 and 2017 (Fig. 1 and Suppl. material 1). *Hemidactylus* geckos were identified in the field, and a small piece of the tail-tip removed and stored in 96% ethanol for genetic analyses. We extracted DNA from these tissues using standard High Salt methods (Sambrook et al. 1989), and then performed a PCR to amplify part of the 16s rRNA gene, using the 16SH and 16SL primers from Palumbi (1996) and the conditions described in Harris et al. (1998). Positive PCR products were cleaned and sequenced in one direction by a commercial company (Genewiz, Germany). The sequences were aligned in BioEdit using clustalW (Thompson et al. 2003), with representative sequences of the same species from GenBank.

Phylogenetic relationships were estimated using a Maximum Likelihood approach, employing PhyML 3.0 (Guindon et al. 2010), both for identifying the most appropriate model of evolution under the AIC criteria (Lefort et al. 2017), and for estimating the phylogeny. Support for the phylogeny was inferred with 1,000 bootstrap replicates. The most appropriate model of evolution identified using PhyML and therefore employed in the analysis was the GTR+I+G model for both datasets. Trees were imported to FigTree v1.4.4 for visualization.

Results

Twenty-one partial 16S rRNA sequences were generated for this study (Accession Numbers PP495142–PP495143, PP495257 and PP495518–PP495523) belonging to the *H. mabouia-mercatorius* complex (19 specimens) and *H. platycephalus* (2 specimens). Each species was analysed in a separate phylogenetic analysis, owing to differences in lengths of comparative sequences from GenBank. For *H. platycephalus* the alignment consisted of 23 sequences (494 bp), including two specimens of *Hemidactylus principensis* which were designated as outgroups. For the *H. mabouia-mercatorius* complex the alignment consisted of 120 terminals (423 bp), including divergent specimens of “*H. mabouia*” from continental Africa used to root the tree following Rocha et al. (2010a). Since all the specimens from Europa Island (n = 15) shared the same haplotype, a single representative individual was included in the analysis.

The two specimens of *H. platycephalus* sequenced from Juan de Nova shared a single haplotype, which differed by one nucleotide from a sample of this species from Mozambique (AY517572, Vences et al. 2004). These formed part of one of the three lineages previously identified by Rocha et al. (2010a), including other samples from the Comoros islands, Zanzibar and Madagascar (Fig. 2, group C). The other two lineages corresponded to clades so far restricted to Zanzibar and Pemba (Fig. 2, group A), and continental East Africa (Fig. 2, group B).

The *H. mabouia-mercatorius* complex was identified on Grande Glorieuse, Juan de Nova, Europa and Tromelin (Fig. 3). The specimen from Juan de Nova was a new haplotype, related to haplotypes previously recovered

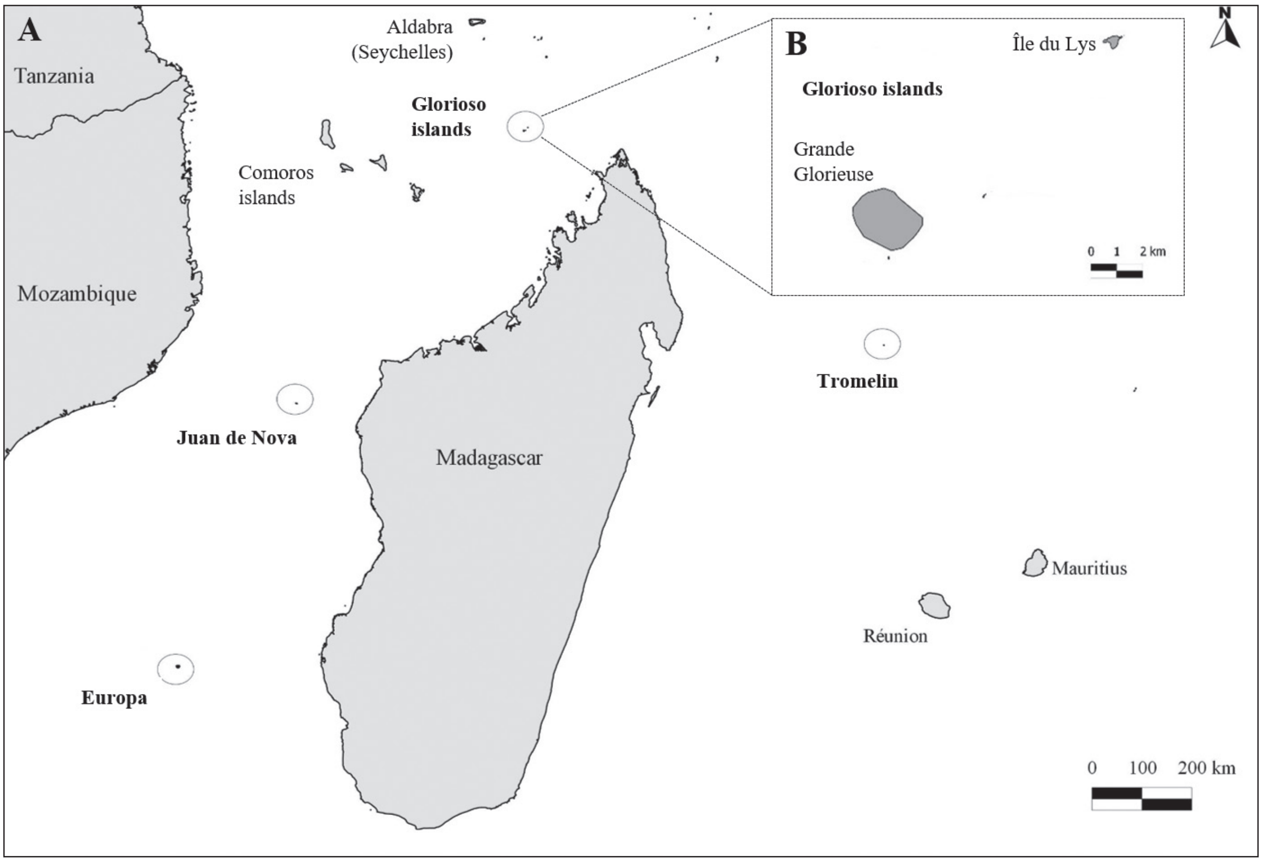

Figure 1. (**A**) The location of the Îles Éparses in the western Indian Ocean and (**B**) details of Glorioso islands.

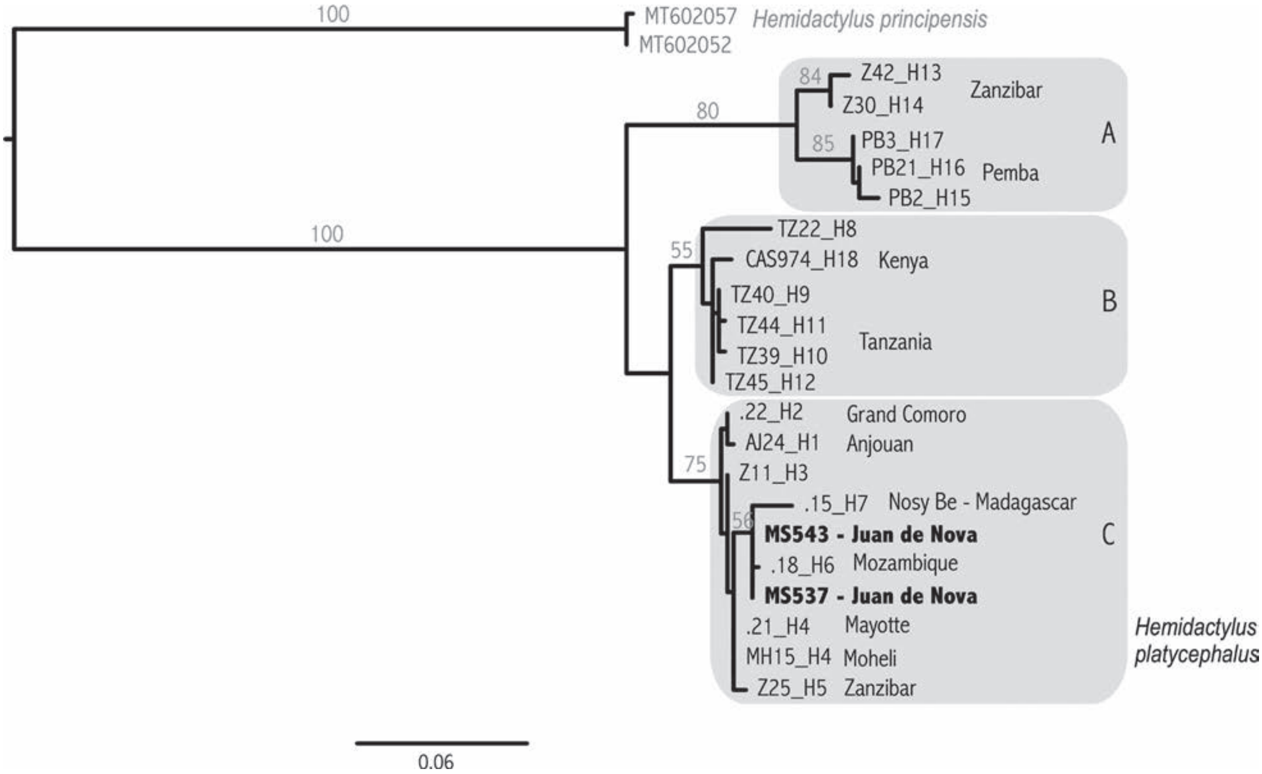

Figure 2. Estimate of relationships between populations of *Hemidactylus platycephalus* based on partial 16S rRNA sequences derived from a maximum likelihood analysis. Numbers on branches indicate bootstrap support (above 50%). Individual labeling follows Rocha et al. (2010a) for previously collected samples (sample_haplotype). Sample codes reflect geographic locations, with full details including GenBank numbers in Suppl. material 1.

from continental Africa and the Comoros islands, as well as Réunion (Group I in Rocha et al. 2010a). Following Agarwal et al. (2021) and Pinho et al. (2023) this lineage represents *H. mabouia sensu stricto*. Specimens from Europa and Tromelin (15 and 1 respectively) shared a new haplotype, related to specimens from Madagascar (Group II in Rocha et al. 2010a, and therefore *H. mercatorius*). Specimens from Grande Glorieuse also all shared a new haplotype, embedded within a diverse clade of *H. mercatorius*, previously identified only from the coralline

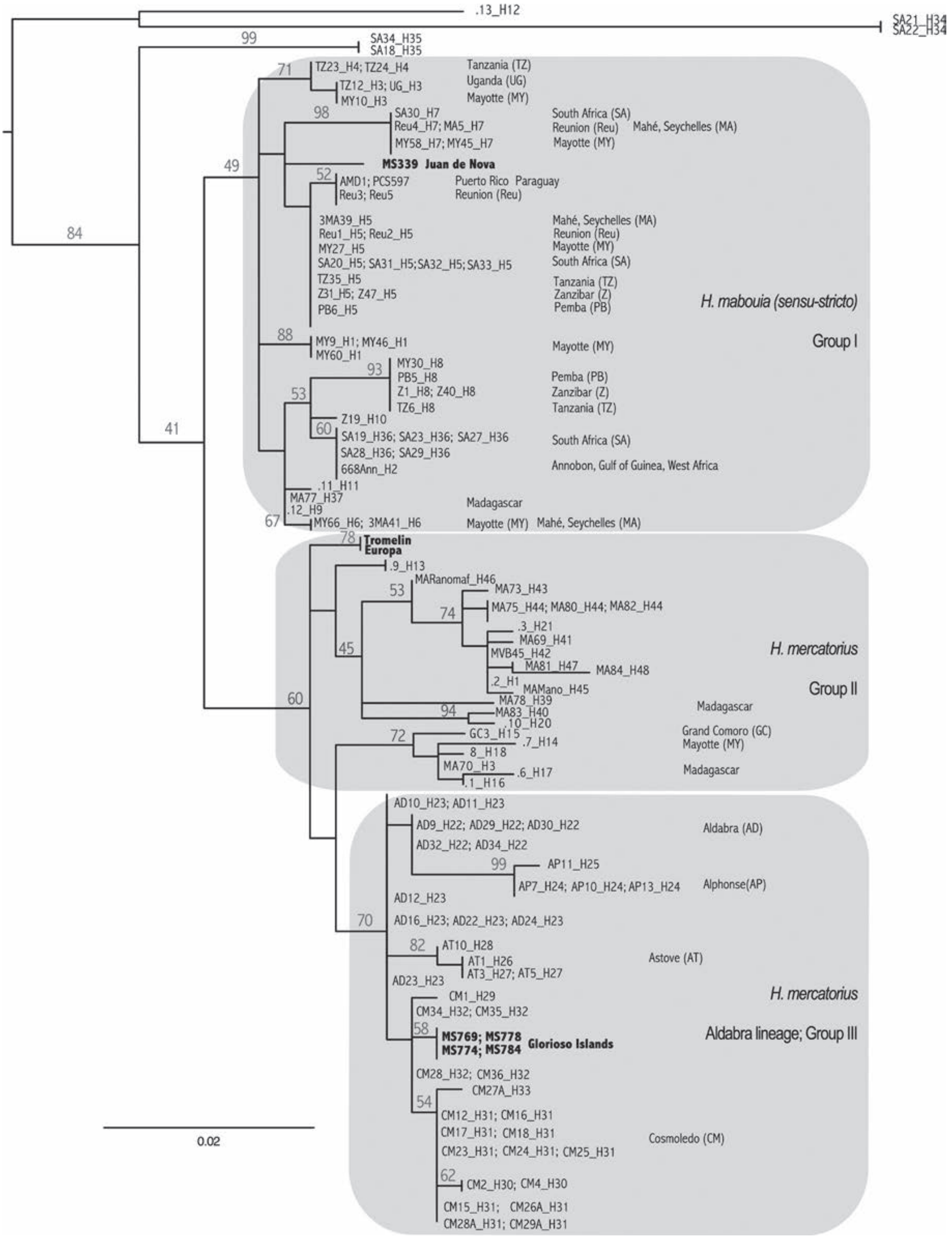

Figure 3. Estimate of relationships within the *H. mabouia-mercatorius* complex based on partial 16S rRNA sequences derived from a maximum likelihood analysis. Numbers on branches indicate bootstrap support (above 50%). Groups I–III (and individuals) are labelled following Rocha et al. (2010a) for previously collected samples (sample_haplotype). Sample codes reflect geographic locations, with full details including GenBank numbers in Suppl. material 1.

archipelago of Aldabra, including Aldabra, Cosmoledo, Astove and Assumption (Group III in Rocha et al. 2010a).

Discussion

Distinguishing native from alien species is a first step towards answering many biogeographic and evolutionary questions, and for addressing key conservation concerns. Attempts to define approaches to identify alien species usually propose various criteria, while recognizing that these are generally indirect evidence and that rather than recognizing alien status based on a single criterion, only the existence of several criteria together can result in a high classification probability (e.g. Orlova-Bienkowskaja 2016). Low genetic diversity, high frequency of known naturalization, unexpected geographic distribution and lack of historical records are all criteria often proposed as indicators for alien status (e.g. Webb 1985).

In the case of *Hemidactylus* geckos in Indian Ocean islands, most of these criteria are not particularly informative. Frequency of naturalization is high for some species, particularly the *H. mabouia* complex, which has "conquered the world", although this contains up to 20 putative species, only one of which is invasive and widely distributed (Agarwal et al. 2021). Similarly, *Hemidactylus* species have undoubtedly reached many islands naturally, so that the geographic occurrence on even relatively young islands cannot be used to discount native status. House geckos tend to be recorded less than more conspicuous mammals and birds, so early historical records are mostly limited to a few reports by naturalists, and even then the notable morphological similarity between many species means that species-level identifications can be suspect if museum specimens were not collected. On the other hand, early records on some isolated islands have been considered to suggest the possibility of early (pre-European) or natural dispersals for some species (reviewed in Rocha et al. 2022). Regarding the Îles Éparses, records indicate "*H. mabouia*" on Glorioso and Juan de Nova since the 1890's, and on Europa since 1903 (reviewed in Sanchez et al. 2019), which while relatively early historically still leave open the possibility of anthropogenic introductions. *Hemidactylus platycephalus* was only identified with certainty on Juan de Nova and Glorioso in the last 25 years, as was *H. mercatorius* on Tromelin (reviewed in Sanchez et al. 2019). This leaves genetic data as one of the few options left to identify potential status on most islands.

Mitochondrial DNA sequences have, in some cases, given compelling additional evidence regarding the status of some populations of *Hemidactylus* on Indian Ocean islands. In the case of the population of *H. mabouia* (initially considered as *H. mercatorius*) from Réunion, three haplotypes were identified from a partial 16S rRNA fragment, two of which were immediately reported as having been found in populations from Africa and the Comoros (Sanchez et al. 2012), while the third (individuals Reu 3 and Reu 5) is identical to specimens since collected in Puerto Rico (GenBank accession KC840516, Diaz-Lameiro et al. 2013). These all belong to the lineage now considered as *H. mabouia sensu-stricto* (probably clade "b", although there is low resolution in the 16S rRNA sequence data to recover the two clades identified by Agarwal et al. (2021) using ND2 sequence data), which is associated with anthropogenic movements and widespread across Africa and the Americas. In this case, the genetic data therefore strongly supports recent, anthropogenic introduction(s). Furthermore, the *H. mabouia-mercatorius* complex (group I of Rocha et al. 2010a) is now clearly identified as *H. mabouia sensu stricto* (Pinho et al. 2023), implying that these populations found in Mayotte (Comoros), Mahé (Seychelles), Zanzibar and Pemba (Tanzania) and some primarily coastal localities of Tanzania and South Africa are highly likely introduced through human-aided transport, which seems to be the predominant pattern of this species. Regarding the Îles Éparses, the finding of the same unique haplotype in specimens from Europa and Tromelin, along with the lack of older records from Tromelin and the large distance between these two islands, also indicates a recent introduction of *H. mercatorius* to Tromelin from Europa – currently logistic support boats land on each island consecutively every two to three years to remove waste material, and this seems a plausible colonization pathway. Likewise, the lack of historical records of *H. platycephalus* on Juan de Nova and the high genetic similarity with specimens from continental Africa indicates a recent introduction. However, the situation of the populations of *H. mercatorius* on Europa and *H. mabouia* on Juan de Nova is less clear-cut, since both islands accommodate specimens with distinct haplotypes, quite different from any currently available for comparisons (Fig. 3). Perhaps this fact is due to a lack of sampling across likely source populations. A similar situation occurs with *Hemidactylus* species in the Maldives, where attempts to illuminate colonization patterns are hampered by incomplete information on the natural variation from continental source populations (Agarwal et al. 2019). Interestingly, Europa seems to have been colonized with *H. mercatorius* from Madagascar, while Juan de Nova with *H. mabouia* from continental Africa, and this same pattern occurs in *Lygodactylus* geckos, with *Lygodactylus verticillatus* from Europa conspecific with Malagasy populations, while *Lygodactylus insularis* from Juan de Nova is embedded within the *Lygodactylus capensis* group, with an African origin (Röll et al. 2023). On the other hand, the skink *Trachylepis maculilabris* from Europa appear closely related to populations from Mozambique (Rocha et al. 2010b), meaning that no clear biogeographical pattern is evident from the reptiles of this island. Further, without more data from potential source populations, the native or introduced status for *Hemidactylus* from these islands remains debatable.

The identification of a new haplotype within *H. mercatorius* from Grande Glorieuse, embedded within the "Aldabra clade" (*sensu* Rocha et al. 2010a), is of particular interest. It could be interpreted as a recent introduction from Aldabra, given that concessions to exploit coconut plantations on the islands were given to Seychelles companies during the 20th Century. On the other hand, a high diversity on Aldabra is quite unexpected given the relatively recent complete inundation of this atoll (around 136,000 years ago, reviewed in Hume et al. 2018). Previous haplotype diversity identified by Rocha et al. (2010a) was already high (12 in total, across the four islands of the Aldabra archipelago sampled), and the finding of another unique haplotype on Grande Glorieuse increases the known diversity within this clade. Many authors of phylogeographic assessments in this region suggest that compared to Aldabra, the other islands of the group are much younger, around only 15,000 years (e.g. Radkey 1996; Warren et al. 2003). However, this evidence seems to be based on geological assessments focused on Aldabra (e.g. Taylor et al. 1979). Korotky et al. (1990) identified multiple marine terraces on Assumption, some of which are higher than those on Aldabra, suggesting these islands have similar ages (Boyden et al. 2021). Having multiple, older islands in relative proximity – Assumption Island is only 27 km from Aldabra – may help explain the diversity identified in this clade, endemic to the archipelago, and might also explain the appearance and extinction of two or even three other gecko species on Aldabra which are present in the fossil record after the inundation about 100,000 years ago (Arnold 1976). While their extinction may have been associated with the formation of the central lagoon on Aldabra around 5000 years ago (reviewed in Hume et al. 2018), their presence after the inundation indicates that Aldabra was being regularly colonized by reptiles over an evolutionary short time period. Overall, given these alternative scenarios, examination of other flora and fauna within the Aldabra archipelago for similar genetic signatures of longer persistence in the area should be performed.

To conclude, on the Îles Éparses, while some signals of recent anthropogenic introductions were identified (*H. platycephalus* on Juan de Nova and *H. mercatorius* on Tromelin), the status of the populations of *H. mabouia* on Juan de Nova and *H. mercatorius* on Europa remains ambiguous. On the other hand, *H. mercatorius* from Grande Glorieuse is part of a clade previously identified on the Aldabra archipelago, adding to this unexpectedly diverse endemic insular lineage. Further sampling in Madagascar and continental Africa, as well as assessment of more taxa from Aldabra, will be needed to further address these complex biogeographical questions.

Acknowledgments

We thank the Préfet des Terres Australes et Antarctiques Françaises (TAAF) for authorization to work in the Îles Éparses, and for issuing permits to conduct our sampling (Permit numbers: Arrêtés préfectoraux TAAF n°2015-19 and n°201726), and the Forces Armées de la Zone Sud de l'Océan Indien (FAZSOI) for transport and logistic supports. We are grateful to Jean-Michel Probst, Luc Gigord, Christian Fontaine and Jean Hivert for their help with sample collection and the Nature Océan Indien NGO for support. The field research was financially supported by the European Union (Project BEST 2.0. n°1173) and the TErres et MErs Ultra-Marines Fund (TEMEUM). Thanks to A. Isabel Ferreira for her help in the laboratory. This work was also supported by the European Union's Horizon 2020 Research and Innovation Programme under the Grant Agreement Number 857251. Thanks to the reviewers for their comments on an earlier version of this manuscript.

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Supplementary material 1

Supplementary DNA sequence data

Authors: D. James Harris, Mickaël Sanchez, Sara Rocha

Data type: docx

Explanation note: The supplementary material includes a full list of all samples included in the analyses, with GenBank accession numbers, sample codes and geographic localities.

Link: https://doi.org/10.3897/herpetozoa.37.e118699.suppl1

OGH Herpetozoa
Austrian Herpetological Society

Herpetozoa 37: 189–195 (2024)
DOI 10.3897/herpetozoa.37.e124331

First description of the female of *Achalinus sheni* (Serpentes, Xenodermidae), with expanded description of this species

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https://zoobank.org/01520209-7D04-4286-9697-9141EF343B1D

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Academic editor: Silke Schweiger ♦ **Received** 31 March 2024 ♦ **Accepted** 26 June 2024 ♦ **Published** 9 July 2024

Abstract

Achalinus sheni Ma, Xu, Qi, Wang, Tang, Huang & Jiang, 2023 was originally described based on only male specimens from Lianyuan City and Nanyue District, Hunan, China. So far, no information on the females of this species is available. After molecular systematics and morphological characters of recently collected specimens from Xinshao County, Hunan, several specimens (3 males and 1 female) were identified as this species. Therefore, we provided supplementary descriptions of the female characteristics of this species in this study. Meanwhile, we extended the description of this species.

Key Words

Morphology, Phylogenetics, Xinshao County

Introduction

Achalinus Peters, 1869, commonly known as the odd-scaled snakes, is the most diverse genus of the family Xenodermidae (Uetz et al. 2022). It contains 28 recognized species, and is widely distributed in eastern and southeastern Asia, ranging from northern Vietnam to southwestern China, and partly into Japan, from which 21 species occur in China. In recent years, with extensive sampling and molecular phylogenetic methods, more than 20 members of the genus *Achalinus* have been discovered and described (Wang et al. 2019; Ziegler et al. 2019; Li et al. 2020; Luu et al. 2020; Miller et al. 2020; Hou et al. 2021; Huang et al. 2021; Li et al. 2021; Ha et al. 2022; Yang et al. 2022; Ma et al. 2023a, 2023b; Yang et al. 2023; Zhang et al. 2023; Li et al. 2024).

Achalinus sheni was described based on five specimens collected in Hunan Province, China: four male specimens (ANU20230012—ANU20230015) from Lianyuan City and one male specimen (CIB 119043) from Nanyue District (Ma et al. 2023b). Molecular phylogeny inferred from the mitochondrial *COI* gene fragment revealed that this new species is most closely related to *A. yunkaiensis*, but it can be distinguished from

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A. yunkaiensis by the following morphological characters: (1) relative length of supraocular and upper anterior temporal (supraocular equal to or longer than anterior temporal, SPOL/ATUL 0.99–1.20 vs. supraocular shorter than anterior temporal, SPOL/ATUL 0.55–0.83); (2) higher ventral + subcaudals counts in males (220–225 vs. 200–212); (3) higher ventral scale counts in males (161–170 vs. 150–162); (4) higher subcaudal scale counts in males (55–61 vs. 49–56). However, to date there have been no morphological comparisons between females of these two species.

During a herpetofaunal survey of Hunan, China in 2022, one female and three male *Achalinus* were collected from Xinshao County, Shaoyang City (Fig. 1). Morphological and molecular phylogenetic analyses revealed that the specimens are *A. sheni*. Here we provide morphological data for the newly collected female specimen of *A. sheni* , and extend the description of this species.

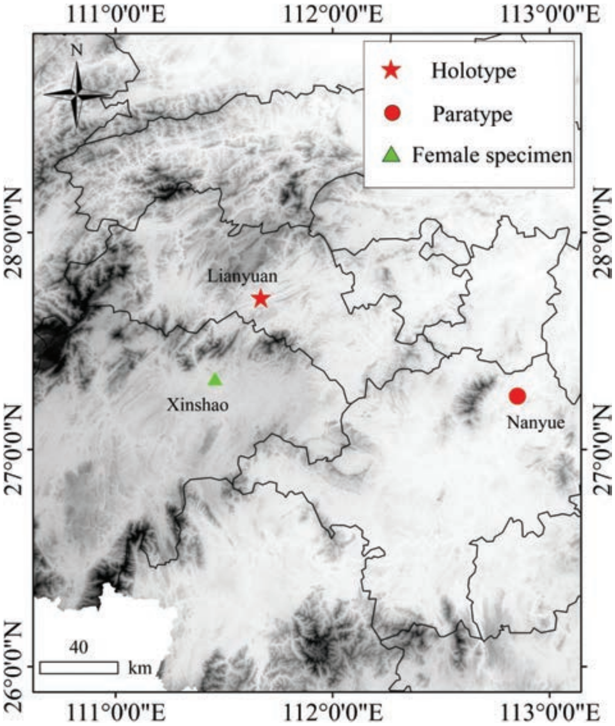

Figure 1. Sampling sites of *Achalinus sheni*: solid red star, the locality of the holotype; solid red circle, the locality of the paratype; solid green triangle, the locality of the newly collected female specimen.

Materials and methods

Sampling

Four odd-scaled snake specimens were collected from Xinshao County, Shaoyang City, Hunan Province, China (HNNU2022001—HNNU2022004). The four specimens were fixed in 75% ethanol and deposited in the Vertebrate Zoology Laboratory, College of Life Science, Hunan Normal University.

Morphological examination

Morphological descriptions followed Zhao (2006) and Ma et al. (2023b). Abbreviations in this study are as follows: snout-vent length (**SVL**): snout-vent length from tip of snout to anterior margin of the cloaca; tail length (**TaL**): tail length from posterior margin of cloaca to tip of tail; total length (**TL**): from snout tip to tail end; head length (**HL**): from the tip of snout to the posterior margin of mandible; head width (**HW**): from the widest part of the head in dorsal view; eye horizontal diameter (**ED**): from the most anterior edge of the eye to the most posterior edge; loreal height (**LorH**): measured from the highest point to the lowest point of the loreal in lateral view; loreal length (**LorL**): from the most anterior edge of the loreal to the most posterior edge of the loreal in lateral view; length of the suture separating the internasals (**LSBI**); length of the suture separating the prefrontals (**LSBP**); length of the supraocular (**SPOL**): horizontal distance between the anterior and posterior tips of the supraocular, and length of the upper anterior temporal (**ATUL**): horizontal distance between the anterior and posterior tips of the upper anterior temporal. We also directly compared the length of the sutures between the internasals and prefrontals (**LSBI vs. LSBP**).

The morphological features and their abbreviations are as follows: loreals (**Loreal**), supralabials (**SPL**), infralabials (**IFL**), the number of infralabials touching the first pair of chin shields (**IFL-1st Chin**), supraoculars (**SPO**), temporals (**TEM**), the number of anterior temporals touching the eye (**aTEM-Eye**), ventral scales (**VEN**), subcaudals (**SC**), cloacal plate entire or divided (**CP**), dorsal scale rows (**DSR**) (counted at one-head-length posterior of head, at midbody, at one-head-length anterior of cloacal plate). Bilateral scale counts were given as left/right.

Phylogenetic analyses

Genomic DNA was extracted from preserved liver tissue using the TIANamp Genomic DNA Kit. The fragment of the mitochondrial DNA gene encoding cytochrome c oxidase subunit I (*COI*) was amplified using the primer pairs Chfm4 and Chrm4 (Che et al. 2012). The PCR products were sequenced at Shanghai Map Biotech Co., Ltd. The homologous sequences of the *Achalinus* species and the outgroups species were downloaded from GenBank (Table 1).

The *COI* sequences (639 bp) were assembled using SeqMan in the DNASTAR software package (Burland, 2000), and compared and aligned using MEGA 7 software (Kumar et al. 2018). The uncorrected pairwise distances (*p*-distance) were calculated in MEGA 7. Maximum likelihood analysis (Nguyen et al. 2015) was executed using IQ-TREE 2 under the best-fit model TIM3 + F + I + G4 selected by Modelfinder according to AIC. Nodal support was estimated by 1,000 bootstrap replicates using the ultrafast bootstrap feature.

Table 1. Localities, voucher information and GenBank numbers for all samples used in this study.

Species	Locality	Voucher NO.	Accession
A. sheni	Lianyuan, Hunan, China	ANU20230013	OR178146
A. sheni	Lianyuan, Hunan, China	ANU20230014	OR178147
A. sheni	Nanyue, Hunan, China	CIB 119043	OR189183
A. sheni	Xinshao, Hunan, China	HNNU2022001	PP854453
A. ater	Huaping Nature Reserve, Guangxi, China	SYSr00852	MN380334
A. dabieshanensis	Fuziling Provincial Reserve, Anhui, China	AHU2018EE0710	MW316598
A. damingensis	Shanglin, Nanning, Guangxi, China	ANU20220009	OP644487
A. dehuaensis	Dehua, Fujian, China	YBU13013	MZ442642
A. emilyae	HoanhBo, Quang Ninh, Vietnam	IEBR4465	MK330857
A. formosanus	Taiwan, China	RN2002	KU529452
A. huangjietangi	Huangshan, Anhui, China	HSR18030	MT380191
A. hunanensis	Huaihua, Hunan, China	CIB119039	OQ848425
A. juliani	HaLang, Cao Bang, Vietnam	IEBRA.2018.8	MK330854
A. meiguensis	Mianyang, Sichuan, China	GP835	MZ442641
A. nanshanensis	Nanshan National Park, Hunan, China	HNNU230903	OR523370
A. niger	Taiwan, China	RN0667	KU529433
A. ningshanensis	Ningshan, Shaanxi, China	ANU20220006	ON548422
A. panzhihuaensis	Yanbian, Sichuan, China	KIZ040189	MW664862
A. pingbianensis	Honghe, Yunnan, China	YBU18273	MT365521
A. quangi	northern Vietnam	sp4	OQ197471
A. rufescens	Hongkong, China	SYSr001866	MN380339
A. spinalis	Badagong Mountains, Hunan, China	SYSr001327	MN380340
A. timi	ThuanChau, Son La, Vietnam	IEBRA.2018.10	MK330856
A. tranganensis	NinhBinh, Vietnam	VNUFR.2018.21	MW023086
A. vanhoensis	VanHo, Son La, Vietnam	VNUFR.2019.13	ON677935
A. yangdatongi	Wenshan Nature Reserve, Yunnan, China	KIZ034327	MW664865
A. yunkaiensis	Dawuling Forestry Station, Guangdong, China	SYSr001443	MN380329
A. yunkaiensis	Dawuling Forestry Station, Guangdong, China	SYS r001502	MN380330
A. yunkaiensis	Dawuling Forestry Station, Guangdong, China	SYS r001503	MN380331
A. zugorum	Bac Me, Ha Giang, Vietnam	IEBR4698	MT502775
Fimbrios klossi	Quang Ngai, Vietnam	IEBR3275	KP410744
Parafmbrios lao	Louangphabang, Laos	MNHN2013.1002	KP410746
Xenodermus javanicus	Sumatera Barat, Indonesia	—	KP410747

Results

Maximum likelihood trees showed consistent topology. The newly collected female specimen clustered with the specimens (including the holotype) of *Achalinus sheni* and demonstrated strong support (ML = 97, Fig. 2). The genetic distance (uncorrected p-distance) between the newly collected female specimen and the specimens (including the holotype) of *A. sheni* was only 0.7–0.8% (Table 2).

Achalinus sheni Ma, Xu, Qi, Wang, Tang, Huang & Jiang, 2023

Specimen examined. HNNU2022001, adult female, collected by Hui Li, Lin Zhou on April 11, 2022, from Xinshao County (27°23'58"N, 111°33'44"E, 420 m a. s. l.), Shaoyang City, Hunan Province, China.

Description of the female specimen. Adult female with a total length of 345.1 mm (SVL 294.8 mm and TaL 50.3 mm), tail relatively short, TL/ToL 0.149, body slender and cylindrical. Head distinct from neck, rostral small, triangular, only the upper tip is visible from above. Head length 9.16 mm, head width 3.95 mm, HL/HW 2.32. Eyes small, eye width 1.10 mm. Length of the suture between the internasals (LSBI 1.40 mm) subequal to the length of the suture between the prefrontals (LSBP 1.31 mm). Frontal pentagonal pointed backwards, much shorter than the parietals; each parietal bordered with an elongated nuchal, with no preoculars or postoculars. Nostril in anterior portion of nasal scale, posterior margin of nostril with a distinct nostril cleft. A single loreal scale present, extending from the nasal to the eye, distinctly wider than high. Temporals 2+2+3. aTMPs elongated, upper aTMP much smaller than the lower aTMP; upper aTMP and lower aTMP in contact with eye, lower aTMP also in contact with parietal scale. Supralabials 6, 4th–5th contact the eye, the last much elongated. A single mental scale present. Two chin shields, the anterior pair longer than the posterior pair. Infralabials 5, the first contacting each other posterior to the mental and anterior to the 1st chin shields, 1st–3rd contact the 1st chin shields. Dorsal scales 23-23-23, strongly keeled, dorsum with an inconspicuous longitudinal vertebral stripe. Ventrals (VEN) 173. Subcaudals (SC) 45, not paired. Cloacal plate (CP) entire (Table 3).

Coloration of the female specimen in life. Scales possess a subtle iridescent quality which gives the dorsum a distinctive reflective brownish-black appearance. Dorsum dark brown and the five innermost dorsal scale rows a little darker, forming an inconspicuous longitudinal vertebral line. Chin shields are tan. On the ventral surface, an off-white shade prevails, with the edges of the ventral scales gradually transitioning from gray-white to black. Ventral side of tail brownish.

Coloration in preservative. (Fig. 3**)** The dorsal surface of the body uniformly brownish-black, slightly tinged with iridescence and the longitudinal vertebral line appears a little darker. Chin shields light brown. Ventrals generally pale brown, darker on both sides, free margins of ventral scales grayish-white. Ventral surface of tail light brown.

Extended diagnosis. Upon examining the additional adult males of *Achalinus sheni* from Xinshao County, it was observed that they possessed fewer ventral scales compared to specimens from the type locality. However, the number of subcaudal scales remained approximately the same for individuals from both locations. Upon inspection of adult female *A. sheni* collected in Xinshao County, it was noted that it exhibited an extremely short tail and consequently fewer subcaudals and more ventral compared to adult male individuals.

Comparison. Compared to male individuals of *A. sheni* and *A. yunkaiensis*, female individuals of both species exhibit similar differences in the number of ventral scales and subcaudals. Compared to males, both female *A. yunkaiensis*

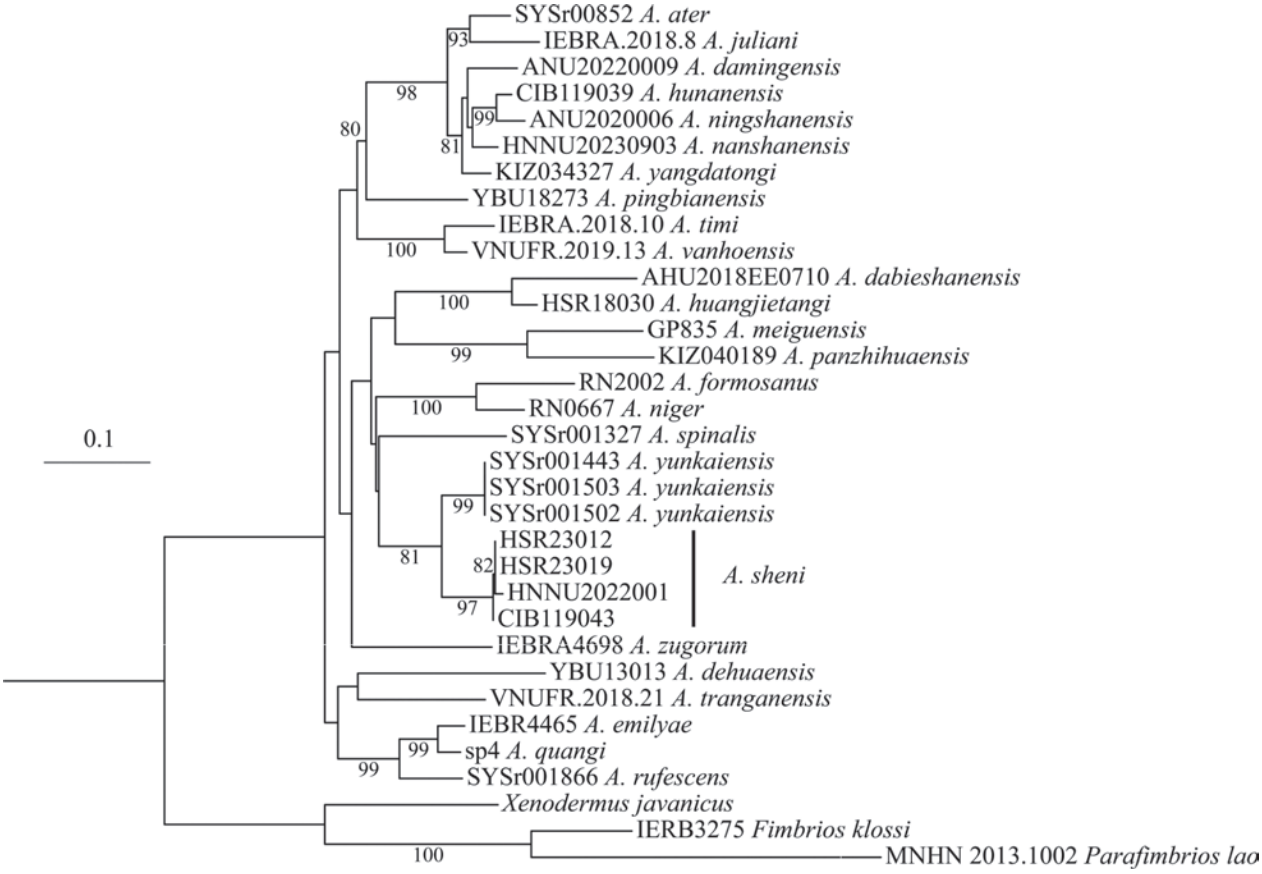

Figure 2. Phylogenetic tree of the genus *Achalinus* inferred from *COI* gene fragments (629 bp) using Maximum Likelihood. The numbers above the branches represent the supporting values.

Table 2. Uncorrected p-distances (%) among *Achalinus* species inferred from mitochndrial *COI* gene.

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**22**	**23**	**24**	**25**	**26**	**27**
1 HNNU2022001																											
2 *A. sheni*	0.7																										
3 *A. sheni*	0.7	0.0																									
4 *A. sheni*	0.8	0.2	0.2																								
5 *A. ater*	13.5	13.2	13.2	13.2																							
6 *A. dabieshanensis*	15.3	15.8	15.8	15.6	14.7																						
7 *A. damingensis*	14.4	14.0	14.0	14.0	8.0	15.8																					
8 *A. dehuaensis*	13.9	13.9	13.9	13.8	17.1	18.4	16.1																				
9 *A. emilyae*	14.2	13.7	13.7	13.5	11.9	17.7	13.4	15.7																			
10 *A. formosanus*	13.2	13.0	13.0	13.0	14.4	19.0	14.9	16.4	14.0																		
11 *A. huangjietangi*	13.6	13.6	13.6	13.3	15.0	8.9	16.3	16.5	14.5	15.6																	
12 *A. hunanensis*	12.5	12.1	12.1	12.3	7.3	16.9	6.1	14.9	13.2	13.7	16.8																
13 *A. juliani*	14.4	14.0	14.0	14.2	7.0	15.8	8.7	15.1	12.9	12.5	14.6	8.8															
14 *A. meiguensis*	14.6	14.1	14.1	13.9	15.4	17.7	16.8	18.1	15.4	15.6	15.2	16.4	16.8														
15 *A. nanshanensis*	13.5	13.9	13.9	14.0	6.7	16.0	5.4	14.7	13.4	15.1	16.6	4.7	8.2	17.7													
16 *A. niger*	12.7	12.5	12.5	12.5	13.5	15.8	14.4	16.2	12.4	9.2	13.9	13.2	12.5	13.9	13.5												
17 *A. ningshanensis*	14.5	14.1	14.1	14.2	7.6	17.2	7.8	16.5	14.1	14.8	17.2	3.4	9.6	17.0	5.1	14.6											
18 *A. panzhihuaensis*	14.6	14.6	14.6	14.6	16.2	16.6	15.5	15.3	16.6	16.0	15.2	16.2	15.5	11.6	15.5	14.4	17.4										
19 *A. pingbianensis*	11.5	11.5	11.5	11.6	11.6	15.3	10.9	15.0	12.8	14.5	13.0	11.1	1.0	16.8	11.5	11.8	11.7	14.9									
20 *A. quangi*	15.1	14.5	14.5	14.3	12.2	18.1	13.2	15.9	3.3	14.2	15.0	13.2	12.9	15.2	13.0	11.9	13.4	16.9	13.8								
21 *A. rufescens*	13.7	13.5	13.5	13.3	12.9	16.9	13.9	14.4	8.0	14.0	14.3	12.1	12.5	17.3	12.2	12.7	12.3	16.0	12.8	7.9							
22 *A. spinalis*	12.0	11.5	11.5	11.3	15.4	16.6	15.4	14.4	14.4	14.0	13.4	13.9	14.4	16.0	14.4	13.7	15.6	15.8	13.2	14.0	13.0						
23 *A. timi*	14.2	13.9	13.9	13.8	13.2	16.4	13.5	15.9	13.4	13.5	14.8	12.0	14.4	15.8	13.9	11.9	13.6	15.5	11.8	13.5	14.2	14.4					
24 *A. tranganensis*	14.0	13.7	13.7	13.7	12.9	15.3	14.4	14.0	12.0	17.1	13.4	14.0	13.9	16.4	13.7	14.5	15.2	16.4	13.2	12.4	11.7	15.1	14.0				
25 *A. vanhoensis*	14.1	13.8	13.8	13.6	13.1	15.5	12.6	16.0	12.2	14.0	14.6	11.5	13.6	15.6	12.4	12.8	12.1	15.5	10.8	12.4	13.8	12.9	5.2	13.3			
26 *A. yangdatongi*	14.0	13.7	13.7	13.8	6.2	16.6	5.6	14.0	12.8	14.4	14.6	5.1	7.3	17.1	4.4	13.7	5.9	15.5	11.3	12.6	11.5	14.2	13.1	12.8	11.3		
27 *A. yunkaiensis*	6.5	6.5	6.5	6.2	13.2	14.9	12.7	15.1	13.5	12.4	12.5	12.0	12.9	15.8	12.7	12.4	13.7	15.7	11.6	14.0	13.7	12.2	14.2	13.9	13.6	12.0	
28 *A. zugorum*	10.9	10.9	10.9	10.8	13.7	15.3	12.9	14.7	12.5	13.4	4.3	11.8	13.4	15.0	13.0	13.2	12.8	15.3	10.8	13.2	13.7	13.4	13.5	12.0	11.9	12.2	11.2

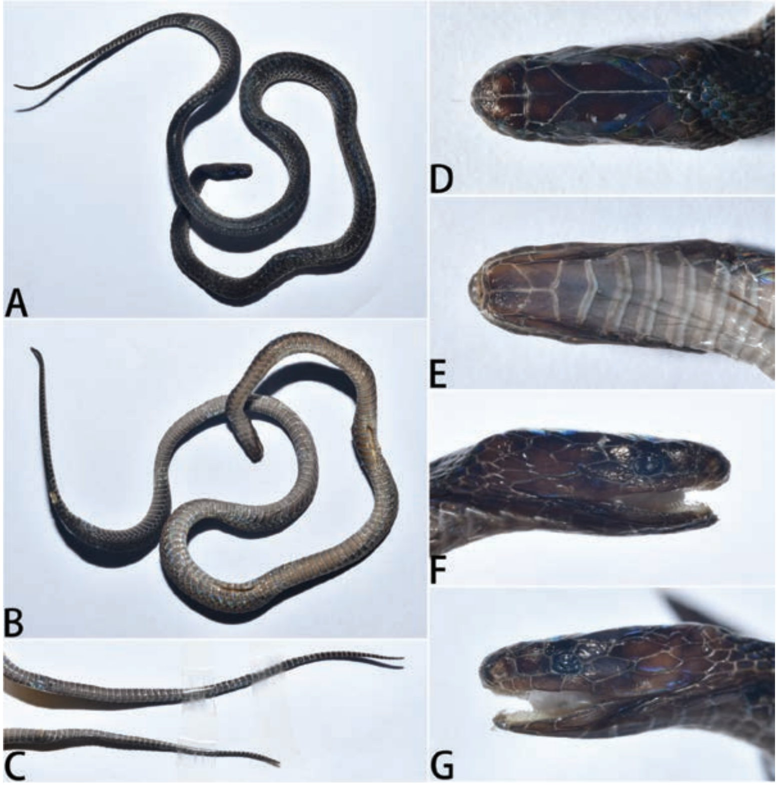

Figure 3. Adult female (HNNU2022001) of *Achalinus sheni*. **A.** Dorsolateral view; **B.** Ventral view; **C.** The tail of the male and female; **D.** Dorsal head view; **E.** Ventral head view; **F.** Light side of head view; **G.** Ventral head view. Photos by Le-Qiang Zhu.

Table 3. Main morphological characters of *Achalinus sheni*.

Voucher number	HNNU2022001	HNNU2022002	HNNU2022003	HNNU2022004
Sex	**Adult female**	**Adult male**	**Adult male**	**Adult male**
SVL	294.8	270.8	239.9	211.5
TaL	50.34	74.3	67.7	59.2
TL	345.1	345.1	307.6	270.7
TaL/TL	0.149	0.215	0.220	0.219
HL	9.16	8.76	7.86	7.65
HW	3.95	4.01	3.94	3.26
ED	1.10	0.97	1.02	0.76
SPL	6	6	6	6
SPL-Eye	4th–5th	4th–5th	4th–5th	4th–5th
IFL	5	5	5/6	5
Chin	2	2	2	2
IFL-1stChin	1st–3rd	1st–3rd	1st–3rd	1st–3rd
Loreal	1	1	1	1
LorH	0.88	0.87	0.93	0.73
LorL	1.38	1.14	1.31	1.00
LorH / LorL	0.63	0.76	0.71	0.73
LSBI vs LSBP	=1	=1	=1	=1
SPO	1	1	1	1
SPOL	1.49	1.15	1.15	1.33
TEM	2+2+3	2+2+3	2+2+3	2+2+3
aTEM-Eye	2	2	2	2
ATUL	1.63	1.41	1.71	1.46
SPOL / ATUL	0.91	0.82	0.67	0.91
DSR	23-23-23	23-23-23	23-23-23	23-23-23
VEN	173	156	156	158
SC	45	61	61	62
VEN+SC	218	217	217	220
CP	Entire	Entire	Entire	Entire

and female *A. sheni* have fewer sublabial scales, which is consistent with the common characteristics of this genus. However, the number of sublabial scales of *A. sheni* is even less than that of female *A. yunkaiensis* (Table 4).

Discussion

Only five male specimens of the *Achalinus sheni* have been described in previous studies. This study reports the first discovery of a female of the same species and provides a detailed description and photograph of the female. In terms of morphological characteristics, the male specimens collected in Xinshao County are close to the holotype specimen from Lianyuan City. Some differences were noted in the female specimen: Tail relatively short, TL/ToL 0.149, which was different from that of the male specimens (0.183–0.224); the tail of the female specimen is suddenly tapered, distinct from the elongated tail of the male specimens.

Xinshao County and Lianyuan City are geographically adjacent, and the climate is not obviously different. *Achalinus sheni* is fossorial and difficult to find. Only one female specimen was collected in this study. Whether the apparent differences between the sexes of this species are consistent, whether there are further differences, and the reasons for these differences have not yet been fully investigated and analyzed.

Table 4 Comparing the differences between *Achalinus sheni* and *Achalinus yunkaiensis* in different regions from a taxonomical perspective.

Species	*Achalinus sheni*			*Achalinus yunkaiensis*
	Lianyuan City & Nanyue District	**Xinshao County**		***Guangdong***
Sex	**Males(n=5)**	**Males (n=3)**	**Female**	**Female**
SVL	121.8–292.2	211.5–270.8	294.8	204–386.3
TaL	27.2–80.3	59.2–74.3	50.3	52–72.8
TL	149.0–371.3	270.7–345.1	345.1	256–448.1
TaL/TL	**0.183 ~ 0.224**	**0.215–0.220**	**0.149**	**0.156–0.204**
HL	10.07–10.95	7.65–8.76	9.16	-
HW	5.96–7.25	3.26–4.01	3.95	-
ED	1.09–1.11	0.76–1.02	1.10	-
SPL	6	6	6	6
SPL-Eye	4th–5th	4th–5th	4th–5th	4th–5th
IFL	**5 (rarely 6)**	**5 (rarely 6)**	**5**	**6**
Chin	2	2	2	2
IFL-1stChin	1st–3rd	1st–3rd	1st–3rd	1st–3rd/4th
Loreal	1	1	1	1
LorH	0.69–0.93	0.73–0.93	0.88	0.74–1.2
LorL	1.29–1.71	1.00–1.31	1.38	1.51–2.2
LorH / LorL	0.53–0.57	0.71–0.76	0.63	0.49–0.55
LSBI vs LSBP	=1	=1	=1	=1
SPO	1	1	1	1
SPOL	1.21–1.59	1.15–1.33	1.49	1.26–1.60
TEM	2+2+3	2+2+3	2+2+3	2+2+3/4
aTEM-Eye	2	2	2	2
ATUL	1.20–1.48	1.41–1.71	1.63	1.93–2.90
SPOL / ATUL	0.99–1.16	0.67–0.91	0.91	0.55–0.65
DSR	23-23-23	23-23-23	23-23-23	23-23-23
VEN	**161–170**	**156–158**	**173**	**144–156**
SC	**55–61**	**61–62**	**45**	**51–55**
VEN+SC	220–225	217–220	218	195–205
CP	Entire	Entire	Entire	Entire

Author contributions

Yi-han Ma: Conceptualization, Writing - original draft. Jia-yu Liu: Data curation, Investigation. Hui Li: Data curation, Formal analysis. Lin Zhou: Investigation. Yu-hao Xu: Methodology. De-yong Peng: Investigation. Zhi-qiang Zhang: Funding acquisition, Methodology. Xiao-yang Mo: Writing - review and editing, Methodology.

Acknowledgements

We thank Le-qiang Zhu for taking photos of the specimen, and thank the driver Xian Zhang for his help in the field investigation process (Lutou Experimental Forest Farm, Central South University of Forestry and Technology).

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ÖGH Herpetozoa
Austrian Herpetological Society

Herpetozoa 37: 197–200 (2024)
DOI 10.3897/herpetozoa.37.e123065

Earthworms as a prey source for the insular snake *Thamnophis sirtalis* (Linnaeus, 1758)

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Academic editor: Johannes Foufopoulos ♦ **Received** 22 March 2024 ♦ **Accepted** 11 June 2024 ♦ **Published** 10 July 2024

Abstract

Insular organisms typically evolve in closed, isolated systems; thus, changes caused by introduced species can drastically affect their ecology. Isle Royale National Park (Michigan, USA) is a remote island in Lake Superior that, until relatively recently, was void of earthworms. Its two native natricine snakes are known to feed on earthworms on the mainland; here, we confirm that eastern gartersnakes (*Thamnophis sirtalis*) are also feeding on earthworms on Isle Royale. It is unknown how this new important dietary source will impact the snake ecology on Isle Royale.

Key Words

diet, eastern gartersnake, Isle Royale National Park, redbelly snake, *Storeria occipitomaculata*

Insular organisms have evolved in isolation from conspecifics on the mainland and in sympatry with other species native to that island. Islands are excellent models for more complex ecosystems and allow for a better understanding of predator-prey relationships because of the comparative reduction in species richness (Simberloff 1974).

Many islands around the world have evolved interesting snakes with unique life histories (e.g., King and Lawson 1997; Boback 2003; Shwiff et al. 2010; Portillo et al. 2019). Whether native or introduced, insular snakes can be subject to important ecological dynamics, including the introduction of a new prey source (King et al. 2006). The introduction of invasive alien species often has dramatic effects on the ecosystem, especially on islands (e.g., King et al. 2006; Russell et al. 2017). Earthworms, which have now become well-established, are not native to the upper Midwest and are believed to have arrived in the United States as early as the 1600s in potting soil or ship ballasts (i.e., Gailing et al. 2012). On average, most earthworms move less than 8 m a year, but they are spread readily by humans, ending up in more remote places because of their usage as live bait for fishing or decomposers for gardening. Estimates suggest about 268 worms per square meter of soil and as many as 81.8 billion worms alone in Manistee County (3320 km^2 in size) in the northern lower peninsula of the U.S. state of Michigan (Freley 2021).

Earthworms can have extensive negative impacts in forested environments. They may remove the surface plant litter rapidly, alter the understory vegetation, and disrupt soil physiology (Hendrix and Bohlen 2002; Craven et al. 2017). Such changes drastically alter the ecosystem (Frelich et al. 2006), especially on islands where space is relatively limited.

It can be difficult to identify an earthworm as a species unless you are able to count the segments between the

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start of the worm and the clitellum (band), measure the total length of the worm as well as the length from the start of the worm to the clitellum (band), and also examine a picture of the underside of the worm; however, all are introduced species in the Upper Midwest. For example, Minnesota, United States, has at least 15 introduced species of earthworms. Ontario, Canada, has at least 15 confirmed invasive species (Holdsworth et al. 2017). In the upper Midwest, the last locations to experience the invasion of earthworms are often islands, including those in Lake Superior.

Even though earthworms are now widespread in the Upper Midwest and several snakes are known to feed on them, little work has been done to document this prey source. Here we confirm that snakes isolated on an island are utilizing earthworms as prey and establish baseline data for the study of this over the next few years.

Methods

Study site

Isle Royale National Park, Keweenaw County, Michigan, United States (47.9763°N, 88.9313°W), is a relatively remote island in western Lake Superior. It is located 29 km from the Minnesota shore, 90 km from the Michigan shore, and 24 km from the Ontario, Canada, shore. Isle Royale was designated a National Park in 1940, and to this day, 99% of it is federally protected wilderness (NPS 2023). The surrounding waters of Lake Superior are vast and cold, making it impossible for many organisms to immigrate to, or emigrate from, the island.

Study species

Isle Royale has two native species of snakes: *Thamnophis sirtalis* (Linnaeus, 1758), popularly known as the eastern gartersnake, and *Storeria occipitomaculata* (Storer, 1839), popularly known as the northern redbelly snake. It is unclear how they came to the island, though both are well-established and widespread; neither is well-studied in this environment. *Thamnophis sirtalis* is most notable on Isle Royale for its highly variable color morphs (Mooi et al. 2011). It has a wide diet, including frogs and toads, rodents, small fish, and invertebrates such as slugs and earthworms (Mullin and Seigel 2009; Virgin and King 2019). *Storeria occipitomaculata* feeds primarily on slugs and occasionally on earthworms (Virgin and King 2019).

Procedure

The following observation was made while visiting Isle Royale National Park: The snake was not captured or handled, and digital documentation of the event was taken.

Results

On 23 June 2023, at 14:54 h, at the South Lake Descor Campground (47.969099, -80.975028), a small (presumably a 2022 neonate) *Thamnophis sirtalis* was found feeding on an earthworm (Fig. 1). This represents the first photographed event of the consumption by a snake on Isle Royale, even though both native species of snakes are documented to feed on earthworms on the mainland.

There is a single record of an eastern gartersnake (*Thamnophis sirtalis*) consuming a *Lumbricus terrestris* Linnaeus, 1758 in what was previously the Ransom settlement, located on the northwestern side of the island near the present-day Daily Farm campground (Adams 1909). During a visit to Isle Royale in July 2023, two large additional “nightcrawler” earthworms were found near Malone Bay. One was on the concrete platform of the duplex housing, and the other was found under a log on the Ishpeming Trail near Siskiwit Lake.

Discussion

Earthworms, not native to the Upper Midwest of the United States, were presumably introduced to Isle Royale National Park in Lake Superior as byproducts of fishing in the area. At this time, we do not know how the introduction of earthworms and their expansion of range are going to impact the two native species of snakes on Isle Royale. It has been shown in other systems that the introduction of an abundant prey source can lead to increases in body size and thus increased reproductive potential in another natricine snake (King et al. 2006; Llewelyn et al. 2010).

The presence and impacts of earthworms on the nearby Boundary Waters Canoe Area Wilderness on the border of Minnesota, United States, and Ontario, Canada, are well documented (Wellnitz et al. 2020). However, there is little information available on the presence of earthworms on Isle Royale *per se*, as well as when they were originally introduced to the island. Similar observations have been made recently by one of the authors (JMR) in proximate locations (Sand Island in the Apostle Islands, Lake Superior, Wisconsin, United States) while surveying for eastern gartersnakes (*Thamnophis sirtalis*). In that case, JMR recovered an earthworm in the regurgitate from a snake caught during an evening rainstorm. At the time, invasive earthworms had not yet been documented on Sand Island in the Apostle Islands. Given the presence of earthworms on these islands in or near Lake Superior, it is not surprising that earthworms are present on Isle Royale.

As stated by Simberloff (1974), in reference to Isle Royale, it is the isolation of the island that leads to the reduced flora and fauna. This allows for observations to be more easily made in reference to how changes impact the ecosystem. If earthworms are widespread and abundant on Isle Royale, they may constitute an important food source for the snakes. Further studies of the island should make an effort to document the presence of earthworms among species to understand how widespread and abundant the invasive species are.

Figure 1. Sequence of a juvenile eastern gartersnake (*Thamnophis sirtalis*) ingesting a non-native earthworm on Isle Royale National Park, Keweenaw County, Michigan, United States of America.

Acknowledgements

JMR thanks the Grand Portage National Monument in partnership with the Grand Portage Band of Lake Superior Chippewa and the 1854 Treaty Authority. JK thanks his wife, Shri Karafa, who patiently waited for him to take pictures during their adventures.

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ÖGH Herpetozoa
Austrian Herpetological Society

Herpetozoa 37: 201–211 (2024)
DOI 10.3897/herpetozoa.37.e120130

Vine snakes (*Oxybelis*) and Sharpnose snakes (*Xenoxybelis*) (Squamata, Serpentes) from lowlands of Bolivia, with first records of *Oxybelis inkaterra* for the country

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Academic editor: Silke Schweiger ♦ **Received** 2 February 2024 ♦ **Accepted** 26 June 2024 ♦ **Published** 10 July 2024

Abstract

We present information on the occurrence of colubrid vine snakes (*Oxybelis*) and dipsadid sharpnose snakes (*Xenoxybelis*) from the lowlands of Bolivia. These genera have been poorly reported from Bolivia and information presented herein includes nine new record provincials from the departments of Beni, Cochabamba, La Paz, Pando, and Santa Cruz, Bolivia. Moreover, we present the first records of *Oxybelis inkaterra* Jadin, Jowers, Orlofske, Duellman, Blair & Murphy, 2021 from Bolivia and we extend the known range of this species by approximately 207 km (Río Sipia, La Paz) and 628 km (Campamento Guacharos, Cochabamba) southeast of the type locality (Puerto Maldonado, Peru) in South America. In addition, we present morphometric information, meristic characters, coloration pattern, ecological aspects and natural history for the three species of vine snakes (*O. aeneus*, *O. fulgidus*, *O. inkaterra*) and two species of sharpnose snakes (*X. argenteus*, *X. boulengeri*) from the Bolivian lowlands.

Key Words

Arboreal, *Oxybelis aeneus*, *Oxybelis fulgidus*, Serpentes, *Xenoxybelis argenteus*, *Xenoxybelis boulengeri*

Introduction

The genera *Oxybelis* Wagler, 1830 (Colubridae) and *Xenoxybelis* Machado, 1993 (Dipsadidae) comprise several widely distributed Neotropical colubrid species commonly known as vine snakes and sharpnose snakes, respectively (Arredondo et al. 2021; Uetz et al. 2023). Several species are sympatric in many regions of South America. However, *Oxybelis* has a much broader distribution that includes a northernmost distribution into the southern United States, whereas the northernmost distribution of *Xenoxybelis* occurs in southern Venezuela (Wallach et al. 2014; Nogueira et al. 2019; Uetz et al. 2023).

The nomenclatural history of the genus *Oxybelis* is long and convoluted (see Jadin et al. 2021). In addition, taxonomy within the genus has been unclear and disputed (Keiser 1974; Machado 1993; Jadin et al. 2019, 2020).

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Keiser (1974) maintained retaining *Oxybelis aeneus* (the type species for the genus) as a single species throughout its extensive range. However, Jadin et al. (2019, 2020) presented compelling data that supported multiple species and considerable diversity; moreover, they proposed an *O. aeneus* complex to include six species with the description of two new species for Central America and northern South America. Additionally, new species within the complex have also been described from Ecuador (Torres-Carvajal et al. 2021) and Peru (Jadin et al. 2021); among them *Oxybelis inkaterra* Jadin, Jowers, Orlofske, Duellman, Blair & Murphy, 2021.

The taxonomy of the genus *Xenoxybelis* has also been unclear and debated (see Melo-Sampaio et al. 2020). The genus was initially described by Machado (1993) and currently includes two species: *Xenoxybelis argenteus* (Daudin, 1803) and *X. boulengeri* (Procter, 1923) (Prudente et al. 2008; Melo-Sampaio et al. 2020; Uetz et al. 2023). Both species (*X. argenteus* and *X. boulengeri*) have been previously included in the genera *Oxybelis* (Peters and Orejas-Miranda 1970; Fugler et al. 1995) and *Philodryas* (Wallach et al. 2014; Nogueira et al. 2019). *Oxybelis* and *Xenoxybelis*, as reflected in their taxonomy, are not related but members of different colubrid families (Colubridae and Dipsadidae). However, they are a good example of convergence due to their arboreal lifestyle (e.g., morphology, coloration, diet, and habitat).

Vine snakes and sharpnose snakes are diurnal and predominately arboreal, although they are occasionally terrestrial (Duellman 1978; 2005; Dixon and Soini 1986; Martins and Oliveira 1998; Santos-Costa et al. 2015; Harrington et al. 2018). In general, species from these genera are quite cryptic, due to their coloration, elongated head, and attenuated slender bodies and long tail (authors' pers. obs.). Prey items include a diversity of small vertebrates, including lizards, birds, amphibians and mammals (Cunha and Nascimento 1978; Henderson 1982; Nascimento et al. 1988; Martins and Oliveira 1998; Duellman 2005; Scartozzoni et al. 2009; Santos-Costa et al. 2015).

Bolivian vine snakes *Oxybelis aeneus* (Wagler, 1824) and *Oxybelis fulgidus* (Daudin, 1803), and sharpnose snakes *Xenoxybelis argenteus* (Daudin, 1803) and *Xenoxybelis boulengeri* (Procter, 1923) are distributed in the Amazonian forests, Yungas, and riparian forests of the floodplains of Bolivia (Fugler et al. 1995; Moravec and Aparicio 2005; Nogueira et al. 2019; Eversole et al. 2021). However, information on the distribution and natural history of these species in Bolivia is extremely limited (Procter 1923; Donoso-Barros 1967; Fugler 1983; Keiser 1989; Fugler et al. 1995; Cadle and Reichle 2000; Cadle et al. 2003; Moravec and Aparicio 2005; Eversole et al. 2021).

Herein we report information on the distribution and natural history for Bolivian vine snakes and sharpnose snakes, including the first confirmed records of *Oxybelis inkaterra* from Bolivia.

Materials and methods

We examined specimens of vine snakes (*Oxybelis aeneus*, *Oxybelis fulgidus*, and *Oxybelis inkaterra*) and sharpnose snakes (*Xenoxybelis argenteus* and *Xenoxybelis boulengeri*) deposited in the four herpetological collections located in Bolivia: 1. Centro de Investigación de Recursos Acuáticos (CIRAH) of the Universidad Autónoma del Beni José Ballivián, Beni, 2. Colección Boliviana de Fauna (CBF) of the Museo Nacional de Historia Natural, La Paz, 3. Museo de Historia Natural Noel Kempff Mercado (MNKR), Santa Cruz, and 4. Museo de Historia Natural Alcide d'Orbigny (MHNC-R), Cochabamba (Tables 1–4).

Scale counts, scutellation, and terminology follow Dowling (1951) and Peters (1964). Measurements were taken using a flexible ruler to the nearest millimeter (snout-vent length, SVL; tail length, TL; in the case of CIRAH after euthanasia). Dorsal scale row counts were taken at three standardized locations; head length behind occiput, midbody, and head length anterior to cloaca and separated by a script (-). Paired subcaudals were counted on one side only, average number of subcaudal and ventral scales in parentheses. In addition to the number of supralabials, the scales that make contact with the orbit are identified in parentheses (right/left). In addition to the number of infralabials, the scales that make contact with the first pair of chin shields are identified in parentheses (right/left).

Specimen identification was determined by comparing and analyzing meristic data, morphometrics, coloration, figures, drawings, and photographs from description and taxonomic information by Daudin (1803), Boulenger (1896), Procter (1923), Peters and Orejas-Miranda (1970), Keiser (1974), Cunha and Nascimento (1978), Duellman (1978, 2005), Nascimento et al. (1988), Keiser (1989), Prudente et al. (2008), Jadin et al. (2020, 2021). We follow the taxonomic nomenclature proposed by Jadin et al. (2020, 2021) and Uetz et al. (2023).

For CIRAH specimens, we determined sex via cloacal probing and photographed each individual following the methodology outlined by Eversole et al. (2019). In addition, each specimen was weighed (g) with an electronic scale (Ohaus model HH 320).

We obtained geographic coordinates in decimal degrees using the Global Positioning System (Garmin eTrex, WGS84). We mapped the distribution of vine snakes and sharpnose snakes using Arc GIS software (ArcMap 10.2) including previous records cited in Tables 1, 3.

Results

We examined nine specimens of *Oxybelis aeneus*, six *Oxybelis fulgidus*, three *Oxybelis inkaterra*, one *Xenoxybelis argenteus* and seven *Xenoxybelis boulengeri* deposited in the Bolivian herpetological collections (Tables 1–4). The *O. inkaterra* record represents the first for Bolivia. We increased new locations of occurrence of these snakes for

Table 1. Previous and additional records of vine snakes (*O. aeneus*, *O. fulgidus*) and first records of *O. inkaterra* for Bolivia.

Department	Province	Locality	Latitude, Longitude	Voucher number	Reference
Oxybelis aeneus					
Beni	Vaca Diez	Tumi Chucua	-11.1333, -066.1667	–	Fugler 1983
Beni	Yacuma	Exaltación community	-13.3095, -065.2486	CIRAH-201	This study
Beni	Yacuma	Totaizal community	-14.8767, -066.3322	CIRAH-290	Eversole et al. 2021
Beni	Yacuma	Totaizal community	-14.8766, -066.3222	CIRAH-408	Eversole et al. 2021
Beni	Yacuma	El Trapiche, EBB	-14.7822, -066.3364	CBF-0948	This study
Santa Cruz	Andrés Ibáñez	Espejillos	-17.8000, -063.1667	–	Fugler et al. 1995
Santa Cruz	Ñuflo de Chávez	Perseverancia	-14.6369, -062.6318	MNKR-302	This study
Santa Cruz	Ichilo	Reserva El Chore	-17.8670, -064.1218	MNKR-1414	This study
Santa Cruz	Andrés Ibáñez	Comunidad en Maque (28 km. SW de Santa Cruz)	-17.9399, -063.3508	MNKR-1579	This study
Santa Cruz	Sara	Santa Rosa del Sara	-17.1060, -063.5956	MNKR-3396	This study
Santa Cruz	Andrés Ibáñez	Potrerillo de Guenda	-17.6706, -063.4584	MNKR-5682	This study
Oxybelis fulgidus					
Beni	Iténez	Bella Vista	-13.2667, -063.7000	–	Quintana and Padial 2003
Beni	José Ballivián	Cumbre del Pilon	-14.5167, -067.5833	–	Fugler et al. 1995
Beni	Vaca Diez	Buen Retiro community	-11.3130, -066.0489	CIRAH-929	This study
La Paz	Iturralde	Ixiamas, Barraca, Santa Rosa, Río Manurimi	-12.1667, -067.5000	MNKR-2120	This study
La Paz	Larecaja	Guanay	-15.5006, -067.8867	CBF-0223	This study
La Paz	Sud Yungas	La Asunta	-16.0333, -067.1667	CBF-0437	This study
La Paz	Franz Tamayo	Chalalán, PNyANMI Madidi	-14.4167, -067.9167	CBF-2345	This study
Pando	Manuripi	San Antonio	-11.6114, -068.2025	CBF-0866	This study
Pando	Nicolás Suarez	Surroundings Cobija (approximate coordinates)	-11.0693, -068.7839	–	Cadle and Reichle 2000
Oxybelis inkaterra					
Cochabamba	Carrasco	Campamento Guacharos El Palmar, PNC	-17.0615, -065.4929	MNKR-3740	This study
La Paz	Franz Tamayo	Río Sipia, PNyANMI Madidi	-14.3619, -068.5417	CBF-4275	This study
Unknown	Unknown	Unknown	–	CBF-3780	This study

Table 2. Meristic characters and scale counts of examined specimens of *Oxybelis* in the Bolivian herpetological collections. For abbreviations, see Materials and Methods. *=incomplete tail.

Voucher number	Sex	SVL (mm)	TL (mm)	Weight (g)	Dorsals	Ventrals	Subcaudals	Cloacal	Loreal	Supralabials (in contact with the orbit)	Infralabials (in contact with the first pair of chin shields)
Oxybelis aeneus											
CIRAH-201	Female	383	227	8.0	17-17-13	184	158	Divided	Absent	9(5,6)/9(5,6)	10(1-5)/10(1-5)
CIRAH-290	–	843	520	94.0	17-17-13	185	156	Divided	Absent	9(4,5)/9(4-6)	10(1-4)/9(1-4)
CIRAH-408	Female	301	165	5.0	17-17-13	185	154	Divided	Absent	9(5,6)/9(4-6)	10(1-4)/9(1-4)
CBF-0948	Female	800	519	–	17-17-13	192	168	Divided	Absent	9(4-6)/9(4-6)	10(1-4)/10(1-4)
MNKR-302	–	770	510	–	17-17-13	188	156	Divided	Absent	8(4,5)/9(5,6)	9(1-4)/10(1-4)
MNKR-1414	–	630	370	–	17-17-13	192	154	Divided	Absent	9(4-6)/9(4-6)	10(1-4)/10(1-4)
MNKR-1579	–	760	510	–	17-17-13	182	152	Divided	Absent	8(4,5)/9(4,5)	10(1-4)/9(1-4)
MNKR-3396	–	575	345	–	17-17-13	190	166	Divided	Absent	9(4,5)/9(4,5)	10(1-4)/9(1-4)
MNKR-5682	–	730	460	–	17-17-13	188	–	Divided	Absent	8(4,5)/8(4,5)	10(1-4)/10(1-4)
Oxybelis fulgidus											
CIRAH-929	Female	1322	553	325.0	17-17-13	217	128*	Divided	Absent	10(5-7)/10(5-7)	10(1-4)/10(1-4)
MNKR-2120	Male	1200	560	–	17-17-13	207	145	Divided	Absent	10(5-7)/10(5-7)	10(1-4)/10(1-4)
CBF-0223	–	1133	564	–	17-17-13	220	151	Divided	Absent	10(5-7)/10(5-7)	10(1-4)/10(1-4)
CBF-0437	Male	1093	587	–	17-17-13	205	155	Divided	Absent	10(5-7)/10(5-7)	10(1-4)/10(1-4)
CBF-2345	–	969	531	–	17-17-13	214	155	Divided	Absent	10(5-7)/10(5-7)	10(1-4)/10(1-4)
CBF-0866	Male	1161	544	–	17-17-13	202	156	Divided	Absent	9(5-6)/9(5-6)	10(1-4)/10(1-4)
Oxybelis inkaterra											
MNKR-3740	Female	580	385	–	17-17-13	181	170	Divided	Absent	8(4,5)/8(4,5)	10(1-4)/9(1-4)
CBF-4275	Male	730	531	–	17-17-13	200	160	Divided	Absent	9(4-6)/9(4-6)	9(1-3)/10(1-4)
CBF-3780	–	810	544	–	17-17-13	197	161	Divided	Absent	8(4-6)/8(4-6)	10(1-4)/9(1-4)

Bolivia and contributed information on some aspects of their natural history.

We compiled localities, geographic coordinates, voucher number (this study) and references of the previous, additional and first records of *Oxybelis* and *Xenoxybelis* for Bolivia (Tables 1, 3), and examined the morphometric and meristic characters of specimens (Tables 2, 4).

Table 3. Previous and additional records of sharpnose snakes (*X. argenteus, X. boulengeri*) for Bolivia.

Department	Province	Locality	Latitude, Longitude	Voucher number	Reference
Xenoxybelis argenteus					
Beni	Cercado	Trinidad, Mamoré River	-14.7833, -064.7833	–	Procter 1923
Beni	Moxos	Villa Fatima community, TIPNIS	-16.4667, -065.9175	MHNC-R 442	This study
Cochabamba	Carrasco	Río Chimore	-16.7167, -064.8167	–	Donoso-Barros 1967
Pando	Federico Roman	Caiman	-10.2167, -065.3667	–	Cadle et al. 2003
Pando	Federico Roman	Piedritas	-9.9500, -065.3333	–	Cadle et al. 2003
Pando	Nicolás Suarez	Bioceanica	-11.1333, -069.3667	–	Moravec and Aparicio 2005
Xenoxybelis boulengeri					
Beni	Cercado	Trinidad, Mamoré River	-14.7833, -064.7833	–	Procter 1923
Pando (probably)	–	–	-12.4919, -068.6422	–	Keiser 1989
Pando	Manuripi	San Francisco community	-11.6193, -069.0959	CIRAH-589	This study
Pando	Manuripi	San Francisco community	-11.6143, -069.1073	CIRAH-608	This study
Pando	Manuripi	Alta Gracia community	-11.5985, -068.2578	CIRAH-624	This study
Pando	Manuripi	Alta Gracia community	-11.5805, -068.2827	CIRAH-670	This study
Pando	Nicolás Suarez	Vera Cruz community	-11.4102, -069.0171	CIRAH-730	This study
Pando	Nicolás Suarez	Vera Cruz community	-11.4066, -069.0198	CIRAH-744	This study
Pando	Manuripi	Ucia community	-11.7454, -068.9755	CIRAH-1086	This study

Table 4. Meristic characters and scale counts of examined specimens of *Xenoxybelis* in the Bolivian herpetological collections. For abbreviations, see Materials and Methods. *=incomplete tail.

Voucher number	Sex	SVL (mm)	TL (mm)	Weight (g)	Dorsals	Ventrals	Subcaudals	Cloacal	Loreal	Supralabials (in contact with the orbit)	Infralabials (in contact with the first pair of chin shields)
Xenoxybelis argenteus											
MHNC-R 442	Female	691	411	–	17-17-15	209	182	Undivided	Absent	6(4)/6(4)	7(1-4)/7(1-4)
Xenoxybelis boulengeri											
CIRAH-589	Male	704	435	22.0	17-17-15	200	173	Divided	1/1	6(4)/6(4)	7(1-4)/7(1-4)
CIRAH-608	Male	692	430	25.0	17-17-15	200	163	Divided	1/1	6(4)/6(4)	7(1-4)/7(1-4)
CIRAH-624	Male	615	384	–	17-17-15	205	183	Divided	1/1	6(4)/6(4)	7(1-4)/7(1-4)
CIRAH-670	Male	728	506	24.9	17-17-15	196	186	Divided	1/1	6(4)/6(4)	7(1-4)/7(1-4)
CIRAH-730	Male	640	340	19.5	17-17-15	197	132*	Divided	1/1	6(4)/6(4)	7(1-4)/8(1-4)
CIRAH-744	Male	668	461	23.5	17-17-15	202	186	Divided	1/1	6(4)/6(4)	8(1-4)/7(1-4)
CIRAH-1086	–	432	261	7.8	17-17-15	209	187	Divided	1/1	6(4)/7(4)	8(1-4)/8(1-4)

Oxybelis aeneus (Wagler, 1824)

Fig. 1A, B

Specimens examined. One subadult female (CIRAH-201) collected at 2307 h on 14 June 2015 from the community of Exaltación. One adult (CIRAH-290) collected at 0225 h on 26 June 2015 and one juvenile female (CIRAH-408) collected at 2312 h on 22 June 2016 from Totaizal community. One adult female (CBF-0948) collected at 1100 h on 07 August 1992 from El Trapiche, Estación Biológica del Beni (EBB). One adult (MNKR-302) collected on 14 April 1990 from Perseverancia. One adult (MNKR-1414) collected on 17 October 1997 from Reserva El Chore. One adult (MNKR-1579) collected on 11 July 1997 from Comunidad en Maque. One adult (MNKR-3396) collected on 07 August 2002 from Santa Rosa del Sara. One adult (MNKR-5682) collected on 05 February 2022 from Potrerillo de Guenda (Table 1, Fig. 3).

Morphometric and meristic characters. Snout-vent length 575–843 mm (adults > 500 mm, n = 7). Tail length 345–520 mm (n = 7). Smooth dorsal scales 17-17-13 rows (100%), without apical pits. Ventral scales 182–192 ($\bar{x}$ = 187). Subcaudal scales 152–168 ($\bar{x}$ = 158). Divided cloacal plate (100%). Loreal absent (100%). Preocular 1 (100%). Postoculars 2 (100%). Temporals 1+2 (100%). Supralabials 8–9 (9/9 in 66% of specimens, 8/9 in 22% and 8/8 in 11%); fourth and fifth contact the orbit (33%), fourth, fifth and sixth contact the orbit (56%), and fifth and sixth contact the orbit (11%). Infralabials 9–10 (9/10 in 56% of specimens and 10/10 in 44%); the first four in contact with the first pair of chin shields (89%) and the first five contact the first pair of chin shields (11%) (Table 2); and usually the fourth, fifth and sixth contact the second pair of chin shields.

Coloration pattern. Upper region of head golden brown to tan; supralabials and ventral surface of head uniform cream color, the color transition is separated by a dark brown preocular line that extends from the nasal scale, under the eye, and toward the anterior region of the body. Black bars or spots present in the anterior region of the body; dorsal and ventral surface of the rest of the body relatively uniform light brown with scattered small black spots (Fig. 1A, B).

Ecological notes. The specimens (CIRAH-201, 290, 408) were found resting on herbaceous plants and tree branches at a height between 0–4 m from the ground during nocturnal searches between 2307–0225 h. The localities where they were found are best described as riparian forests (secondary and tertiary forests) of the Mamoré River sub-basin.

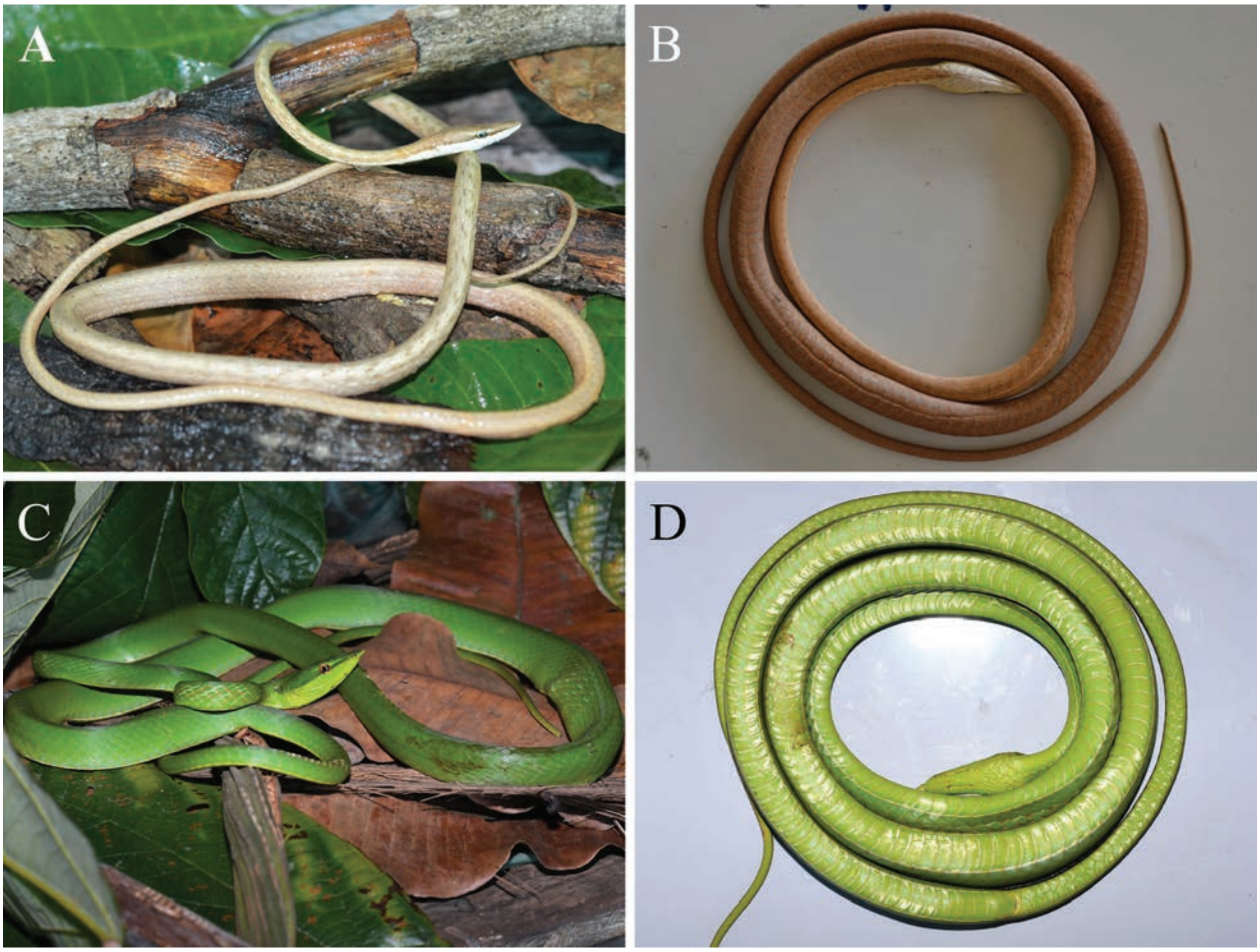

Figure 1. Vine snakes from Bolivia. **A, B.** Dorsal (CIRAH-408) and ventral (CIRAH-290) view *Oxybelis aeneus*, Totaizal community, Yacuma, Beni; **C, D.** Dorsal and ventral view *Oxybelis fulgidus* (CIRAH-929), Buen Retiro community, Vaca Diez, Beni. Photos by Cord Eversole (**A–D**).

Oxybelis fulgidus (Daudin, 1803)

Fig. 1C, D

Specimens examined. One adult female (CIRAH-929) collected at 1145 h on 24 June 2022 from Buen Retiro community. One adult male (MNKR-2120) collected on 01 March 1999 from Ixiamas, Barraca, Santa Rosa, Río Manurimi. One adult (CBF-0223) collected on 10 July 1986 from Guanay. One adult male (CBF-0437) collected on 02 June 1990 from La Asunta. One adult (CBF-2345) collected on 31 January 2007 from Chalalán, Parque Nacional y Area Natural de Manejo Integrado Madidi (PNyANMI Madidi). One adult male (CBF-0866) collected on 07 October 1995 from San Antonio (Table 1, Fig. 3).

Morphometric and meristic characters. Snout-vent length 969–1322 mm (adults, n = 6). Tail length 531–587 mm (n = 6). Smooth dorsal scales 17-17-13 rows (100%), vertebral and paravertebrals keeled, without apical pits. Ventral scales 202–220 ($\bar{x}$ = 211). Subcaudal scales 145–156 ($\bar{x}$ = 152). Divided cloacal plate (100%). Loreal absent (100%). Preocular 1 (100%). Postoculars 2 (100%). Temporals 1+2 (100%). Supralabials 9–10 (10/10 in 83% of specimens and 9/9 in 17%); fifth, sixth and seventh contact the orbit (83%) and fifth and sixth contact the orbit (17%). Infralabials 10 (100%); the first four contact the first pair of chin shields (100%) (Table 2); and fourth, fifth and sixth in contact with the second pair of chin shields.

Coloration pattern. Upper region of the head green; supralabials and ventral surface of head yellowish green, the color transition is not separated by any line; it is evident from the rostral to the last supralabial. Dorsal surface of body uniform green; yellowish-green ventral surface with two yellow ventrolateral lines extending from the throat to the tail (Fig. 1C, D).

Ecological notes. The specimen CIRAH-929 was found capturing a bird in the crown of a pacay tree (*Inga* sp.) at an approximate height of 5.5 m from the ground. Found in a rural village, typical of Amazonian Bolivia, surrounded by secondary Amazonian forest where the harvesting of Brazilian nuts (*Bertholletia excelsa*) and the açaí palm (*Euterpe oleracea*) are common.

Oxybelis inkaterra Jadin, Jowers, Orlofske, Duellman, Blair & Murphy, 2021

Fig. 2A–D

First record. Bolivia.

Specimens examined. One adult female (MNKR-3740) collected on March 2005 from Campamento

Figure 2. First record of *Oxybelis inkaterra* (CBF-4275), Río Sipia, Franz Tamayo, La Paz, Bolivia. **A, B.** Dorsal and ventral view (surface mottled with dense black spots); **C.** Bottom of the head black spots; **D.** Eyespot on ventrals scales. Photos by Mauricio Ocampo (**A**) and Gustavo Rey (**B–D**).

Guacharos, El Palmar, Parque Nacional Carrasco (PNC). One adult male (CBF-4275) collected on 24 June 2015 from Río Sipia, PNyANMI Madidi. One adult (CBF-3780) date and location unknown (Table 1, Fig. 3).

Morphometric and meristic characters. Snout-vent length 580–810 mm (adults, n = 3). Tail length 385–544 mm (n = 3). Smooth dorsal scales 17-17-13 rows (100%), without apical pits. Ventral scales 181–200 ($\overline{x}$ = 193). Subcaudal scales 160–170 ($\overline{x}$ = 164). Divided cloacal plate (100%). Loreal absent (100%). Preocular 1 (100%). Postoculars 2 (100%). Temporals 1+2 (100%). Supralabials 8–9 (8/8 in 67% of specimens and 9/9 in 33%); fourth and fifth contact the orbit (33%) and fourth, fifth and sixth contact the orbit (67%). Infralabials 9–10; generally the first four contacting the first pair of chin shields (Table 2) and fourth, fifth and sixth contact the second pair chin shields. Supraocular and prefrontal of similar length; posterior border of internasals extends beyond posterior edge of first supralabial; and presence of small scales between the second pair of chin shields (Fig. 2C).

Coloration pattern. Upper region of head is brown with dark brown to black mottling, black spots on posterior edge of nasal, and on preocular; black mottling on temporals forming an irregular postocular stripe that extends to second or third ventral; supralabials with mottling on borders, infralabials heavily mottled; mental, first pair of infralabials, and chin shields black with white spots (more intense in specimens CBF-3780 and MNKR-3740). Dorsal scales mottled with black and brown pigment in all rows; on anterior third of the body, some scales have heavy black pigment on their borders and irregular transverse bands; anterior ventrals heavily mottled becoming fine stippling posteriorly; some ventrals mottled with scattered black spots anteriorly; posteriorly, these spots encircled with white pigment to form eyespot markings; some of these markings also occur on the ventral and lateral portions of the tail (Fig. 2A–D).

Ecological notes. Specimen CBF-4275 was accidentally severed into two pieces by local guides while they were clearing work trails near the camp. The area has xeric and thorny vegetation, with representatives from the Bromeliaceae, Cactaceae, and Araceae families. This particular individual, feeling threatened, remained motionless, mimicking one of the branches of the shrub it was on. Unfortunately, this behavior caused it to go unnoticed by the guide, resulting in the unfortunate accident.

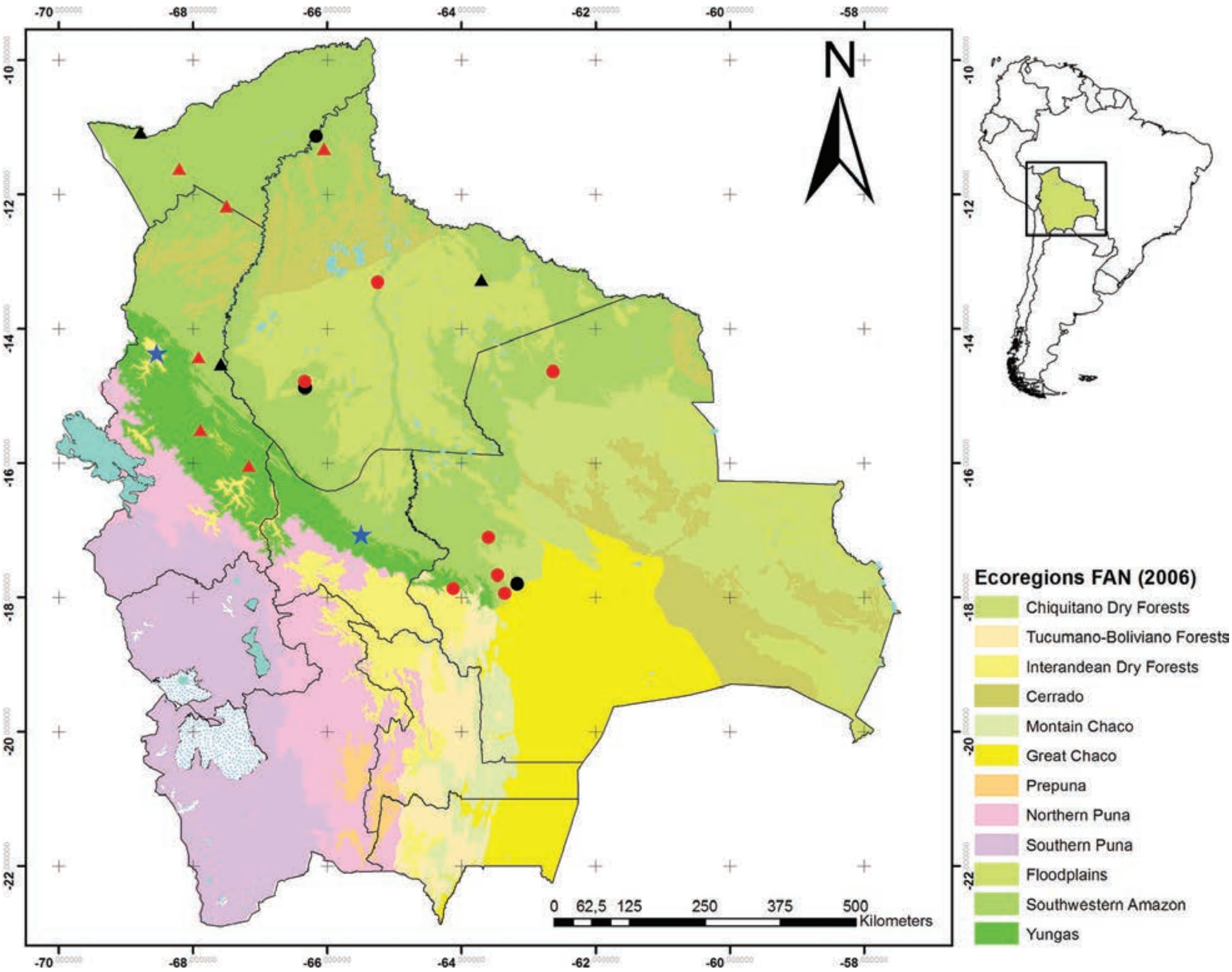

Figure 3. Distribution of the vine snakes in Bolivia. *Oxybelis aeneus* (points), *Oxybelis fulgidus* (triangles), and *Oxybelis inkaterra* (blue stars, first records). Red symbols correspond to additional records.

Xenoxybelis argenteus (Daudin, 1803)

Fig. 4A, B

Specimen examined. One adult female (MNHC-R 442) collected at 2315 h on 17 August 2001 from Villa Fatima community, Territorio Indígena y Parque Nacional Isiboro Sécure (TIPNIS) (Table 3, Fig. 5).

Morphometric and meristic characters. Snout-vent length 691 mm. Tail length 411 mm. Smooth dorsal scales 17-17-15 rows, without apical pits. Ventral scales 209. Subcaudal scales 182. Undivided cloacal plate. Loreal absent. Preocular 1. Postoculars 2. Temporals 1+1+2/1+2+2. Supralabials 6; fourth contacting the orbit. Infralabials 7; the first four contacting the first pair of chin shields (Table 4) and fourth and fifth contact the second pair of chin shields.

Coloration pattern. Upper center region of head brown; dorsolateral region of the head and supralabials light brown green, separated by broad grayish-green band, from nasal, crosses the eye, and extends to the body; ventral surface of the head yellowish green with scattered black points. Dorsal surface of the body light-greenish brown with two thin greenish-brown lateral bands; ventral surface bright yellowish green anteriorly and light green posteriorly with two lateral green bands (Fig. 4A, B).

Ecological notes. The specimen was found during the night resting on a branch of Piperaceae at a height 1.9 m off the ground in a secondary forest close to a stream.

Xenoxybelis boulengeri (Procter, 1923)

Fig. 4C, D

Specimens examined. Two adult males (CIRAH-589, 608) collected at 2318 and 2112 h on 28 and 30 June 2017 from San Francisco community. Two adult males (CIRAH-624, 670) collected at 2122 and 0036 h on 01 and 04 July 2017 from Alta Gracia community. Two adult males (CIRAH-730, 744) collected at 2332 and 2229 h on 04 and 05 June 2019 from Vera Cruz community. One juvenile (CIRAH-1086) collected at 1943 h on 07 July 2023 from Ucia community (Table 3, Fig. 5).

Morphometric and meristic characters. Snout-vent length 615–728 mm (adults > 500 mm, n = 6). Tail length 340–506 mm (n = 6). Smooth dorsal scales 17-17-15 rows (100%), without apical pits. Ventral scales 196–209

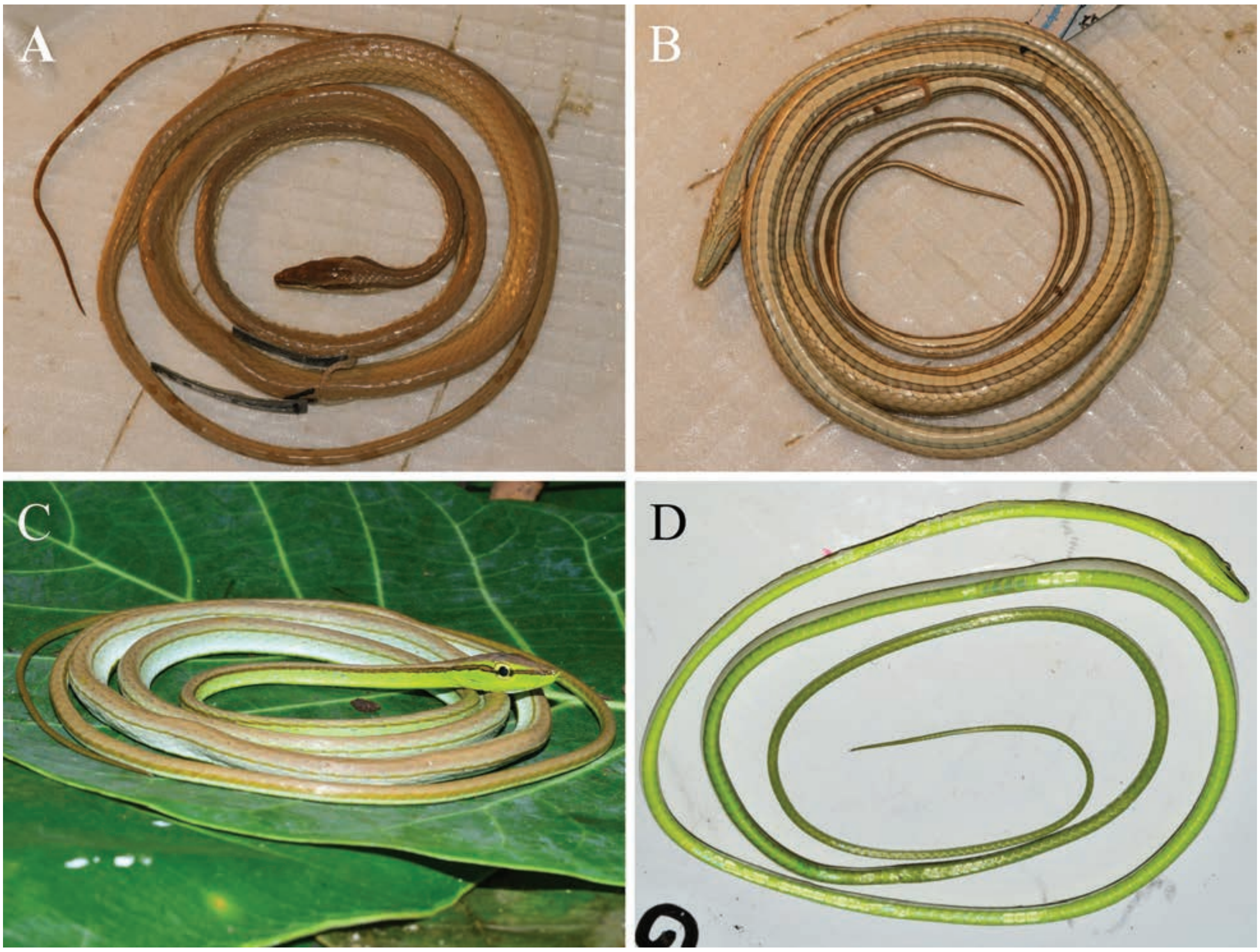

Figure 4. Sharpnose snake from Bolivia. **A, B.** Dorsal and ventral view *Xenoxybelis argenteus* (MHNC-R 442), Villa Fatima community, Moxos, Beni; **C, D.** Dorsal (CIRAH-670) and ventral (CIRAH-624) view *Xenoxybelis boulengeri*, Alta Gracia community, Munuripi, Pando. Photos by Gabriel Callapa (**A, B**) and Cord Eversole (**C, D**).

($\bar{x}$ = 201). Subcaudal scales 163–187 ($\bar{x}$ = 180). Divided cloacal plate (100%). Loreal 1 (100%). Preoculars 1–2 (1/1 in 71% of specimens, and 2/2 in 29%). Postoculars 2–3 (2/2 in 57% of specimens, and 3/3 in 43%). Generally temporals 1+1(2)+2 and 1+2+3 or less. Supralabials 6–7 (6/6 in 86% of specimens and 6/7 in 14%); fourth contacting the orbit. Infralabials 7–8 (7/7 in 57% of specimens, 7/8 in 29% and 8/8 in 14%); the first four contacting the first pair of chin shields (100%) (Table 4) and fourth and fifth contact the second pair of chin shields (100%).

Coloration pattern. Upper center region of head brown; dorsolateral region of the head and supralabials yellowish green, separated by a dark brown band with black edges, from nasal, crosses the eye, and extends to the front of the body; ventral surface of the head yellowish green. Dorsal surface of the body with two thin dark greenish-brown lateral bands, the broad vertebral band light greenish-brown, the broad lateral bands light green (much brighter on the anterior part of the body); ventral surface uniform yellowish-green (Fig. 4C, D).

Ecological notes. The specimens were found resting (coiled) on tree branches between 0.5–2 m above the ground, during nocturnal searches between 1943–0036 h. The localities where they were found correspond to primary and secondary Amazonian forests of the Manuripi and Tahuamanu river sub-basin.

Discussion

Previous records of vine snakes (*O. aeneus* and *O. fulgidus*) collected in Bolivia include the departments of Beni, La Paz, Pando, and Santa Cruz (Fugler 1983; Fugler et al. 1995; Cadle and Reichle 2000; Quintana and Padial 2003; Nogueira et al. 2019; Eversole et al. 2021). This study increases the museum voucher material collected in lowlands of Bolivia by 13 additional records of *O. aeneus* (n = 7) and *O. fulgidus* (n = 6) for the departments of Beni, Cochabamba, La Paz, Pando, and Santa Cruz (Table 1, Fig. 3). It is likely that some records presented in this study correspond to the southern distribution limit of both species.

Furthermore, we report the first records of *O. inkaterra* for Bolivia, with specimens from the department of Cochabamba and La Paz (one specimen does not have a specific locality) and it constitutes the eighth and ninth locality of the species across its range (Table 1, Fig. 3). *Oxybelis inkaterra* was recently described by Jadin et al. (2021) within the *O. aeneus* complex, with specimens from Peru. Jadin et al. (2021) indicated that this species probably also occurs in Bolivia, Colombia and Brazil. Additionally, Torres-Carvajal et al. (2021) reported *O. inkaterra* from Ecuador. The specimens reported herein confirm the presence of *O. inkaterra* in Bolivia and extend the known distribution by approximately 207 km (Río Sipia) and 628 km (Campamento Guacharos, both localities located in protected areas) southeast of the type locality (Puerto Maldonado, Peru).

Previous records of sharpnose snakes (*X. argenteus* and *X. boulengeri*) collected in Bolivia include the departments of Beni, Cochabamba, and Pando (Procter 1923; Donoso-Barros 1967; Keiser 1989; Fugler et al. 1995; Cadle et al. 2003; Moravec and Aparicio 2005). Our report adds eight new records for *X. argenteus* (n = 1) and *X. boulengeri* (n = 7), all from the Amazonian forests of the department of Beni and Pando respectively (Table 3, Fig. 5), considering that the type locality of *X. boulengeri* is located to the south in the riparian forests of the Mamoré River, Trinidad, Beni (Procter 1923). *Xenoxybelis boulengeri* is considered rare and poorly represented in scientific collections (Prudente et al. 2008).

The meristic and morphological variation of *O. aeneus* has been well supported by Jadin et al. (2020) in its distribution range of the Amazon basin. The characters of the examined specimens of Bolivian *O. aeneus* (Table 2, Fig. 1A, B) are congruent with the description of the species by Jadin et al. (2020), and the color pattern (in life) matches the description of the species by Keiser (1989) and Jadin et al. (2020).

The meristic characters and color pattern (in life) of the examined specimens of *O. fulgidus* (Table 2, Fig. 1C, D) are congruent with the description of the species by Daudin (1803) and Boulenger (1896), and other related publications (Peters and Orejas-Miranda 1970; Nascimento et al. 1988; Cole et al. 2013; Fraga et al. 2013; Curlis et al. 2020). In addition, we contribute meristic data of the examined specimens, information scarcely available in the available literature. It is likely that the reduced number of subcaudals (128 scales) in the specimen CIRAH-929 is the result of the specimen having lost part of its tail at some point during its life.

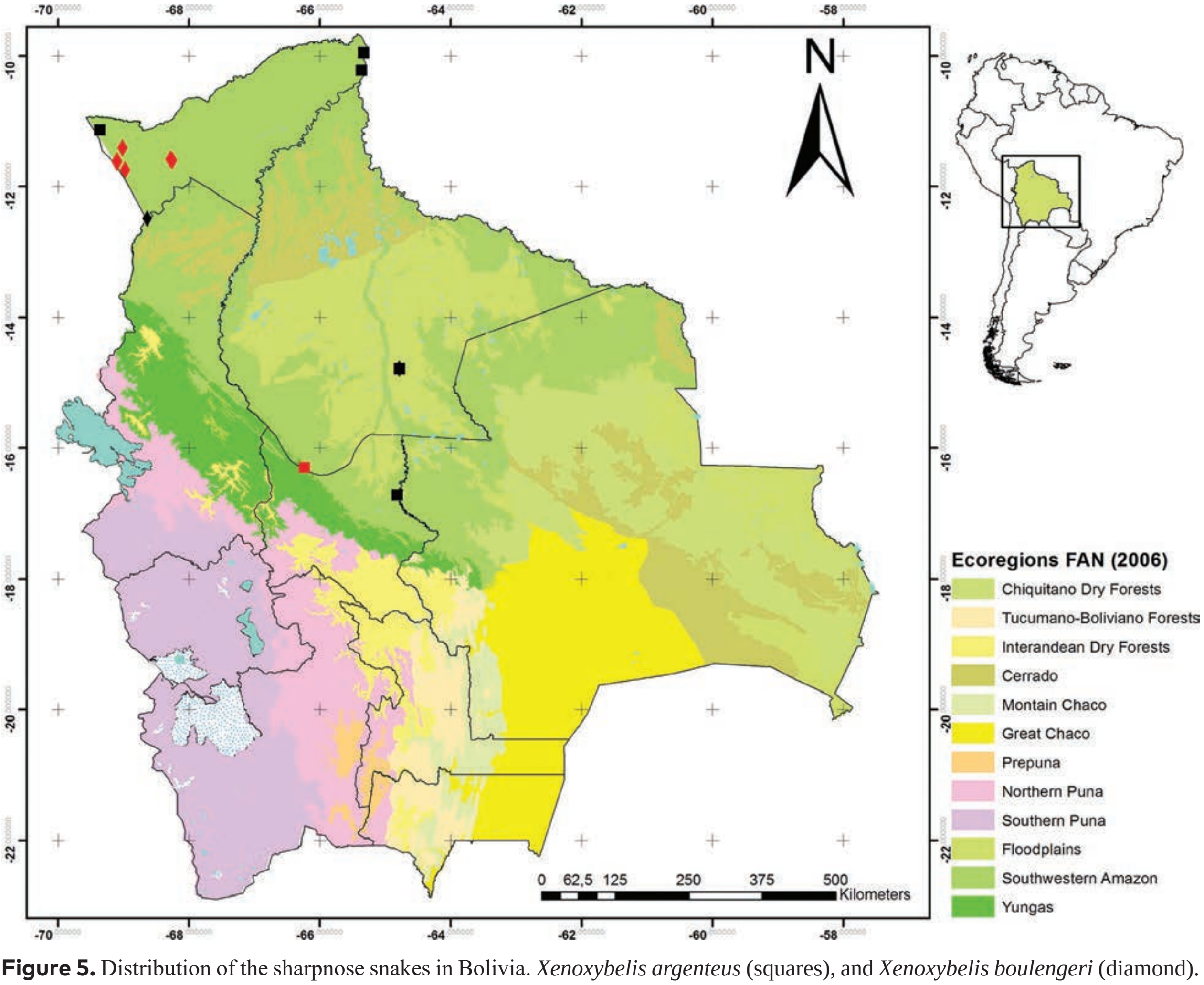

Figure 5. Distribution of the sharpnose snakes in Bolivia. *Xenoxybelis argenteus* (squares), and *Xenoxybelis boulengeri* (diamond). Red symbols correspond to additional records.

Specific meristic characters and coloration of *O. inkaterra* specimens reported in our study (Table 2, Fig. 2A–D) are congruent with the original description of the species by Jadin et al. (2021). However, specimen CBF-4275 has few eyespots in the posterior ventral and subcaudal region of the body; this is less evident in specimen MNKR-3740. Furthermore, this last specimen presents two supralabials that contact the orbit (fourth and fifth), unlike the other two specimens examined (fourth, fifth and sixth contact the orbit).

Our examined specimen of *X. argenteus* (Table 4, Fig. 4A, B) is consistent with the description and identification of the species by Daudin (1803) and Duellman (1978) respectively. In addition, we contribute with meristic data of the examined specimen. The absent loreal scale and undivided cloacal scale are specific characters of the species.

The meristic characters and color pattern (in life) of the examined specimens of *X. boulengeri* (Table 4, Fig. 4C, D) are congruent with the description of the species (Procter 1923; Keiser 1989; Duellman 2005; Prudente et al. 2008). However, our data on ventral scales (196–205) and divided subcaudal scales (132–186) of male specimens are slightly lower than those reported by Prudente et al. (2008). It is likely that the reduced number of subcaudals (132) counted in this study is the result of a specimen also having lost part of its tail at some point in its life. Furthermore, unlike Prudente et al. (2008) who described one preocular, we counted two preoculars in 29% (n=2) of the specimens examined.

The mimicry of *Oxybelis* and *Xenoxybelis* with their environment is characteristic of this group of snakes, which is why it is very difficult to observe and capture them during the day. As a result, 91% of the specimens examined (CIRAH) were collected during the night, generally resting on branches or leaves of bushes at a height between 0–4 m from the ground. Only one specimen was found at ground level.

These new and first additional reports of *Oxybelis* and *Xenoxybelis* represent contributions that can be used to improve the understanding of their distribution (Figs 3, 5) and aspects of their natural history in Bolivia and throughout their range in South America. In addition, they constitute valuable voucher specimens for Bolivian herpetological collections.

Acknowledgments

We thank the local guides of the different communities of Beni, Cochabamba, La Paz, Pando, and Santa Cruz for their collaboration during the active searches for snakes. We acknowledge the management and staff of the herpetological collections of the Museo Nacional de Historia Natural - La Paz, Museo de Historia Natural Noel Kempff Mercado - Santa Cruz, and Museo de Historia Natural Alcide d'Orbigny - Cochabamba. To the Director and protection team of the Reserva Nacional de Vida Silvestre Amazónica Manuripi. To the Dirección General de Biodiversidad y Áreas Protegidas for the collection permits for scientific samples. Lastly, we thank Dr. Robert Jadin for the suggestions and confirmation of the *O. inkaterra* specimens from Bolivia.

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ÖGH Herpetozoa
Austrian Herpetological Society

Herpetozoa 37: 213–226 (2024)
DOI 10.3897/herpetozoa.37.e116376

Direct fire-induced reptile mortality in the Sierra Morelos natural protected area (Mexico)

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Academic editor: Yurii Kornilev ♦ **Received** 27 November 2023 ♦ **Accepted** 2 July 2024 ♦ **Published** 19 July 2024

Abstract

Little is known about fire as an agent of direct animal mortality, and specifically, there is controversy regarding the effects of fire on reptiles. In the Sierra Morelos natural protected area in Mexico, both unplanned (e.g., man-made illegal and accidental burns) and prescribed burns occur during the dry season. In this study, we assessed direct fire-induced reptile mortality in the Sierra Morelos natural protected area by comparing live and dead individuals. Of the 14 reptile species reported in the area, seven were found dead due to fire. One-third of the individuals found in 2021 (34% of 169 ind.) and 2022 (33% of 33 ind.) were killed by fire. The mean density of detected dead individuals was 1.60 ± 2.99 individuals/ha in 2021 and 0.31 ± 0.27 individuals/ha in 2022. Mortality densities were similar in areas subjected to prescribed burns and in areas affected by unplanned burns (W= 37.5, p = 0.4383). Since our survey likely underestimated the actual mortality caused by fire, our findings show the important direct impact of fire on the reptile community of the Sierra Morelos natural protected area and support the notion that anthropogenic fire can represent a serious risk for reptiles in fragile ecosystems.

Key Words

carcass count, fire impact, fire management, prescribed burning

Introduction

Fire is a fundamental ecological process in many ecosystems and shapes their structure, composition, and function (Bond and Keeley 2005; Bowman et al. 2009; Pausas and Keeley 2009; He et al. 2019; McLauchlan et al. 2020). Some species are adapted to natural fire regimes (Keeley et al. 2011; Pausas and Parr 2018) and can benefit from fire and the environmental heterogeneity it creates (Parr and Andersen 2006; He et al. 2019). People have used fire to modify environments for millennia (Bowman et al. 2011); however, human activities are now changing patterns of fire at a global scale (Kelly et al. 2020; Bowman et al. 2020). Extreme droughts have increased the frequency, duration, and intensity of fires in many regions of the world due to human-induced climate change, and ecosystems worldwide are becoming more stressed due to warmer climates (Duane et al. 2021). The fire season has lengthened for 25.3% of the Earth's vegetated surfaces, leading to an increase in the intensity and frequency of wildfires and an unprecedented amount of burned area (Jolly et al. 2015; Duane et al. 2021; Garcia et al. 2021). These changes in fire regimes are changing the biodiversity patterns of local communities (McKenzie et al. 2004;

Duane et al. 2021) and threatening species with extinction across the globe (Kelly et al. 2020).

Fire can affect species populations through direct and indirect effects (Whelan 2002; Engstrom 2010). The direct effects of fire include mortality during a fire event (Whelan 2002; Smith et al. 2012; Jordaan et al. 2020; Tomas et al. 2021) due to smoke inhalation (Lyet et al. 2009; Jordaan et al. 2020), radiant heat, or direct consumption by flames (Jolly et al. 2022). The indirect effects of fire are related to changes in habitat suitability in recently burned areas, such as reductions in food and shelter, which may increase species susceptibility to predation (Conner et al. 2011; Leahy et al. 2016; Santos et al. 2022); alteration of microclimatic conditions, which may increase overheating risk (Legge et al. 2008; Ferreira et al. 2016a); or threats related to the successional dynamics that fire initiates (Whelan 2002; Engstrom 2010). Species survival to fire events depends on functional traits (Santos et al. 2016), such as dispersal ability, size, microhabitat preference or evolutionary exposure to fire, and adaptation to local fire regimes (Santos and Cheylan 2013; Pausas and Parr 2018; Nimmo et al. 2021; Jolly et al. 2022). Vertebrates that disperse over large expanses, such as birds and large mammals, are often less vulnerable to direct mortality caused by fire, whereas most herpetofaunal species have more limited abilities to evade fire (Greenberg et al. 2018). Additionally, physical conditions such as age, reproductive status, fat reserves, or ecdysis may increase individuals' susceptibility to direct fire effects (Nimmo et al. 2021; Jordaan and Steyl 2023).

There is controversy regarding the effects of fire on reptiles; some studies have reported positive effects of fire on reptile communities (Langford et al. 2007). For example, it has been suggested that fire can temporally increase nutrient availability or stimulate seed release in some plant species, leading to an increase in arthropods, which may benefit insectivorous reptiles (Pausas and Parr 2018; Smith 2018), or that fire can create open areas with better thermal quality for reptiles (Bury 2004). Most importantly, some authors suggest that direct negative impacts of fire are negligible for reptiles (Means and Campbell 1981; Ford et al. 1999; Russell et al. 1999; Greenberg and Waldrop 2008), especially low-intensity fires (Floyd et al. 2002; Costa et al. 2013; Jolly et al. 2022), and that direct mortality of herpetofauna from prescribed burns is rare (Harper et al. 2016; Greenberg et al. 2018; Certini et al. 2021).

On the other hand, many studies report important negative effects of fire on reptiles. For example, Popgeorgiev (2008), Smith et al. (2012), Tomas et al. (2021), Santos et al. (2022), and Ballouard et al. (2023) reported high direct mortality due to natural/unplanned fires, whereas Lyet et al. (2009), Cross (2015), and Jordaan et al. (2020) reported high direct mortality in prescribed burns. Popgeorgiev and Kornilev (2009) reported a lower abundance of four reptile species in burned areas than in unburned adjacent areas. McLeod and Gates (1998), Valentine and Schwarzkopf (2009), and Santos and Cheylan (2013) reported lower reptile abundance and diversity in frequently burned areas, while Santos and Poquet (2010), Abom and Schwarzkopf (2016), and Ferreira (2016b) reported changes in community composition with frequent fires, even in extremely fire-prone ecosystems such as those found in Australia, Argentina, the Iberian Peninsula, South Africa, and USA. Most of these assertions about the impacts of fire on reptile populations have been assessed based on changes in animal population size before and after fire and do not distinguish between survival/mortality and immigration/emigration, creating uncertainty regarding the proportion of animals that survived the passage of fire (Jolly et al. 2022). In fact, there is surprisingly little knowledge of fire as an agent of direct animal mortality (Nimmo et al. 2021; Tomas et al. 2021; Jolly et al. 2022). Systematic searches for carcasses after fires (Popgeorgiev 2008; Jordaan et al. 2020; Tomas et al. 2021; Ballouard et al. 2023) can enhance our understanding of the direct impact of fire on animal populations. This approach can aid in determining the most suitable fire management practices for wildlife conservation in various ecosystems.

In Mexico, approximately 90% of recorded fires have anthropogenic causes (Trejo 2008). This is particularly concerning due to the increasingly warm and dry conditions in the context of a country highly vulnerable to climate change (Seager et al. 2009). The natural protected area of Sierra Morelos State Park (PESM by its acronym in Spanish) represents 78% of the green areas within Toluca city (Gobierno del Estado de México 2013), comprising major habitats for resident and migratory wildlife. The PESM contains a great diversity of vertebrate species, including at least 12 mammals, 184 birds, 14 reptiles, and two amphibians (www.inaturalist.org). In the PESM, many fires occur every dry season. However, some of them are prescribed burns conducted as part of the natural protected area management program. This paper focuses on estimating the death toll among reptiles directly caused by fires based on data collected in the field by accounting for live and dead individuals.

Materials and methods

Study site

The PESM is located north of Toluca city (19°20'00"N–19°17'47"N, 99°39'00"W–99°43'25"W), approximately 63 kilometers southwest of Mexico City (Fig. 1). The PESM ranges in elevation from 2,630 to 3,040 m above sea level (Sierra-Domínguez et al. 2018). The dominant climate is temperate subhumid, with summer rains. The dry season spans from November to May, and the rainy season spans from June to the end of October. The average annual rainfall from the nearest weather station (located less than 1 km away in the community of Calixtlahuaca) is 840 mm. The wettest month is July, with 167 mm of rain, whereas the driest month is December, with 9.6 mm of rain. Due to the high elevation, daily temperature fluctuations are very pronounced throughout the year. The annual average temperature oscillates between 4.9 and 22.3 °C (Servicio Meteorológico Nacional 2021).

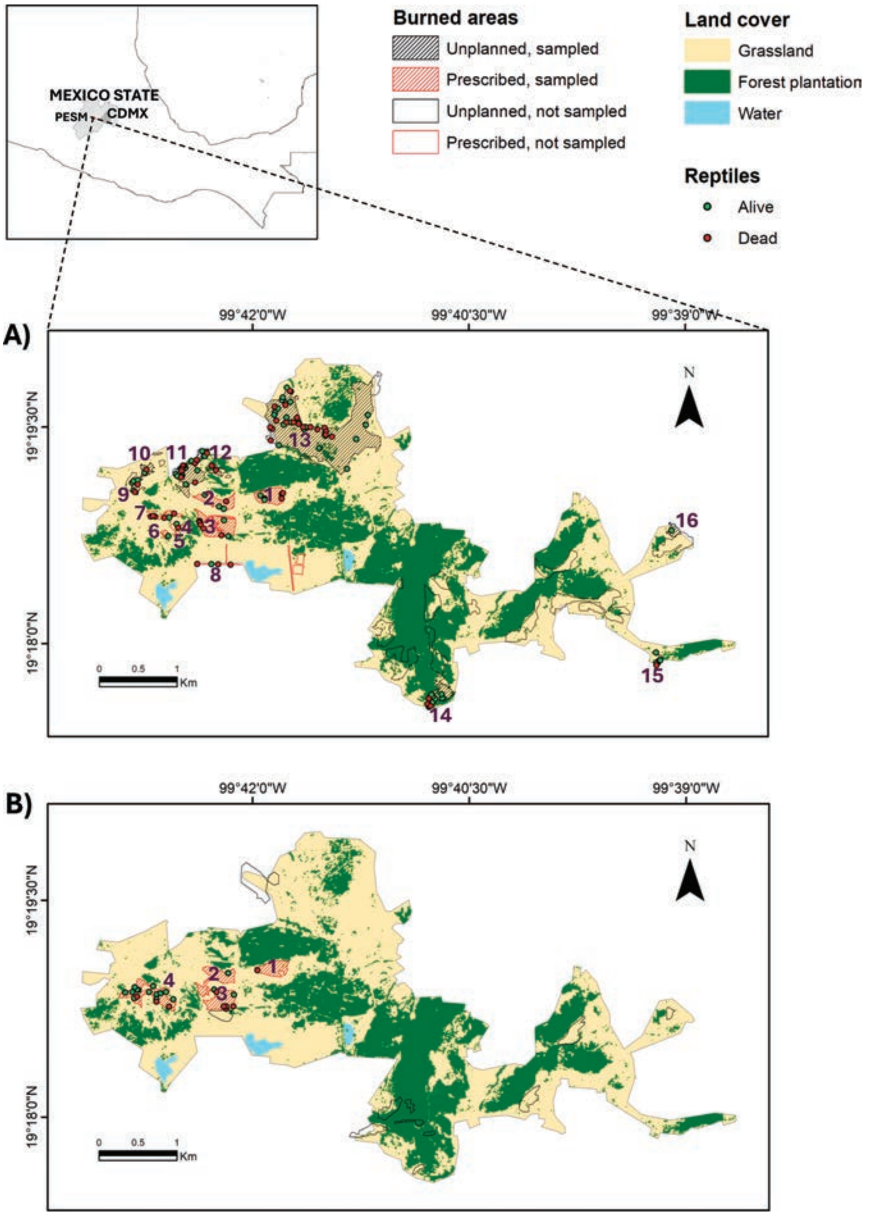

Figure 1. Burned areas in the PESM during the dry season in 2021 (**A**) and 2022 (**B**) and the reptiles found within them. The burned areas surveyed (marked with stripes) were delineated in the field with GPS and were used to calculate the density of dead and live reptiles. The burned areas not surveyed (without stripes) were delineated using satellite images. Red areas were prescribed burn sites as part of a fire management program in the natural protected area, whereas black areas were burned for reasons unrelated to the fire management program.

PESM was declared a natural protected area in 1976 with an original extent of 394.96 ha (Gobierno del Estado de México 1976). In 1981, an additional area of 860.13 ha was incorporated into the park (Gobierno del Estado de México 1981), reaching 1,255 ha. When the natural protected area was originally declared, approximately 12.65 ha of human settlements (isolated buildings separated by cultivated terraces and backyards) were already present within the polygon. Since that time, the urban built-up area has expanded in the PESM as cultivated land and backyards were replaced by new buildings (Sierra-Domínguez et al. 2018).

The current landscape of the PESM consists of a small plain and multiple hills covering the municipalities of Toluca (98% of the PESM) and Zinacantepec (2%) (Olvera-Viscaino 2018). The hills originated during the Miocene and are formed by highly permeable volcanic rocks that favor water infiltration (Niño-Gutiérrez et al. 2007), thus playing an important hydrological role. In contrast, the soil composition of the plain has a high clay content, which favors the flooding of the land during the rainy season and the formation of lagoons. Most of the PESM is covered by grassland and shrubland ecosystems. The forest cover is composed of introduced species that form forest stands, mainly *Cupressus lusitanica*, *Eucalyptus* sp., and *Pinus radiata*, which are considered fire-prone species, particularly the latter two (McWethy et al. 2018). There are also native tree species in the area, such as *Buddleja cordata*, *B. microphylla*, wild black cherry (*Prunus serotina*), Mexican hawthorn (*Crataegus mexi-*

cana), shrub oaks (such as *Quercus frutex*), and arboreal oaks (*Q. mexicana* and *rugosa*), but they mostly grow isolated rather than as forest stands, with the exception of shrub oaks (Sierra-Domínguez et al. 2018). Scientific information about the biodiversity of Sierra Morelos is scarce; to date, the records published in iNaturalist probably constitute the most reliable information on diversity in PESM and consist of 441 species of plants, 62 fungi, 542 arthropods, and 213 vertebrates. Fourteen reptile species have been reported in the area.

Study species

Of the 14 species reported for the PESM, five belong to the genus *Thamnophis* (Garter snakes, Colubridae family). The most common *Thamnophis* species in the PESM are *T. scaliger* (adult snout-vent length, SVL of 29–38 cm), *T. eques* (adult SVL of 39.5–92 cm), and *T. scalaris* (adult SVL of 34.5–50 cm) (Reguera et al. 2011; Manjarrez et al. 2007, 2014). They are slow-moving, and ground-dwelling species that can usually be found in grasslands and can use natural cavities or burrows on the ground. The lined Toluca earth snake (*Conopsis lineata*) is also a ground-dwelling snake from the Colubridae family that can be found in grasslands and forests (Sunny et al. 2023), usually under rocks. The Mexican dusky rattlesnake (*Crotalus triseriatus*) is also present in the area (Sunny et al. 2019; Rubio-Blanco et al. 2024) and can usually be found in bunchgrass, such as *Muhlenbergia* sp. Four reptile species present in the PESM belong to the *Sceloporus* genus (Phrynosomatidae family); the most common is the torquate lizard (*S. torquatus*), a lapidicolous species with an adult SVL of 8.4 to 9.7 (Ortiz et al. 2001). The graphic spiny lizard (*S. grammicus*) is smaller and much less abundant in the area and can usually be found in fallen logs. The Mexican plateau horned lizard (*Phrynosoma orbiculare*, also from the Phrynosomatidae family) is a lizard that typically reaches an adult SVL of 6.5 to 8.5 cm (Hernández-Navarrete 2018). This species is classified as threatened in Mexico under NOM-059-SEMARNAT-2010 and inhabits open areas and grasslands, using bunchgrass for perching and shelter (Gómez-Benítez et al. 2021). The transvolcanic alligator lizard (*Barisia imbricata*), which belongs to the Anguidae family, has adults with SVLs ranging from 9.38 to 9.7 cm (Woolrich-Piña et al. 2021). It is a ground-dwelling species commonly found among bunchgrasses (Sunny et al. 2017).

Methodology

The methodology consisted of mapping the burned areas in 2021 and 2022 within the PESM and searching on the ground for live and dead reptiles throughout the entire extent of the burned areas after the fires occurred.

Assessing burned areas:

We sampled burned areas shortly after the fires occurred (from the same day up to three weeks; see Table 1). To assess the surveyed area, we recorded the perimeter of the sampled areas using GPS. Unburned areas within the burned area polygon were also registered and excluded. The area of the surveyed polygons was calculated using a Geographic Information System in UTM Zone 14 N, WGS84. To map all the other burned areas that we could not survey, Sentinel-2 satellite images were acquired at the end of the dry season in 2021 and 2022. We displayed

Table 1. Burned areas surveyed (prescribed or unplanned) identified according to Fig. 1, number of dead and live reptiles found, area burned, time between the fire and the survey (during the first week, second week, or third week), perimeter, and relationship between area and perimeter (fire shape) for each burned area surveyed.

Burned area id	Prescribed/Unplanned	Dead	Alive	Area (ha)	Dead/ha	Survey after fire	Perimeter (km)	Fire shape
1 (2021)	Prescribed	2	4	5.88	0.34	Third week	1.01	5.80
2 (2021)	Prescribed	1	3	5.41	0.18	Third week	1.47	3.68
3 (2021)	Prescribed	5	3	9.16	0.55	Second week	3.97	2.31
4 (2021)	Prescribed	1	1	0.29	3.43	Second week	0.24	1.20
5 (2021)	Prescribed	0	0	0.05	0.00	Second week	0.10	0.55
6 (2021)	Prescribed	0	1	0.67	0.00	First week	0.44	1.53
7 (2021)	Prescribed	5	3	2.01	2.49	First week	0.84	2.40
8 (2021)	Prescribed	3	1	0.24	12.26	Second week	1.66	0.15
9 (2021)	Unplanned	2	2	1.55	1.29	First week	0.57	2.70
10 (2021)	Unplanned	1	3	1.84	0.54	Second week	1.03	1.79
11 (2021)	Unplanned	10	15	10.38	0.96	Second week and third week	2.68	3.87
12 (2021)	Unplanned	2	3	1.65	1.21	First week	0.60	2.75
13 (2021)	Unplanned	20	46	73.73	0.27	First, second and third week	6.10	12.08
14 (2021)	Unplanned	5	19	5.11	0.98	First week	1.41	3.64
15 (2021)	Unplanned	1	5	0.91	1.09	First week	0.57	1.61
16 (2021)	Unplanned	0	2	1.71	0.00	First week	0.78	2.19
1 (2022)	Prescribed	1	1	5.49	0.18	First week	1.34	4.10
2 (2022)	Prescribed	0	2	4.76	0.00	First week	1.10	4.31
3 (2022)	Prescribed	5	2	9.16	0.55	First week	2.34	3.91
4 (2022)	Prescribed	5	17	9.53	0.52	Second week	3.01	3.17

the images in RGB colors in a Geographic Information System with the following band combinations: shortwave infrared (band 12: R), near-infrared (band 8: G), and green (band 3: B). We visually outlined the burned areas and calculated their extent. While we knew when the fire occurred at some sites, for others, the timing was uncertain. Thus, we used the Sentinel-2 satellite images to approximate the dates of the fires in cases of uncertainty. This approach allowed us to determine the time between the fire and the sampling.

Prescribed burned areas (conducted as a fire management program of the PESM) were differentiated from unplanned burned areas (e.g., man-made illegal or accidental burns) because the former were very similar in shape, extent, and location both years. In addition, we corroborated prescribed burns with local people and with the authorities of the PESM. The main objective of these prescribed burning practices was to preserve areas planted with non-native trees. While prescribed burning practices have been conducted in the PESM since at least 2013, in 2021 and 2022, the amount of burned area under this type of management increased considerably (from an average of 2.65 ha per year between 2013 and 2020 to 35.9 ha in 2021).

Searching for reptiles

We carried out the surveys from the end of January to the beginning of April 2021 and in January 2022 between 09:30 h and 15:00 h (except on one occasion when the fire started as we were leaving, and we stayed to extinguish it and survey the burned area at approximately 16:00 h). The sampling was conducted on sunny days, which are most common during the dry season. Since most fires occurred in grasslands, the majority of the surveys were conducted in this ecosystem. Only sites 12 and 14 were extensively covered by trees (cedars and eucalyptus, respectively).

Usually, during the first two weeks following a fire (but in some cases, up to three weeks), we sampled the entire burned area in search of reptiles. The burned area was divided among two to four people with experience in surveying herpetofauna. We walked across the entire area in a zigzag pattern, maintaining a distance of approximately two meters between passes to ensure that no areas were overlooked. In large burned areas, we needed to conduct surveys over several days. In 2021, the surveys were conducted over 18 days (approximately 100 hours; equivalent to 300 person-hours), and in 2022, they were conducted over four days (approximately 22 hours; equivalent to 66 person-hours).

When we found a reptile in the burned area, we identified the species, noted whether it was dead or alive, took a photograph, recorded the coordinates using GPS, and documented the type of habitat where it was found. When possible, we displaced live and/or dead individuals outside the burned area to avoid counting them twice. Carcasses were not collected because they represent a food resource for predators. Live animals were released near the edges of burned areas so that they could avoid short- and medium-term fire effects but could eventually return to the area. When needed, we poured clean water over the survivors to cool them and remove the ashes. The density of carcasses was obtained by dividing the number of carcasses by the area surveyed (the area recorded with GPS). Animals that died from a cause other than fire were excluded from the analyses and tables presented here. For example, one *Sceloporus torquatus* and one *Thamnophis* sp. were excluded because they seemed to have been crushed.

We conducted a Mann-Whitney U test to compare the density of dead individuals in prescribed burned areas versus unplanned burned areas. Finally, we assessed the relationship between the density of dead reptiles and burn shape (area/perimeter).

Results

During the 2021 dry season, 196.91 ha were affected by fire in the PESM (Table 2). Of these, 35.9 ha were prescribed burns, according to PROBOSQUE (a decentralized public entity from the State of Mexico affiliated with the Ministry of Agriculture; see Suppl. material 1), and occurred during the last half of January. The area sampled consisted of 120.60 ha and was divided into 16 sampling sites (23.72 ha in prescribed burned areas, with 8 sampling sites; Fig. 1A). In 2022, 63.33 ha were affected by fires in the natural protected area (Table 2). From this total, at least 28.95 ha, where we conducted our search (four sampling sites, Fig. 1B), were in prescribed burned areas, where the fire occurred on January 27th.

Table 2. Total burned area (ha), sampled area, prescribed burning area reported by authorities, and prescribed burning sampled area for 2021 and 2022 in the PESM.

	2021	2022
Total burned area (ha)	196.91	63.33
Sampled area (ha)	120.60	28.95
Prescribed burning area reported by authorities (ha)	35.9	No Data
Prescribed burning sampled area (ha)	23.72	28.95

Of the 14 reptile species reported for the PESM in iNaturalist (with research grade quality), we found nine (dead or alive) in the burned areas sampled: three garter snake species (*Thamnophis eques*, *T. scalaris*, and *T. scaliger*), the Mexican plateau horned lizard (*Phrynosoma orbiculare*), the torquate lizard (*Sceloporus torquatus*), the graphic spiny lizard (*S. grammicus*), the Mexican dusky rattlesnake (*Crotalus triseriatus*), the transvolcanic alligator lizard (*Barisia imbricata*), and the lined Toluca earth snake (*Conopsis lineata*). Two of the missing species belong to the genus *Sceloporus* and have been reported only once for the PESM. Another missing species is *T. melanogaster*, which is highly associated with aquatic habitats. *Thamnophis* species were classified only at the genus level due to the inability to distinguish the species of some burned carcasses. Of the 14 species reported for the PESM, seven were found dead by fire (Figs 2, 3, Suppl. material 2).

One-third of the individuals found in 2021 (34% of 169 ind.) and in 2022 (33% of 33 ind.) were killed by fire (Table 3). The overall mortality density (total dead individuals across the total sampled area) was 0.48 ind./ha (mean density ± SD = 1.60 ind./ha ± 2.99) for 2021 and 0.38 ind./ha for 2022 (mean = 0.31 ind./ha ± 0.27). In 2021, site 8 had the highest mortality density (12.26 ind./ha), and in 2022, site 3 had the highest mortality density (0.55 ind./ha) (Table 1). The mortality density in prescribed burned areas during 2021 was 0.72 reptiles/ha (mean = 2.41 ind./ha ± 4.18), whereas in unplanned burned areas during 2021, it was 0.42 reptiles/ha (mean = 0.79 ind./ha ± 0.47). During 2022, all the surveys were carried out in a prescribed burned area, where the mortality density was 0.38 ind./ha (mean = 0.31 ind./ha ± 0.27) (Table 3, Fig. 4). In 2021, 53% of the reptiles found in the prescribed burned areas surveyed were dead, whereas 30% of those found in unplanned burned areas were dead. In 2022, 33% of the reptiles found in prescribed burned areas were dead (Fig. 4). We found no significant differences in the densities of dead individuals between unplanned and prescribed burned areas ($W = 37.5$, $p = 0.4383$). We did not find a significant relationship between the density of dead reptiles and area/perimeter ($R^2 = 0.0528$, p value = 0.168).

In 2021, 84% of the carcasses were found on burned grass, 9% on leaf litter, 5% on rocky surfaces, and 2% on bare soil, whereas 35% of the survivors were found on burned grass, 25% on rocky surfaces, 11% on bare soil, 10% in/under agave plants, 8% on leaf litter, 6% in burrows, and 5% in fallen logs. One surviving *P. orbiculare* was found in a bird's nest on the burned grass. The taxa with a higher density of carcasses were *P. orbiculare*, with 0.17 dead ind./ha (mean = 0.30 dead ind./ha ± 0.51), and *Thamnophis* sp., with 0.16 dead ind./ha (mean = 0.84 dead ind./ha ± 3.05) (Table 3). The most common species found alive was *S. torquatus*, with 61 individuals, and *P. orbiculare*, with 24 (four of which were visibly injured). On two occasions, we arrived at the fire site while it was still burning and successfully prevented some animals (three *P. orbiculare*) from entering the fire. In 2022, all carcasses were found on burned grass, while 58% of the survivors were found on burned grass, 32% on a rocky surface, and 11% on bare soil. The species with the highest density of carcasses was *P. orbiculare*, with 0.14 dead individuals/ha (mean = 0.13 ± 0.09). The most common species found alive was *P. orbiculare*, with 11 individuals (two of them visibly injured).

Discussion

In this study, we assessed direct fire-induced reptile mortality in the Sierra Morelos natural protected area by comparing live and dead individuals.

Percentages of dead and live reptiles

A substantial proportion (more than 30%) of all reptiles found in burned areas between 2021 and 2022 were dead. Although the surveys were conducted on sunny days, minor changes in climatic conditions during the samplings should be considered a potential source of bias in the relationship between live and dead animals. Additionally, our findings should be interpreted with caution, as some carcasses may have been eaten by predators/scavengers, and some survivors may have abandoned the site, especially fast-moving species such as *S. torquatus*. However, this seems more difficult for slow-moving reptiles such as *P. orbiculare* or *Thamnophis* sp., which were often found in bad physical conditions, e.g., slower than normal, dehydrated, and stained with ash.

Table 3. Number of dead and live reptiles, and density of carcasses (total and mean with standard deviation) found during the dry seasons of 2021 and 2022.

2021	Dead	Alive	Total	Dead/ha
Barisia imbricata (Anguidae)	8	4	12	0.07 (0.12 ± 0.30)
Conopsis lineata (Colubridae)	2	2	4	0.02 (0.22 ± 0.86)
Crotalus triseriatus (Viperidae)	0	4	4	0.00
Phrynosoma orbiculare (Phrynosomatidae)	20	24 (4 injured)	44	0.17 (0.30 ± 0.51)
Sceloporus grammicus (Phrynosomatidae)	0	1	1	0.00
Sceloporus torquatus (Phrynosomatidae)	9	61	70	0.07 (0.12 ± 0.21)
Thamnophis sp. (Colubridae)	19	15	34	0.16 (0.84 ± 3.05)
TOTAL	**58**	**111**	**169**	**0.48 (1.60 ± 2.99)**
2022	**Dead**	**Alive**	**Total**	**Dead/ha**
Barisia imbricata (Anguidae)	2	2	4	0.07 (0.05 ± 0.11)
Conopsis lineata (Colubridae)	0	2	2	0.00
Crotalus triseriatus (Viperidae)	0	1	1	0.00
Phrynosoma orbiculare (Phrynosomatidae)	4	11 (2 injured)	15	0.14 (0.13 ± 0.09)
Sceloporus grammicus (Phrynosomatidae)	0	0	0	0.00
Sceloporus torquatus (Phrynosomatidae)	3	5	8	0.10 (0.08 ± 0.16)
Thamnophis sp. (Colubridae)	2	1 (injured)	3	0.07 (0.05 ± 0.11)
TOTAL	**11**	**22**	**33**	**0.38 (0.31 ± 0.27)**

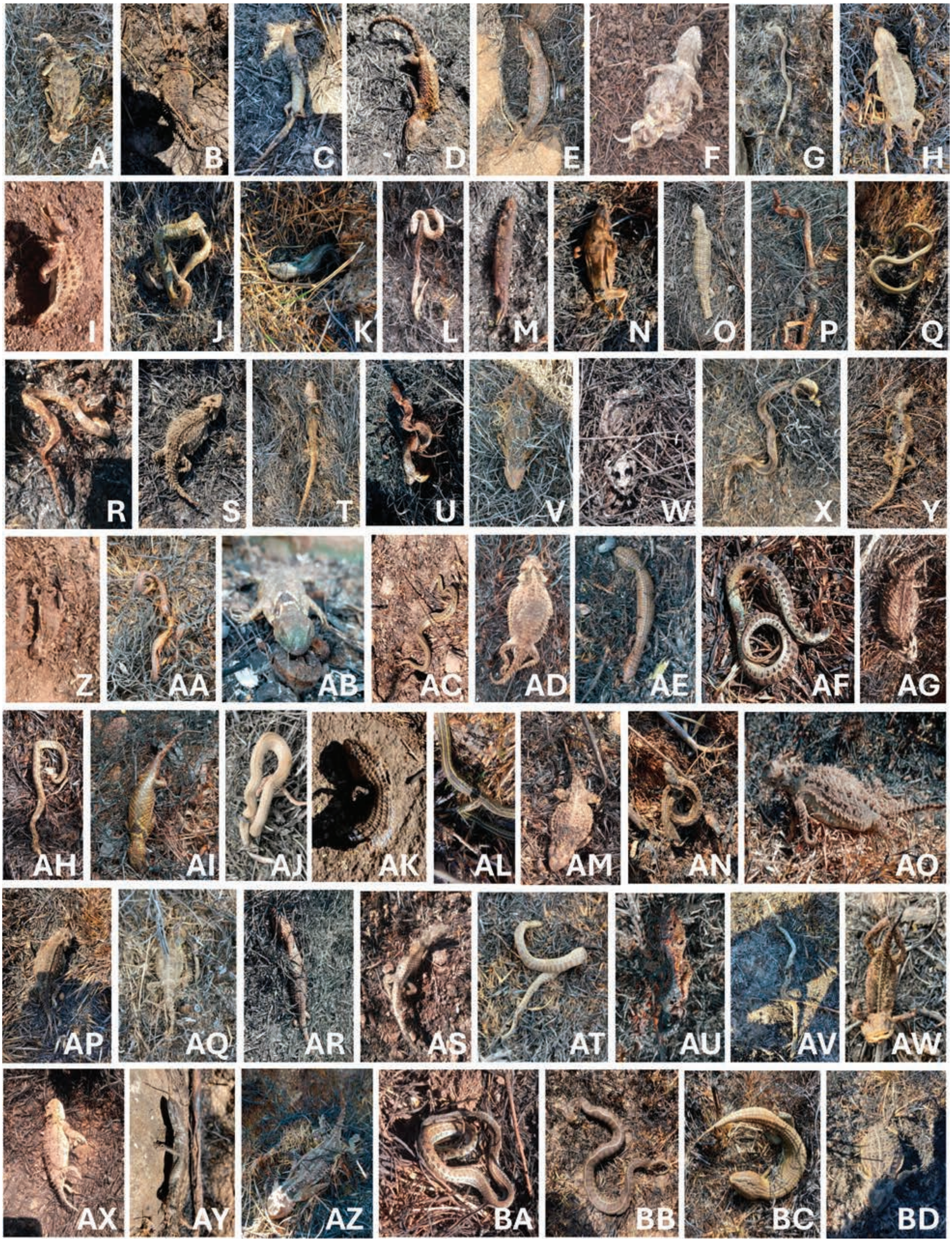

Figure 2. Representative pictures of carcasses found in burned areas in 2021 and 2022. Each picture corresponds to a different individual. The letters A, B, F, H, I, N, S, V, W, Z, AD, AG, AM, AO, AP, AQ, AX, AZ, and BD correspond to *P. orbiculare*; the letters C, E, K, M, O, AE, AK, AR, and BC correspond to *B. imbricata*; the letters D, T, Y, AB, AI, AS, AU, AW, and AY correspond to *S. torquatus*; the letters J, L, P, Q, R, U, X, AA, AC, AF, AH, AL, AN, AT, AV, BA, and BB correspond to *Thamnophis* sp.; and the letters G and AJ correspond to *C. lineata*.

Figure 3. Enlarged pictures shown in Fig. 2 and referenced in the text. The letter W corresponds to a completely calcinated *Phrynosoma orbiculare* that fell apart when we took it. The letter AH corresponds to *Thamnophis scalaris*, almost intact in the middle of a burned surface. The head up suggests death by asphyxia. The letter Q corresponds to *Thamnophis eques* slightly burned above a hole, whereas the letter K corresponds to *Barisia imbricata* slightly burned with half of its body inside a burrow.

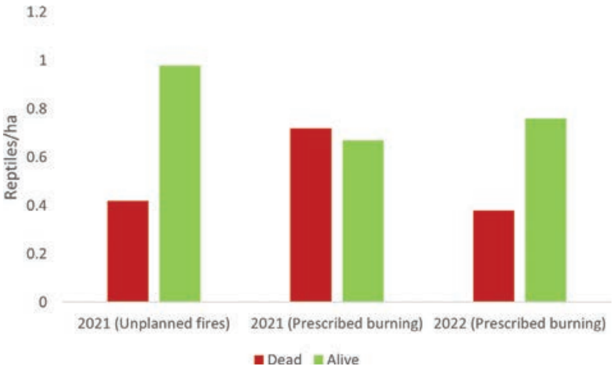

Figure 4. Individuals per hectare found dead and alive in 2021 unplanned fires, 2021 prescribed fires, and 2022 prescribed fires.

Mortality densities

The density of dead reptiles found in our study area is lower than that of other studies, such as in the Pantanal wetland in Brazil, where Tomas et al. (2021) estimated 2.48 dead small reptiles/ha—a value described as astonishing by the authors—and Lapalala wilderness, South Africa (7.77 dead reptiles/ha; Jordaan et al. 2019). Nevertheless, the biodiversity in the PESM is much lower than the biodiversity at these study sites. For example, the Pantanal is the largest wetland in the world, with 113 reptile species reported (including more than 30 species of snakes; Alho 2008), whereas the PESM is a small natural protected area (1,255 hectares) with a low level of protection, situated within a densely populated city and heavily impacted by anthropogenic activities (exotic forest plantations, water pollution, illegal garbage dumps). In the context of lower and more vulnerable biodiversity, the mortality densities found at our study site may alert us to the serious risk that anthropogenic fires can pose to native herpetofauna in fragile ecosystems. At least, our findings contrast with those of several studies that report direct fire-induced mortality in reptiles as rare, negligible, or nonexistent (Costa et al. 2013; Harper et al. 2016; Greenberg et al. 2018; Certini et al. 2021; Jolly et al.

2022). Our findings are rather in line with studies that have already warned about the impacts of fire on reptile populations (Popgeorgiev 2008; Smith et al. 2012; Abom and Schwarzkopf 2016; Jordaan et al. 2019, 2020; Santos et al. 2022; Ballouard et al. 2023) and on vertebrates in general (Berlinck et al. 2021; Tomas et al. 2021).

Detection of individuals

We consider that the mortality densities obtained can be highly underestimated: carcasses are difficult to detect within a burned area, some may be covered by ashes, and some may have been completely calcined (Fig. 3). Some initial survivors, especially those that were injured, may have died after the survey due to wounds or indirect fire effects, and some animals may have died underground or in other refugia; inhalation of hot or toxic gasses produced by fire has been suggested as a source of mortality for snakes that have fled underground (Durbian 2006), making the mortality of these animals nearly impossible to determine. Additionally, surveys were conducted by area (searching across the entire area in a zigzag pattern) rather than by transect, which leaves the possibility that some sites in the largest burned areas were not surveyed. Moreover, it was not always possible to sample the areas during the first week after the fire, extending into the second and, in some cases, the third week; thus, some carcasses may have been eaten by predators/scavengers. For example, Ballouard et al. (2023) reported the disappearance of 20% of carcasses during the second week after a fire. Thus, the overall impact on reptile communities should be considered higher than our counts. Even if mortality by fire has been underestimated in our study, our results still show the major deleterious effects of fire on the reptile community of the PESM.

Mortality rates among species

Phrynosoma orbiculare was the most common carcass found. The high number of carcasses can be attributed to the abundance of this species in certain areas of the natural protected area and its slow movement (Presch 1969), which makes it vulnerable to fire. Fair and Henke (1997) also expressed concerns about fire-induced mortality in Texas horned lizards (*Phrynosoma cornutum*), and other slow-moving lizard species, such as the Australian thorny devil (*Moloch horridus*), have also been reported dead after fire (Smith et al. 2012). The density of *P. orbiculare* carcasses found in 2021 (0.17 ind./ha, mean = 0.30 ind./ha ± 0.51) is similar to that reported for other slow-moving species, such as *Testudo hermanni* (0.19 ind./ha) (Ballouard et al. 2023). In contrast, *S. torquatus*, another lizard of the Phrynosomatidae family that is similar in size and very abundant in the area, experienced markedly lower mortality. This is likely due to its greater speed and ability as a lapidicolous species, enabling it to climb logs, shrubs, agaves, and rock walls.

Thamnophis sp. carcasses were also among the most commonly found. Although they can retreat to underground burrows, ground-dwelling and slow-moving species are more vulnerable to fire than lapidicolous species. Other studies have reported severe fire-induced mortality in snakes (Durbian 2006; Lyet et al. 2009; Cross 2015). For example, small snakes represented 55% of the dead vertebrates recorded in wildfires that hit the Brazilian portion of the Pantanal wetland in 2020 (Tomas et al. 2021). *Thamnophis* sp. were the only species among which some dead individuals (three) were found without signs of burns. These individuals also did not exhibit any other signs of visible damage and were found on a completely burned surface (Fig. 3). A study carried out in the PESM reported that *T. scalaris* can retreat in underground hollows or burrows deeper than one meter (Mundo-Hernández et al. 2017), and studies with better-known *Thamnophis* species from other regions, such as *T. sirtalis*, reported that they use two-meter-deep hollows (Shine and Mason 2004). The dead *Thamnophis* individuals found in our study with no visible burns may have been below ground during the fire and came out after the flames consumed the vegetation and passed by; however, they probably died as a consequence of smoke inhalation or noxious gas inhalation. This is consistent with the findings of other studies on the effects of fire on fossorial reptiles, where dead reptiles were found on the soil surface, some in positions suggesting these animals attempted to reach the surface (Jordaan et al. 2020). Such observations have led to the hypothesis that burrowing reptiles are not necessarily protected from fire effects (Engstrom 2010). Deaths by smoke inhalation and inhalation of noxious gases produced during burning in association with anoxia have been reported for snakes (Durbian 2006; Lyet et al. 2009; Jordaan and Steyl 2023) and other reptiles (Jordaan et al. 2019; Jordaan and Steyl 2023). Finally, we found a relatively high number of *B. imbricata* carcasses (10), considering that this species is not very abundant in the PESM, which suggests that this ground-dwelling species may also be highly vulnerable to fire.

Differences between sampled burned sites

Site 13 had the highest number of dead reptiles (20 individuals). The fire was caused by stubble burning, which went out of control. However, the mortality density at this site was lower than in other sites. This may be because the zigzag survey pattern is insufficiently thorough for such a large area. The highest mortality density was found at site 8, which was a firebreak located alongside a canal that carries water during the wet season. We found three *Thamnophis* sp. carcasses in this small firebreak. This may be due to the greater density of *Thamnophis* near the canal than in other areas without water bodies. We found no relationship between carcass density and fire shape. This could be attributed to the limitations of the simple measure (area/perimeter), which does not account for landscape elements such as the presence and distribution of rocky areas, agave plants, and other potential refuges. These elements could serve as more effective indicators for explaining variations in mortality density across sites. For example, Hale et al. (2022) and Robinson et al. (2013) found that the presence of refugia in burned areas is a key factor that explains wildlife resistance to fire. Other variables not measured in this study, such as wind direction and speed during the fire, as well as the severity of the fire, are likely to have also impacted mortality rates at each study site. Slower and lower-intensity fires would have provided greater opportunities for animal survival.

Prescribed burning vs. unplanned fires

Prescribed burning is the intentional use of fire under specific environmental conditions (e.g., during the dormant season) to achieve various land management goals. It has been reported as an appropriate management tool to benefit herpetofauna by restoring a historical mosaic of successional stages (Russell et al. 1999) and by generating more open habitats with greater insolation (Matthews et al. 2010). However, our study questions these views. We report no statistically significant differences in the densities of dead individuals between unplanned and prescribed burned areas. This may be because, in our study, both prescribed and unplanned fires occurred during the same season (the fire-prone season), and it is likely that the fire severities were similar. As evidence, an individual of *P. orbiculare* was found completely calcinated in a prescribed burned area.

Some studies suggest that prescribed fire effects on herpetofauna can be reduced by conducting burning practices during winter, when many terrestrial herpetofaunal species are inactive and are likely below ground or otherwise insulated from high temperatures and the potential desiccating effects of fire (Harper et al. 2016). However, in our study, all the carcasses found in the prescribed burned areas were from fires conducted during the cold and dry seasons (January and February). We must consider that although the climate of our study site is classified as temperate, it exhibits important differences compared to temperate climates at higher latitudes. At night, temperatures can drop below zero degrees Celsius, but during the hottest hours of the day, they can reach more than twenty degrees. Fires occurring when reptiles are less active can cause less mortality only if inactive reptiles are protected from fire. At our study site, reptiles are less active during the cold and dry seasons but are not completely inactive, and they are not necessarily hidden at a sufficient depth as in colder climates. For example, when *P. orbiculare* is less active, it shelters under bunchgrass or leaf litter (Gómez-Benítez et al. 2021), increasing its vulnerability to fire compared to when it is active (e.g., in areas with less vegetation coverage, looking for ants).

Management implications

On the basis of our findings, we seriously question prescribed burning practices in the PESM and in similar ecosystems of the region, e.g., grasslands that share a similar reptile community composition. Prescribed burning has been carried out in the same grassland areas of the PESM during consecutive years, causing deaths in the same reptile populations, with the main objective of preserving areas with planted trees that are not native and, moreover, fire-dependent (which is related to the promotion of tree planting in the country as a "green" policy that does not adhere to ecological criteria, stemming from the misconception that grasslands or shrublands are less important than forests). Whelan (2002) affirms that populations of many species are less threatened by a single high-intensity fire event than by a sequence of lower-intensity fires in close succession. In the study by Abom and Schwarzkopf (2016), reptile numbers in native grass were found to decline after fire and failed to return to previous numbers with revegetation even in extremely fire-prone, often-burned environments, suggesting that longer periods without burning may be beneficial to reptile assemblages. Furthermore, annual fires disrupt the natural succession of vegetation and hinder regrowth, even for fire-adapted species such as native *Quercus* populations (Trejo 2008).

In the PESM, unplanned fires (e.g., man-made illegal and accidental burns) continue to occur every year despite prescribed burning practices. Unplanned fires are related to a variety of factors, including the use of fire in agriculture and grazing, the practice of trash burning in the vicinity of precarious settlements, the presence of remaining campfires, and the disposal of cigarettes. The practice of systematic 'weed clearance' in natural and semi-natural areas around settlements has led to burning every vacant lot, roadside, and land between crops every year, and is likely the main contributor to unplanned fires in the region, as these fires often spread out of control. Both planned and unplanned fires may have a cumulative impact on the most affected reptile species, such as *P. orbiculare* (which is an endangered species in Mexico), *Thamnophis* sp. (which also suffer from direct killing by humans), and *B. imbricata* (which is less abundant in the natural protected area). The recovery of these populations will also be hindered by the fact that the PESM is a small natural protected area surrounded by urbanized land and agricultural fields and disconnected from other natural environments. This situation can potentially affect reptile diversity and the community composition of PESM in the long term.

Stopping prescribed burns as part of the PESM management program is important. However, if we aim to protect these reptile species, it is also necessary to halt the unplanned fires that have been observed to burn a greater proportion of the natural protected area annually.

More systematic surveys that quantify direct fire-induced reptile mortality and reptile densities before and after the fires in the PESM and similar ecosystems in the region may allow for better direct comparisons of mortality rates and the direct impacts of fires on reptile populations. With the objective of lowering the occurrence and extent of anthropogenic fires and preserving the native biodiversity in the PESM, we suggest several actions be considered. 1) Maintaining a well-distributed bike path network in the area, as bike paths can act as firebreaks. 2) Rotating the existing small-scale livestock farming, as it can reduce fuel accumulation while reducing grazing pressure in specific areas of the PESM. 3) Progressively replacing exotic and flammable plantations of *Pinus radiata* and eucalyptus with native vegetation, as the resins of these commonly planted species are highly flammable, increasing the likelihood and rate of wildfire propagation (McWethy et al. 2018). 4) Implementing a collaborative network and awareness campaign to enhance vigilance across multiple communities during the dry season. 5) Restoring biophysical structures in the landscape, such as agaves, which serve as refuges for some reptiles during fires. 6) Conducting active postfire searches for reptiles, pouring some water over them, and relocating survivors to safer areas outside the burned zone.

Acknowledgments

We are grateful to the subject editor, Yurii Kornilev, for his careful and detailed review and to the anonymous reviewers for their comments. A.G.F. is on her postdoctoral stay at the Universidad Nacional Autónoma de México (UNAM: CJIC/CTIC/5052/2021). The following study was conducted with a CEPANAF sampling permit: 221C0101A-0171/2021. We thank Salus Fuentes for helping us with some sampling. A. S: Adahy Olun Contreras-García cuando íbamos al Sierra Morelos a pasear me inspiraste a hacer este estudio. Espero que pronto podamos regresar a pasear, te extraño mucho hijo.

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Supplementary material 1

Historical report of fires and preventive activities in the Sierra Morelos State Park carried out by Probosque

Authors: Andrea González-Fernández, Stephane Couturier, Rodrigo Dotor-Diego, Ricardo Martínez-Díaz-González, Armando Sunny

Data type: pdf

Link: https://doi.org/10.3897/herpetozoa.37.e116376.suppl1

Supplementary material 2

Enlarged pictures shown in Fig. 2

Authors: Andrea González-Fernández, Stephane Couturier, Rodrigo Dotor-Diego, Ricardo Martínez-Díaz-González, Armando Sunny

Data type: pdf

Explanation note: Each picture corresponds to a different carcass found in burned areas in 2021 and 2022.

Link: https://doi.org/10.3897/herpetozoa.37.e116376.suppl2

ÖGH Herpetozoa
Austrian Herpetological Society

Herpetozoa 37: 227–229 (2024)
DOI 10.3897/herpetozoa.37.e122349

First record of *Hemidactylus turcicus* (Linnaeus, 1758) from Piperi Island, Northern Sporades, Greece

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Academic editor: Andreas Maletzky ♦ **Received** 5 March 2024 ♦ **Accepted** 23 April 2024 ♦ **Published** 26 July 2024

Abstract

New records of species distribution help advance our understanding of species biogeographical dynamics and, potentially, local adaptations. Here, we report for the first time the presence of the Mediterranean house gecko (*Hemidactylus turcicus*) on a small, isolated Aegean island from which only one other reptile species has been previously documented. This discovery contributes new distribution data, enriching future assessments of both the species and its habitat.

Key Words

distribution, Gekkonidae, Mediterranean, reptiles

Introduction

The "Mediterranean" or "Turkish" house gecko, *Hemidactylus turcicus* (Linnaeus, 1758), is a nocturnal and insectivorous lizard that frequents habitats with rocks and boulders and even house walls (Valakos et al. 2008). It is a member of the family Gekkonidae and one of the most widespread gecko species around the Mediterranean basin (Rato et al. 2011). Genetic uniformity among most of the Mediterranean populations, in contrast to the high genetic diversity of the species in the Levant area, indicates a recent, rapid, and probably human-mediated dispersal throughout the Mediterranean Basin (Carranza and Arnold 2006). Due to human-induced transportation, the species has been introduced to the New World since 1915, initially in Florida (Fowler 1915), then spreading to North, Central, and South America (Martínez-Hernández et al. 2017; Weterings and Vetter 2018; Ruhe and Laduke 2019). Such human translocations complementing its incredible hitchhiking abilities have led to a steady increase in its geographic range, yet recent distribution models based on climate and elevation data predict an even greater future expansion (Ceia-Hasse et al. 2014).

In Greece, the species ranges across the mainland and on most Aegean and Ionian islands; however, it is notably scarce or absent at higher elevations, possibly due to its intolerance for low temperatures (Pafilis et al. 2020). While numerous records of *H. turcicus* for both the mainland and most of the islands are available (e.g., Cattaneo 1997; Foufopoulos 1997; Carranza and Arnold 2006; Mario 2010; Uhrin and Benda 2018), new reports come to enhance our knowledge on the species range and biology being reported (Itescu et al. 2016; Cattaneo 2018; Sindaco and Rossi 2020). During the course of a herpetological survey on 9 July 2023, in the Northern Sporades (Fig. 1), we visited Piperi (39°20.900'N, 24°19.300'E), a rather small, isolated, and uninhabited island northeast of Alonissos with a surface area of 4,3 km^2, for three consecutive days. The island is an integral part of the National Marine Park of Alonissos and the Northern Sporades. Within Zone A, it is the park's core zone for the protection of the monk seal (*Monachus monachus*), and the island remains off-limits to the public unless a special permit is obtained for research purposes (Karantoni et al. 2023). Ondrias (1968) compiled the first checklist of reptiles and amphibians in Greece, interestingly omitting any mention

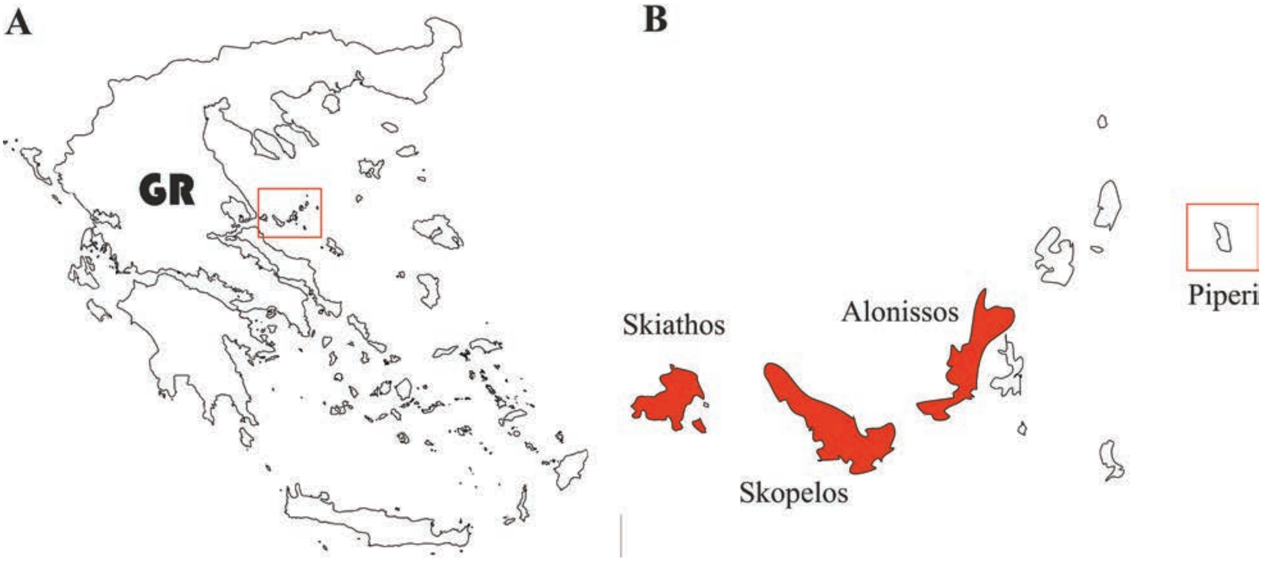

Figure 1. A. Map of Greece (GR) showing the location of the Northern Sporades Island Cluster in the Aegean Archipelago; **B.** The known distribution of the species in the Northern Sporades Islands is shown in red, and on the top right is Piperi Island (the sampling site).

of *H. turcicus* on Piperi Island despite having visited it. Likewise, dedicated surveys carried out by Grillitsch and Tiedemann (1984) or extensive literature reviews by Chondropoulos (1986), Foufopoulos and Ives (1999), and Lymberakis et al. (2018) reported only the endemic saurian subspecies *Podarcis gaigeae weigandii*, without mentioning the presence of any other lizard species.

On 9 July 2023, at approximately 22:15 hours, on a pile of limestone rubble remnants of a collapsed stone wall situated next to the island's abandoned but regularly maintained church (39°20.343'N, 24°19.192'E, 290 m a.s.l.), one adult *H. turcicus* individual was found. Further field work resulted in the observation of two more individuals hidden between stone rubble within a 50-meter radius of the first specimen. More individuals were found within 20 minutes, indicating the presence of a dense population of *H. turcicus* in the area (Fig. 2). We hypothesize that it is the nocturnal nature of the Turkish Gecko that prevented other researchers from observing it during their expeditions that were carried out during the daytime (Hitchcock and McBrayer 2006).

For each specimen, a voucher photograph was taken, and a tissue sample from the first individual was collected and deposited at the Zoological Museum of the Aristotle University of Thessaloniki, Greece (ZMAU30.10.74.1). No other gecko species were detected on the island during our expedition. Overall, this new record expands our knowledge of the species' distribution and the fauna composition of the isolated island of Piperi. Further research is needed to identify the demographic characteristics and phylogenetic affiliations of this population.

Figure 2. Adult *Hemidactylus turcicus* (Linnaeus, 1758), on the church wall in Piperi island, Northern Sporades, Greece.

Funding

This research was funded by the Natural Environment and Climate Change Agency (NECCA) with the Hellenic Foundation for Research and Innovation (H.F.R.I.) under the call "*Actions to protect, conserve, and promote biodiversity. Field studies of endemic, endangered, and nationally important species of Greece*" (Project Number: 012628) as well as the "*2nd Call for H.F.R.I. Research Projects to Support PostDoctoral Researchers*" (Project Number: 00167).

Acknowledgments

The experimental and field work was carried out under the special permits issued by the Greek Ministry of Environment (Α.Π. ΥΠΕΝ/ΔΔΔ/9190/247). We would like to thank Ilias Strachinis for a pre-peer review of the manuscript.

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OGH Herpetozoa
Austrian Herpetological Society

Herpetozoa 37: 231–256 (2024)
DOI 10.3897/herpetozoa.37.e125965

The herpetofauna of the Sporades Islands (Aegean Sea, Greece): New discoveries and a review of a century of research

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Academic editor: Günter Gollmann ♦ **Received** 23 April 2024 ♦ **Accepted** 27 June 2024 ♦ **Published** 31 July 2024

Abstract

The Sporades are one of the most biologically important archipelagos in the Aegean Sea (Greece) and have received priority conservation over the last 50 years. However, despite numerous early efforts, its herpetofauna is only partially described, resulting in many distributional gaps that have prevented adequate understanding and management of the resident species communities. We review one century of bibliography from the Northern Sporades and combine this information with a review of museum specimens and insights from numerous extensive field surveys over the last near-decade to provide for the first time a comprehensive picture of the reptiles and amphibians of the archipelago. We report here on 26 new island records and find that the herptile communities of the region are largely derived from the herpetofauna of the nearby Thessaly mainland, with only a few introduced taxa. There is also a small but significant set of endemic taxa in the archipelago. Island species richness declines with decreasing island size and increasing duration of island isolation. Herptile communities on smaller islands are progressively nested subsets of the communities on larger islands. The presence of reptile species depends sensitively on the condition and management of native ecosystems. While non-aquatic species maintain largely healthy populations, most populations are under pressure from the combined effects of rampant tourist development, the destruction and degradation of rare wetland habitats, and the abandonment of traditional agricultural landscapes. We provide recommendations regarding sustainable management of the local reptile and amphibian populations.

Περίληψη

Οι Βόρειες Σποράδες είναι από βιολογικής άποψης ένα από τα σημαντικότερα νησιωτικά συγκροτήματα του Αιγαίου και έχουν τεθεί υπό ιδιαίτερη νομική προστασία εδώ και σχεδόν μισό αιώνα. Ωστόσο, παρά τις πολυάριθμες προσπάθειες, η ερπετοπανίδα της περιοχής δεν έχει μελετηθεί επαρκώς, με αποτέλεσμα να υπάρχουν πολλά κενά στις κατανομές των ειδών τα οποία εμποδίζουν την ολοκληρωμένη κατανόηση καθώς και τη σωστή διαχείριση των βιοκοινωνιών της περιοχής. Εδώ παρατίθεται μια κριτική ανασκόπηση όλης της υπάρχουσας βιβλιογραφίας ενώ ταυτόχρονα έχει γίνει μια εκτενής εξέταση όλων των σημαντικών μουσειακών συλλογών από την περιοχή. Οι πληροφορίες αυτές, σε συνδυασμό με τα αποτελέσματα σχεδόν μιας δεκαετίας έρευνας πεδίου προσφέρουν, για πρώτη φορά, μία ολοκληρωμένη εικόνα των ερπετών και των αμφιβίων του αρχιπελάγους. Η ερπετοπανίδα των νησιών έχει ουσιαστικά θεσσαλική προέλευση και συμπεριλαμβάνει μόνο λίγα ξενικά είδη. Υπάρχει επίσης ένας μικρός αλλά ση-

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μαντικός αριθμός από ενδημικά τάξα. Ο αριθμός των ειδών της ερπετοπανίδας ενός νησιού ελαττώνεται με τη μείωση του μεγέθους του νησιού, καθώς και με την αύξηση της διάρκειας απομόνωσης του. Συνεπώς τα ανατολικά νησιά (μετά την Κυρά Παναγιά) έχουν λίγα είδη αλλά και σχετικά υψηλότερο ποσοστο ενδημισμού. Ενώ τα περισσοτερα ήχερσαία είδη διατηρούν σε μεγάλο βαθμό υγιείς πληθυσμούς, όλα τα υδρόβια είδη βρίσκονται υπό πίεση λόγω της έντονης υποβάθμισης και καταστροφής των λίγων υγροτόπων της περιοχής. Η διατήρηση παραδοσιακών αγροτικών δραστηριοτήτων σε συνδυασμό με ικανά επίπεδα βόσκησης ευνοεί τα περισσότερα είδη της ερπετοπανίδας στα μεγάλα νησιά των Σποράδων. Σε αντίθεση, σε μικρές νησίδες η υπερβόσκηση έχει αρνητικές επιδράσεις στα τοπικά είδη. Δίνονται συστάσεις ως προς την ορθολογική διαχείριση των διαφόρων ενδιαιτημάτων του αρχιπελάγους.

Key Words

Amphibia, biodiversity, conservation, Greek islands, herpetofauna, island biogeography, reptilia

Λέξεις-κλειδιά

Αμφίβια, βιοποικιλότητα, διατήρηση, ελληνικά νησιά, ερπτικός, νησιωτική βιογεωγραφία, Ερπετά

Introduction

The Northern Sporades (or simply Sporades) are a chain of islands located off the eastern coast of Thessaly, Greece. They constitute one of the major archipelagos of the Aegean Sea, and have long been recognized for their attractive landscapes and their significance as a biodiversity repository (Kamari et al. 1988; Myers et al. 2000; Iliadou et al. 2020; Konaxis 2020). Though the herpetofauna of the islands has been noted for its richness (Lymberakis et al. 2018), it remains understudied, and the islands' reptile and amphibian communities have never been reviewed in a comprehensive fashion.

The herpetofauna of the Sporades is, from a biogeographic perspective, a fairly coherent assemblage, being primarily affiliated with the nearby eastern Greek mainland, though there is also a notable endemic element. We consider here the main chain of the archipelago, which contains over 45 islands and satellite islets, though only Skiathos, Skopelos, and Alonnisos, the three largest islands, are presently inhabited (Fig. 1). To the southeast of the main Sporades chain is the Skyros cluster, which is located on a separate shelf, and has likely been isolated for a longer period (Dermitzakis 1990). Thus we excluded Skyros and its nearby satellite islets from this study as they are not only comparatively well-known, but also biogeographically distinct, and have been traditionally considered separately (Tiedemann and Mayer 1980; Chondropoulos 1986, 1989; Broggi 2006; Pafilis et al. 2013). The only exception is the island of Piperi, which, despite belonging to the Skyros shelf, is considered here together with the rest of the Sporades due to its immediate geographic proximity and shared management regime as part of a marine protected area.

The herpetofauna of the islands has been shaped strongly by human activities. The islands have a long history of sustained human occupation dating back to the Paleolithic period, with evidence of early activities including animal husbandry and agriculture (Sampson 2006). While human population size and attendant landscape impacts have varied over the millennia as the result of conflict and historical vagaries, it is clear that anthropogenic agro-pastoral activities have been ongoing on the Sporades for millennia (Ginalis 2018). However, the effects of human presence on the local habitats and herptiles have been unevenly distributed across the cluster, with occupation on the smaller islands being much more tenuous and with relatively few impacts beyond religious activities, and small ruminant husbandry (Barouda et al. 2023). In the last century, the ecology of the islands, and the herpetofauna, by extension, have been shaped by three distinct but interacting factors. First, a sustained rural exodus, especially from the smaller islands, has led to the abandonment of the agricultural landscape. Second, the proliferation of mass tourism across the Aegean Basin (Kizos et al. 2007) has had profound impacts on natural habitats, particularly on the western islands. Third, the advance of the modern conservation movement has resulted in the establishment of flagship protected areas in the Sporades (Konaxis 2020). Two Natura 2000 sites lie within the archipelago (GR1430009, GR1430004), with the largest one (GR1430004) constituting the National Marine Park of Alonnisos and Northern Sporades (MPA). The authors are in direct communication with the governmental management team of the MPA, the Ministry of Environment, as well as other NGOs that operate in the Sporades. While the MPA has mainly focused on conservation and protection of marine ecosystems, we expect our work will help in the future direction of conservation, restoration, and further research of the terrestrial ecosystems in the MPA and wider archipelago.

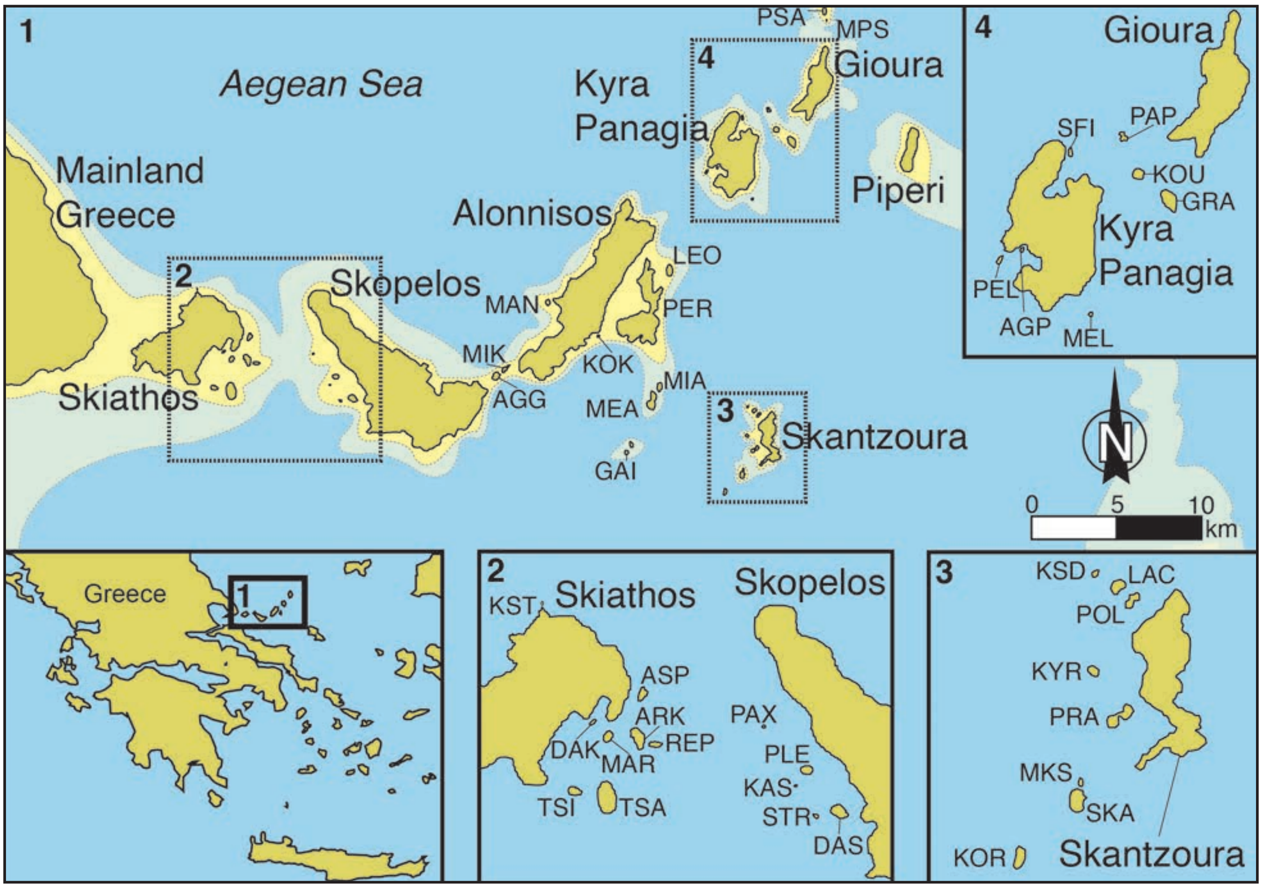

Figure 1. Map of the Sporades study area, including island acronyms. Full faunal information can be found in Tables 1, 2. and additional island information is in Appendix 1: Table A3. Dotted lines surrounding present -day islands indicate 80 m and 120 m isobaths. Light gray/blue indicates land area exposed from approximately 20 kya (at the Last Glacial Maximum) until ca. 14 kya. Light yellow indicates land area that was still exposed at 14 kya but that has become submerged since then (Kalb 2021).

History of herpetological research in the region

The Sporades Islands have had a fairly long history of herpetological field exploration, predominantly by western European scientists. Indeed, up until the very end of the 20th century, herpetological investigations on the islands were almost completely dominated by German-speaking biologists. Commencing in the 1920s and `30s, and through the 1950s, short visits by F. Werner, O. Cyrén, and O.v. Wettstein provided important but limited information on the species communities inhabiting the islands, as well as their basic taxonomic affiliations (Werner 1930, 1938; Cyrén 1935, 1941; Wettstein 1953, 1957a, b, c).

Following these early expeditionary investigations and motivated by the lack of herpetological knowledge in the area, T. Schultze-Westrum and W. Weigand spent extended periods of time on the islands and obtained more fine-grained information on the resident reptiles and amphibians. Their fieldwork, culminating in a substantial series of specimens now residing at the Collection of the Zoological Museum A. Koenig in Bonn, resulted in several foundational publications (Buchholz and Schultze-Westrum 1964; Gruber and Schultze-Westrum 1971; Gruber 1974; Beutler and Gruber 1977; Mayer and Tiedemann 1980) and provided the backbone of herpetological understanding of the Sporades Islands. This basic information was subsequently incorporated into broader-scope review works such as Ondrias (1964), Chondropoulos (1986, 1989), Foufopoulos and Ives (1999), Legakis (2004), Roca et al. (2009), Foufopoulos et al. (2011), etc.

In the last 30 years, however, additional, brief visits by predominantly non-academic herpetologists have resulted in several additional publications, mostly descriptive in nature, that have filled out many knowledge gaps (Bergman 1995; Cattaneo 1997, 1998, 2010; Broggi 2010, 2020; Grano et al. 2013; Passarge 2019; Kalogiannis 2020, 2021). Last but not least, regional-scope laboratory studies utilizing molecular approaches have shed light on the taxonomic affiliations of many of the regional reptile taxa (e.g., Poulakakis et al. 2005; Roussos 2015; Kornilios et al. 2020), though only Poulakakis et al. (2005) explicitly included Sporades samples in the analyses. Consequently, additional molecular studies from the islands are needed, as a fine-grained understanding of the genetic structure and diversity among the reptile populations in the Sporades remains unknown.

This expanding, but relatively disorganized information has created the need for a comprehensive review of the herpetological literature of the archipelago. In this work, we: 1) report on a plethora of new records of species we documented during numerous field trips to the Sporades over the last seven years; 2) review, summarize, and integrate this new information with the existing literature records on the herpetofauna of the region, with the aim of creating a body of information that can be used as a baseline for future studies; 3) provide new field ecology information, as well as conservation and management recommendations for the archipelago's reptile and amphibian communities.

Materials and methods

The Sporades Islands: geology, climate, ecology

The Sporades Islands consist of a diversity of geological substrates, including sedimentary deposits and volcanic rocks. Most of the islands are composed of various forms of limestone, with the exception of Psathoura and Mikropsathoura, which are volcanic in origin (Jacobshagen and Wallbrecher 1984). These fissured and well-draining substrates result in a comparative paucity of surface wetlands with unfavorable effects on amphibians and hydrophilic reptiles. The climate is Mediterranean, characterized by wet winters and warm, dry summers (Iliadou et al. 2020). Though summers in the Sporades can be long and dry, mean annual precipitation is significantly higher and mean annual temperatures are significantly lower than in island groups further south, such as the Cyclades, with important ramifications for the species occurring there (Harris et al. 2020).

From a paleogeographic perspective, most of the western Sporades (west of approximately the 24°E longitude line) are land-bridge islands that were connected to the adjacent Thessalian mainland during the Last Glacial Maximum ~20 kya, when sea levels were about 120 m lower than today (Bintanja et al. 2005). However, even within this continental group, substantial variation exists in the extent of connectivity and insularity throughout the Pliocene and Pleistocene glacial and interglacial periods (Rohling et al. 2014). While Skiathos and its satellite islets have had only a relatively short period of isolation from Thessaly, being connected until 8.5 kya, the rest of the major chain islands (Skopelos, Alonnisos, Mikros Adelphos, and Megalos Adelphos) became separated from the mainland and each other approximately 14–15 kya (Kalb 2021). In contrast, the eastern islands of the main chain (Skantzoura, Kyra Panagia, Gioura, Psathoura, and Piperi) have land bridges submerged below 120 m, and have been isolated for much longer periods of time (> 200 kya) (Kalb 2021).

The natural habitat on the Sporades Islands has been shaped extensively by millennia of human activities. Undisturbed vegetation cover can be relatively dense compared to other, more arid, Aegean islands. The original woodland, likely dominated by *Quercus* sp., was largely reduced and degraded by wood cutting, agricultural activities, and livestock husbandry (Ginalis 2018). As a result, large areas of the main three islands have been converted to a matrix of terraced agricultural slopes, olive groves, and seasonal pasturelands, creating a heterogeneous landscape that is conducive to many typical lowland Mediterranean reptiles and amphibians (Papanastasis et al. 2009; Zakak et al. 2015; Băncilă et al. 2023). Today, in the western Sporades and especially on Skopelos and Skiathos, large areas are also dominated by pine forest (*Pinus* sp.), perhaps due to past forest fires and the abandonment of agricultural areas. In contrast, while vegetation in the eastern Sporades tends to be lower, any relict forest patches consist of Kermes Oaks (*Quercus coccifera*) and Holm Oaks (*Q. ilex*), as pines are absent (with the exception of Piperi). Medium-sized islands support at most tall maquis (dominated by *Juniperus turbinata* on the Skantzoura cluster, *Q. coccifera* everywhere else), while smaller islets, where soils are shallower, support generally only coastal heaths (non-spinose phrygana).

Field visits

We surveyed the herpetofauna of the islands during multiple field expeditions in the period between 2016 and 2024. In addition, one of the authors (SK) lives on the islands and has been conducting impromptu herpetological searches for several years. We used both diurnal and nocturnal surveys, covering all major vegetation types, as well as a variety of distinct microhabitats across the whole archipelago. Whenever possible, we document new records with specimens or photographs, which we have deposited in official museum collections. Beyond ecological and presence data, we also collected information on local reptile names (see Appendix 1: Table A2). All islands of the archipelago were visited, often numerous times, with only 5 islets not visited to date (Gaidaronisi, Manolas, Mikroskandili, Lachanou, and Kassidis). All visited islands harbored at least one species of reptile, with the exception of Kastronisia, a small islet near Skiathos where no reptiles were detected. See Appendix 1: Table A3 for a list of both official and alternative island names.

Results

Island diversity, endemism, and abundance

We summarize our findings as well as past findings on species occurrences in Tables 1, 2. Species richness decreases across the archipelago (Fig. 2), from the youngest to the oldest islands with regards to insularity, and from the largest to the smallest islands in terms of area. Since the archipelago is a chain of islands, and submerged land bridges get deeper towards the more terminal end of the chain in the east, decreasing richness generally follows a west-to-east geographic pattern, as the oldest islands to the east have probably experienced more species extinctions over their longer period of isolation. Subspecific taxa of lizards (e.g., *P. erhardii ruthveni*, *M. kotschyi fuchsi*) have been described from this archipelago based on morphology, and these taxa are more frequently represented on the smallest Sporades islands, resulting in higher percent endemism on the smallest islets (Fig. 3).

Squamates are the most speciose group in the Sporades, with eight lizard and eight snake species. The most widespread lizard species are Kotschyi's gecko (*M. kotschyi* – 30 islands) and the Aegean wall lizard (*P. erhardii* – 40 islands). Lizards with the most limited distributions in the Sporades are the Skyros wall lizard (*P. gaigeae* – 1 island) and the European glass lizard (*P. apodus* – 2 islands). The most widely spread snake species are the Caspian whipsnake (*D. caspius* – 5 islands), the nose-horned viper (*V. ammodytes* – 5 islands), and the four-lined rat snake (*E. quatuorlineata* – 5 islands). The snake species with the most limited distributions in the Sporades are the cat-eyed snake (*T. fallax* – recorded only from Alonnisos, Skopelos, and Peristera), the Balkan whipsnake (*H. gemonensis* – only recorded from Tsougria and Aspronisi), the Eastern Montpellier snake (*M. insignitus* – recorded from Skiathos and Skopelos), and Dahl's

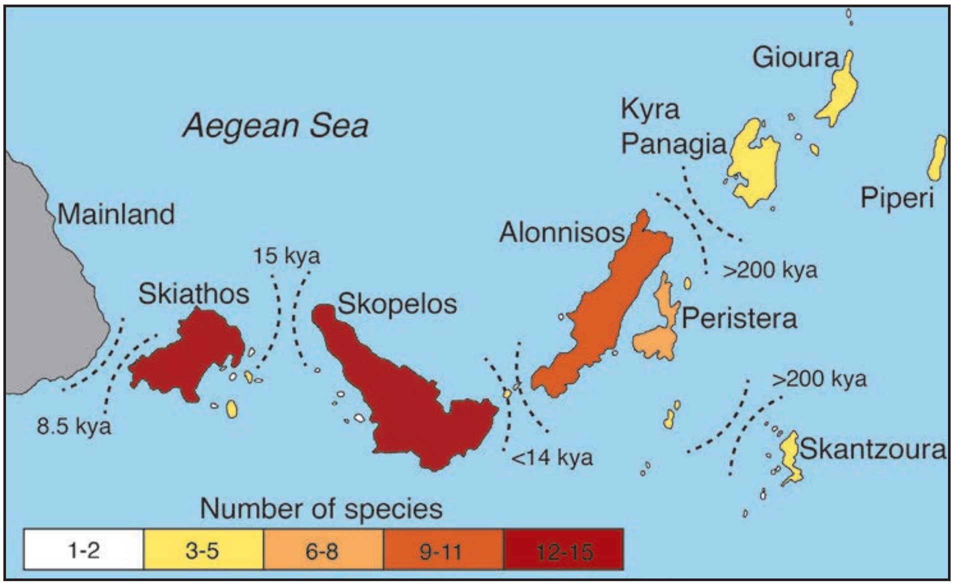

Figure 2. Species richness in the Sporades. Color represents the number of species found on an island. The number of species per island decreases with island size. Species richness also declines from west to east as the duration of island isolation increases.

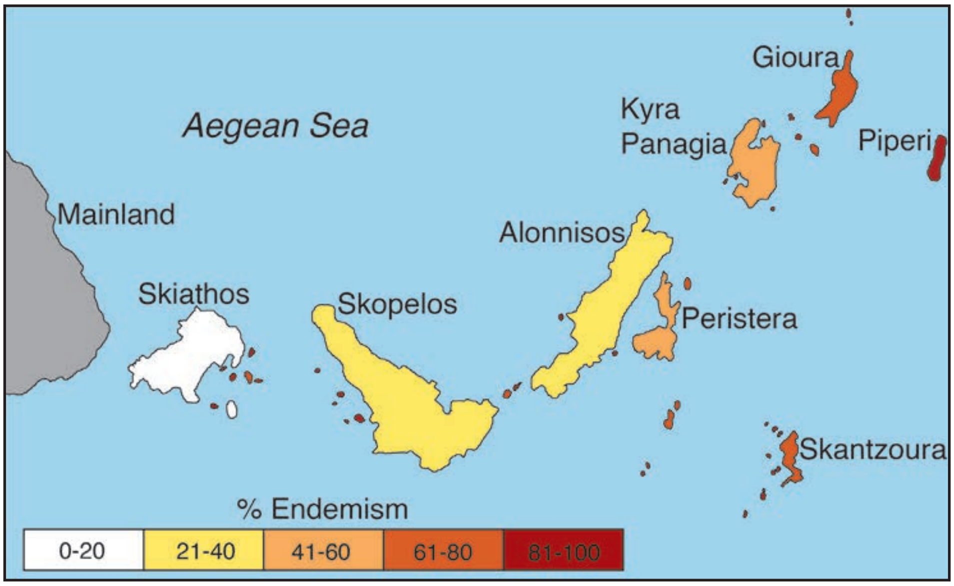

Figure 3. Levels of island endemism of the herpetofauna in the Sporades, expressed as the number of endemic taxa relative to the total number of species on an island (endemic is defined here as any island lineage described as discrete in the taxonomic literature at either the species or the subspecies level, e.g., *P. gaigeae*, *P. erhardii ruthveni*, *M. kotschyi fuchsi*). Older and more isolated islands (on the eastern end of the Sporades chain) have a stronger endemic element. Smaller islands also have high endemism scores, likely because relatively hostile conditions allow only for the persistence of old, locally-evolved taxa.

whipsnake (*P. najadum* – only recorded from Skiathos). Overall, there appear to be some checkerboard distributions of snakes across the Sporades suggestive of competitive intraspecific relationships. Amphibians and turtles are the least common groups of herpetofauna in the Sporades and are most at risk of local extinction due to habitat loss across the archipelago. Amphibians occur only on the young, westernmost large islands of Skiathos and Skopelos, where they are relatively abundant near surface waters. Though anapsids occur on the four largest islands, they are the rarest group of herpetofauna in the Sporades, and terrapins are declining as crucial wetland habitat disappears.

Species accounts

Amphibians

Amphibians have a marginal presence on the islands, with only three species from three families (Bufonidae, Hylidae, and Ranidae) historically recorded from the three largest Sporades (Skiathos, Skopelos, and Alonnisos). However, all amphibians are now likely extinct from Alonnisos, and their future on Skiathos and Skopelos is uncertain. Despite years of searching, we have no new records to report for amphibians.

***Bufotes viridis*, green toad (Laurenti, 1768)**

The green toad is known only from Skopelos, where it was first reported by Werner (1930) and subsequently Cattaneo (1998). We made several observations of this species in the eastern part of Skopelos, often somewhat close to inhabited places (e.g., near the main town and around the meadows of Staphylos), where there is standing or flowing water in the form of wells, flooded fields, and canals. Adults were very active on warm, humid nights during the breeding period. The largest count of individuals was made on 25th March 2022, at 19:40 h, with an air temperature of 16 °C, when about 100 calling males were observed in a flooded field near Staphylos. During a visit to the same spot in April 2023, the fields were completely dry, and only a few calls were heard in the distance. Roadkilled individuals were also frequent on the main road crossing the agricultural plain of Staphylos, south of Skopelos town, and a few adults were found under stones in the same area. Despite the seemingly healthy population on Skopelos, toads are largely dependent on the few suitable breeding spots scattered in a handful of degraded locations that are heavily influenced by human activity and are therefore under the threat of decline.

***Hyla arborea*, European tree-frog (Linnaeus, 1758)**

We confirm the presence of the European tree-frog, *Hyla arborea*, on Skiathos. Until now, this species was only reported by Cattaneo (1997), who discovered a single roadkilled individual. We found the European tree-frog to be common on Skiathos, where it is widespread across the island and mainly inhabits humid, lowland habitats such as slow-flowing streams and their estuaries, flooded fields, vernal pools, and marshes. Particularly dense populations also breed in man-made structures such as abandoned swimming pools, fountains, and canals around the airport area and in the settlement of Troulos. We heard large choruses in Megalos Aselinos and Vromolimnos during the breeding season, especially after rainfalls when males occupied and called from any form of stagnant water. Reproduction takes place between February and April. It seems that the presence of several swimming pools in inhabited areas has contributed to the large population of this species on Skiathos, since tree-frogs possess adhesive toe pads and can easily climb smooth, vertical human-made walls. Despite extensive searching, we cannot confirm a doubtful report with no photo or specimen of *H. arborea* from Alonnisos (Broggi 2010).

***Pelophylax kurtmuelleri*, Balkan marsh frog (Gayda, 1940)**

We confirm the presence of the Balkan marsh frog on Skopelos and Skiathos, where it has also been recorded in the past (Cattaneo 1997, 1998). The marsh frog tends to have a patchy distribution, but can be abundant in suitable habitats. On Skopelos, some individuals were observed in a small seasonal pond located in the Ditropo area and in the coastal marsh of Milia. In Milia, we heard large choruses during spring, and good numbers of the species seem to exist. In the Ditropo pond, which is mostly dry during the warmer months, we observed young froglets during the summer of 2021, indicating successful reproduction. Furthermore, a single adult was seen in a small artificial ditch at the water dam near Panormos. Marsh frogs are more widespread on Skiathos, where we have found them in good numbers in the marsh of Vromolimnos and in the streams in Troulos, Megalos Aselinos, and Lechouni. The stream of Lechouni hosted the largest population recorded so far, with hundreds of individuals of various life stages seen in a single visit. Marsh frogs also used to occur in good numbers on Alonnisos, at least up until the 1980's, but have died out since the late 2000's, most likely due to the loss of breeding habitat. This is evident in several reports by locals who say that frogs were widespread across numerous freshwater springs on the island (Grillitsch and Tiedemann 1984; Broggi 2010). In recent times, the intense exploitation of flowing water used for irrigation purposes on Alonnisos has dried out most of the island's streams. Additionally, the traditional wells and ditches used for agriculture have been replaced by modern ground pumps that can pull significantly more water (Broggi 2010). We detected no evidence of amphibians surviving on Alonnisos despite systematic surveying over several years.

Lizards

Lizards are the most abundant group of herpetofauna in the Sporades. To date, seven lizard species spanning four families have been confirmed in the Sporades:

Ablepharus kitaibelii (Scincidae), *Pseudopus apodus* (Anguidae), *Hemidactylus turcicus*, and *Mediodactylus kotschyi fuchsi* (Gekkonidae), as well as *Lacerta trilineata*, *Podarcis erhardii ruthveni*, and *Podarcis gaigeae* (Lacertidae). We report here on 16 new island records for *M. kotschyi* (12), *H. turcicus* (3), and *P. apodus* (1). Additionally, we report on one ambiguous museum record for *Chalcides ocellatus*.

***Ablepharus kitaibelii*, snake-eyed skink (Bibron and Bory, 1833)**

The snake-eyed skink, *A. kitaibelii*, has a wide distribution across most of the Aegean Sea islands (Chondropoulos 1986). In the Sporades, this species has only been observed on the three larger islands of Skiathos, Skopelos, and Alonnisos (Cyren 1935; Cattaneo 1997; Broggi 2010), and we confirm the presence of *A. kitaibelii* on these islands. During our expeditions, we often found this small, delicate species on the ground in leaf litter, maquis, olive groves, meadows, and especially pine woodlands. We usually encounter *A. kitaibelii* during early morning hours, with overcast conditions, and during the winter, given that its habitat is apt to heat up rapidly during the warmer months. Due to its small size and secrecy, *A. kitaibelii* may seem rare but is actually quite common on Skiathos, Skopelos, and Alonnisos.

***Chalcides ocellatus*, ocellated skink (Forskål, 1775)**

We report here on an earlier, unpublished record of *C. ocellatus* from Skopelos. An adult specimen from the island was deposited in the Natural History Museum of Vienna by a collector (O. Reiser in the 1890s, Voucher Natural History Museum of Vienna 19419/1). However, the species was not mentioned in the travel account, and Reiser's Sporades visit was bookended by visits to other areas of Greece harboring *C. ocellatus*, so doubt exists as to the validity of the record (Reiser 1905). Much of the ocellated skink's contemporary range is the result of historical introductions, generating a discontinuous and patchy distribution (Speybroeck et al. 2016). This species has been found on other Aegean islands, but was likely transported there by humans and successfully established. Despite searching, we have never encountered *C. ocellatus* in the Sporades. Assuming that Reiser's record is not erroneous, and given the long gap since it was observed and the lack of any other specimens or recorded observations of this species in this region, we expect that *C. ocellatus* is not a native species and does not currently occur in the Sporades.

Specimen: Skopelos (NHMW 19419/1).

***Pseudopus apodus*, European glass lizard (Pallas, 1775)**

The only species from the slow -worm family Anguidae found in the Sporades is the European glass lizard. It was first reported by Bergman (1995) on the island of Skiathos, and we report here on a new record of the species from a photograph taken by a citizen on Skopelos (July 2009; Theodora Tsimpo). We can also confirm *P. apodus*' presence on Skiathos, where we observed a few individuals of the species on slopes with dense shrubs in the eastern part of the island. *Pseudopus apodus* is usually found in dense brambles and in grassy areas with some canopy cover on Skiathos (Cattaneo 1997), though this taxon is known to also occupy fairly dry, stony habitats if some vegetation cover is available (Speybroeck et al. 2016). Due to its unique morphology as a legless lizard, this species is difficult to confuse with other herpetofauna in this region.

Photographic voucher: Skopelos (NHMC 80.3.20.52).

***Hemidactylus turcicus*, Mediterranean house gecko (Linnaeus, 1758)**

We report three new island records of the Mediterranean house gecko from Kyra Panagia, Peristera (Fig. 4H), and Arkos. In addition, the species was also recently documented on Piperi (Daftsios et al. 2024). Previously, this species was only known from five islands in the Sporades: the three main islands of Skiathos (Cattaneo 1997), Skopelos (Gruber 1974), and Alonnisos (Grillitsch and Tiedemann 1984), and the small recently uninhabited islands of Tsougria (Grano et al. 2013) off the southeast coast of Skiathos, as well as Pappous near Kyra Panagia (Gruber 1974). We found *H. turcicus* in a variety of warm, rocky habitats, hiding under rocks during the day. It is especially common near areas of human activity and settlements such as buildings, agricultural areas, dry stone walls, piles of rubbish, and ruins. The Mediterranean house gecko originated in the Near East and is considered to have been introduced widely across the warmer areas of the Mediterranean Basin (Moravec et al. 2011). Its distribution in the Sporades maps closely to human habitation, and even among the smaller islets, it occurs only on those with regular human presence. Pappous Islet, for example, was a skete, and Tsougria was settled until the mid -1900s. Even our new record from Arkos is based on an individual found near building materials brought to the island for the construction of a beach bar.

Photographic voucher: Kyra Panagia (NHMC 80.3.87.303), Peristera (NHMC 80.3.87.304).

***Mediodactylus kotschyi*, Kotschy's gecko (Boettger, 1888)**

We report here 12 new island records for Kotschy's gecko, *M. kotschyi*, from the islets of Tsougriaki, Arkos, Daskalonisi, Paximadi, Agios Georgios, Mikronisi, Polemika, Kyrgiagos, Prasso, Skandili, Korakas, Pelerissa, and Piperi (Table 2). Although reliable records of this species are still missing from some islands, this is probably the vertebrate with the widest distribution in the archipelago, being able to survive on small islets with scarcely any vegetation that is otherwise reptile-free (e.g., Daskalonisi by Skiathos or Kassidis and Paximadi by Skopelos). Interestingly, *M. kotschyi* has not yet been

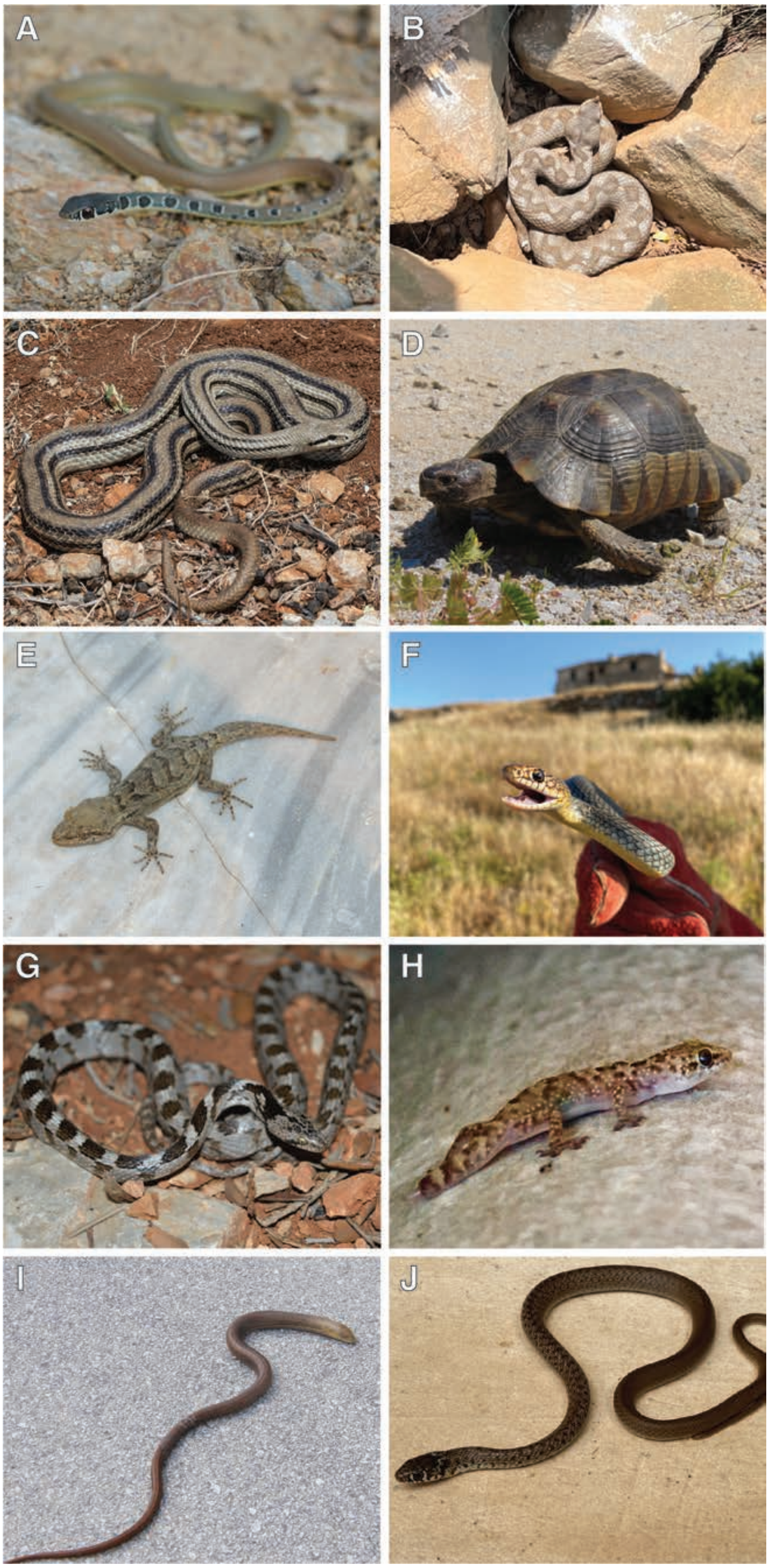

Figure 4. Images of species for which we present new records or newly confirmed occurrences. **A.** *Platyceps najadum* from Skiathos; **B.** *Vipera ammodytes* from Peristera; **C.** *Elaphe quatuorlineata* from Gioura; **D.** *Testudo marginata* from Skopelos; **E.** *Mediodactylus kotschyi* from Skandili; **F.** *Dolichophis caspius* from Skantzoura; **G.** *Telescopus fallax* from Alonnisos; **H.** *Hemidactylus turcicus* from Peristera; **I.** *Pseudopus apodus* from Skopelos; **J.** *Hierophis gemonensis* from Tsougria.

found on Skiathos itself, perhaps because of the dense vegetation and plethora of water, or some other ecological or historical factor. This species has an irregular distribution across the Aegean and is relatively rare on the mainland, and Skiathos is more mainland-like than the rest of the Sporades.

Kotschy's gecko is a small Palearctic "naked-toed" gecko (Beutler 1981). It is easily recognized by its gray coloration, dark chevron dorsal patterning, and thin, kinked toes that lack adhesive toe pads (see Fig. 4E). The populations of most Sporades islands have been taxonomically assigned to *M. kotschyi fuchsi*, which is morphologically distinct from the mainland *M. k. bibroni*. This taxon can be distinguished by the general lack of preanal pores (an average of 0.6 in *M. k. fuchsi* versus 3.8 in *M. k. bibroni*); the larger number of ventral scale rows (an average of 35.8 in *M. k. fuchsi* versus 24 in *M. k. bibroni*); and the larger number of separate tubercles on the hind thigh (an average of 6 in *M. k. fuchsi* versus 3.4 in *M. k. bibroni*) (see Beutler and Gruber 1977; Beutler 1981 for further morphological details). *Mediodactylus k. fuchsi* is an island endemic that is restricted to the eastern Sporades islands up to, and including, the Alonnisos cluster. The remaining populations, Skopelos and westward, are considered by Beutler and Gruber (1977) to be transitional to the mainland *M. k. bibroni*. Beyond these lineages, the populations from the extralimital Skyros archipelago have been assigned to *M. k. schultzewestrumi* (Beutler and Gruber 1977). The taxonomic affiliation of the intermediate Piperi population remains to be determined. While these subspecies have been described based primarily on morphology, it is not clear whether they will hold up in a more holistic species delimitation framework that incorporates molecular data. Prior studies that have inferred evolutionary relationships with one or a few genes have synonymized many subspecies in the Aegean islands to the species *M. kotschyi* from the European continental shelf (Kasapidis et al. 2005; Kotsakiozi et al. 2018).

While Kotschy's gecko is primarily crepuscular, we commonly encountered it basking on rocks, stone walls, or trees in the first hours of the morning sun and last hours of daylight, even during the hot summer months. This species favors dry, rocky habitats and can reach high densities on dry stone walls, though we also observed *M. kotschyi* on trees and wooden telephone poles. On smaller, uninhabited, predator-free islets, it is more typically encountered low to the ground under rocks or debris. Our observations suggest that the density of this species is inversely proportional to the size of the island, achieving the highest densities on small rocky islets. This may be both the result of island competitive release, as well as susceptibility to predation on larger islands (Itescu et al. 2017).

Specimens: Tsougriaki (NHMC 80.3.85.1970), Mikros Adelphos (NHMC 80.3.85.1971-1972), Skantzoura (NHMC 80.3.85.1973-1975), Prasso (NHMC 80.3.85.1976-1979), Kyra Panagia (NHMC 80.3.85.1980-1981), Gioura (NHMC 80.3.85.1982-1984), Grammeza (NHMC 80.3.85.1985)

Photographic voucher: Skandili (NHMC 80.3.85.2012), Korakas (NHMC 80.3.85.2011).

***Lacerta trilineata*, Balkan green lizard (Bedriaga, 1886)**

The Balkan green lizard occurs on the three largest and westernmost islands of the Sporades, as well as on two uninhabited Skiathos satellite islets: Tsougria and Arkos. In 1938, Werner claimed that he observed both *Lacerta trilineata* and *L. viridis* on Skiathos (Werner 1938). However, no observations of *L. viridis* have been reported since, and at present it is considered an erroneous observation. We have no new records of *Lacerta* to report from our expeditions, and we confirm the presence of *L. trilineata* on Skiathos, Skopelos, Alonnisos, Tsougria, and Arkos.

Adult *L. trilineata* from the Sporades has a vivid, bright green coloration that can be speckled with black or bright blue scales. Individuals from Skiathos are noticeably smaller and seem to have a slenderer head than adults from the mainland, Skopelos, and Alonnisos. The typical throat color we observed on the islands is bright yellow, but in some males, a small area of the throat is light blue. Additionally, adult *L. trilineata* have dramatically different coloration than juveniles, the latter of which have light grey/brown dorsal coloration often with three bright pale-yellow lines running in parallel down the dorsum, though about 4/5 of juveniles in the Sporades have the uniform morph, as based on 100s of field observations by us and noted by others (Cattaneo 1997). Adults can be difficult to find, as they generally escape quickly into dense vegetation when approached by humans. Typically, we found this species basking or climbing high up on shrubs, reeds, and stonewalls. *Lacerta trilineata* is exceptionally common on Skiathos, where it is the most abundant lizard species and is found in virtually all habitat types. Fewer observations were made on Skopelos and Alonnisos, mostly from agricultural areas and olive groves.

***Podarcis erhardii*, Aegean wall lizard** (Bedriaga, 1882)

The Aegean wall lizard is, together with Kotschy's gecko, the most abundant reptile in the Sporades (Kalogiannis 2020) and occurs on at least 40 islands in the region. We have no new records to report for *P. erhardii*, despite searching islands like Tsougria and Kastronisia, where we would expect to find wall lizards despite the previous lack of records. The only islands where *P. erhardii* has not been observed, and is presumed absent, are Tsougria, the Kastronisia islets, Daskalonisi, Paximadi, Kassidis, and Piperi. Furthermore, we were not able to confirm the species on Skiathos, despite a past record by Bergman (1995), likely of a marginal population of introduced individuals. Notably, this species is absent from Piperi, where it is replaced by an endemic subspecies of the Skyros wall lizard, *P. gaigeae weigandi* (Gruber and Schultze-Westrum 1971). Though many subspecies of *P. erhardii* have been described from the Aegean Sea, only *P. e. ruthveni* (Werner 1930) is presently recognized in the Sporades (see Gruber 1986 for discussion). *Podarcis e. ruthveni* can be distinguished from mainland *P. e. riveti* based on dorsal scalation, as *P. e. ruthveni* has more dorsal scales than *P. e. riveti*. In the Sporades, *P. erhardii* is phenotypically variable, both within and between islands (Cyren 1941).

Few studies have quantitatively assessed morphological variability between island populations of wall lizards in the Sporades, though we confirm here some patterns that have been noted by others prior (Cyrén 1941; Gruber and Schultze-Westrum 1971). Morphologically, wall lizards from Skopelos were relatively small and had the longest legs, while lizards from the easternmost islands were larger and had shorter, more stout legs. This species also varies considerably in dorsal coloration from island to island. Lizards from the western islands tend to be brown, while lizards from the eastern islands are dusky gray-green (Fig. 5). Thus, with progressive distance from the mainland, lizards get larger, darker, more stout, and have fewer scales, especially east of the 24°E longitude line, marking the "old" islands located off the continental shelf. Cyrén (1941) also noted a similar pattern and found a morphological distinction between the older island populations east of the line and the western populations encapsulated under the presently defunct subspecies *psathurensis* vrs. *skopelensis*. Gruber and Schultze Westrum (1971) note that *P. erhardii* has on-average larger body sizes on smaller rather than on larger islands, presumably because of marine subsidies (Stadler et al. 2023). Only the very smallest rock islets without substantial seabirds are exceptions to this rule (e.g., Mikroskandli). Colorwise, we noticed a tendency for *P. e. ruthveni* to follow the general substrate pattern, with lighter -colored animals on limestone and relatively darker ones on dark, volcanic substrate, like on the easternmost islands of Psathoura and Mikropsathoura. We observed larger blue and aquamarine dorsolateral color patches on small island lizards. The venter of individuals is typically one-colored throughout the archipelago, ranging from dirty white to yellowish, orange, and reddish on Kokkinonisi (a small island across from the Kokkinokastro peninsula of Alonnisos). This lizard species has polymorphic throat coloration, and individuals can be either orange, yellow, white, or a mosaic combination of two of these colors (Brock et al. 2020). Based on our observations, white -throated morphs are by far the most common on every island in the Sporades. With the exception of Alonnisos, we only found orange, white, and mosaic orange-white individuals during our expeditions and note that these are the only throat color morphs currently documented in georeferenced photographs on iNaturalist (www.inaturalist.org), suggesting that the yellow throated morph is largely absent from this region.

Like other species of *Podarcis, P. e. ruthveni* is an ecological generalist and can occur in almost any habitat in the Sporades, including agricultural and urban areas. As its common name suggests, we frequently find this lizard in high densities on dry stone walls characteristic of the region, and it can thrive around humans unless feral cats are present (Krawczyk et al. 2019).

Schultze-Westrum and others note that all islands in this region that sustain the shrub *Pistacia lentiscus* are inhabited by *P. erhardii* (Buchholz and Schultze-Westrum 1964; Gruber and Schultze-Westrum 1971), and even refer to it as a ‘true bush lizard’ (‘richtiggehende Buscheidechse’). We agree that it achieves the highest densities in relatively open habitat with *P. lentiscus*, which provides cover without completely shading out the ground as *Pinus* forest does. Distributional data also suggest that in the absence of anthropogenic refugia, the species can be susceptible to the presence of diurnal snake predators. For example, *P. erhardii* is absent from Tsougria while occurring on neighboring Tsougriaki (aka Mikrotsougria), perhaps because of the presence of *H. gemonensis*, which is an effective *Podarcis* predator (Speybroeck et al. 2021). This effect may be exacerbated by competition by the syntopic *Lacerta*, which, by virtue of its size, is not as susceptible to *H. gemonensis* predation.

On small, uninhabited islands, we usually find this species basking on rocks or under larger, evergreen shrubs and other vegetation during the heat of the day. Notably, this species was extremely common on every island it was found on, even on the smallest uninhabited islands with very few resources. As small-bodied secondary consumers, these lizards play an important ecological role as they eat and are eaten by a variety of animals. On the Sporades, the species harbors relatively simple generalist parasitic helminth communities, the complexity of which declines with island area (Roca et al. 2009). Though wall lizards are primarily insectivorous, they can thrive on tiny islands due to their flexible diets that include plant material, large venomous arthropods, and other conspecifics (Brock et al. 2014; Madden and Brock 2018; Patharkar et al. 2022). On some islands like Strongyllo (or Kyriagos [near Skantzoura]), these lizards are associated with *Falco eleonorae* nests and appear to feed on falcon prey leftovers (Schultze-Westrum 1961). Lizards from small islands appear to be more inquisitive, have shorter flight initiation distances, and appear also to be slower runners than on the larger islands, similar to *P. erhardii* in the Cyclades (Brock et al. 2015; Semegen 2018). Additionally, a recent study found that average clutch size varies considerably between island populations proportionally to the number of predators, ranging from a minimum of 1.6 eggs on Mikropsathoura to a maximum of 2.6 eggs on Skopelos (Foufopoulos et al. 2023).

Specimens: Tsougriaki (NHMC 80.3.51.2981-2982), Agios Petros (NHMC 80.3.51.2983-2984), Grammeza (NHMC 80.3.51.2985-2986), Aspronisi (NHMC 80.3.51.2987-2988), Mikros Adelphos (NHMC 80.3.51.2989), Kyra Panagia (NHMC 80.3.51.2990-2991), Gioura (NHMC 80.3.51.2992-2993), Skatzoura (NHMC 80.3.51.3037).

Figure 5. P*odarcis erhardii ruthveni* from Aspronisi (L) and from Gioura (R). Although considerable variation exists within island populations, these individuals typify the differences between the animals from the large, younger western islands on the main Sporades chain versus those from the older, eastern islands, which tend to have shorter legs and toes, bigger torsos, and a more dusky, gray-green coloration.

***Podarcis gaigeae*, Skyros wall lizard** (Werner, 1930)

The Skyros wall lizard is a narrow-range endemic species with a distribution centered on the Skyros island cluster, southeast of the focal area. In our study area, the species occurs only on the island of Piperi, which is inhabited by the endemic subspecies *P. g. weigandi* (see Gruber and Schultze-Westrum 1971). The individuals we observed on Piperi were normally sized for a *Podarcis* and did not exhibit gigantism of *P. gaigeae* populations from some Skyros satellite islets (Pafilis et al. 2009; Runemark et al. 2015). Thus, individuals from Piperi were similar in size to *P. e. ruthveni*, but had generally darker body coloration, often bright green backs, and dark dorsal reticulation. Gruber and Schultze-Westrum (1971) noted five criteria that distinguish *P. gaigeae* from *P. erhardii* in the Sporades: 1. Coloration: *P. gaigeae* has a strong green component to their dorsal coloration compared to *P. e. ruthveni*, which is more grey-brown to olive-brown. 2. Patterning: *P. gaigeae* has dark occipital spots, whereas *P. e. ruthveni* lacks occipital spots. 3. Femoral pores: *P. gaigeae* has more femoral pores, on average, compared to *P. e. ruthveni*. 4. Scalation: In *P. gaigeae*, the scutum masseteric abuts directly on the scuta supratemporalia more often than in *P. e. ruthveni*, and 5. Morphology: *P. gaigeae* has a relatively larger masseteric diameter than *P. e. ruthveni*.

Similar to other *Podarcis* species, *P. gaigeae* is a generalist that can be found in almost every habitat type. On Piperi, this species was most abundant in open areas near the settlement and at forest margins near the rocky coast, and less abundant in closed forested areas.

Snakes

The Sporades harbor eight snake species from three families: *Dolichophis caspius*, *Elaphe quatuorlineata*, *Hierophis gemonensis*, *Platyceps najadum*, *Telescopus fallax*, and *Zamenis situla* (all Colubridae), *Malpolon insignitus* (Psammophiidae), and *Vipera ammodytes* (Viperidae). We report here on nine new island records for: *D. caspius* (2), *E. quatuorlineata* (2), *T. fallax* (2), *V. ammodytes* (1), *Z. situla* (1), and *H. gemonensis* (1). We also observed a non-viperid snake species on the islet of Agios Georgios (between Skopelos and Alonnisos), but the species could not be identified.

***Dolichophis caspius*, Caspian whipsnake (Gmelin, 1789)**

We recorded the species for the first time on the island of Skatzoura (Fig. 4F) and the nearby islet of Prasso, which likely confirms the unspecified snake observation by Buchholz and Schultze-Westrum (1964) from there. Four large individuals were found on Skatzoura near stonewalls close to abandoned buildings, and an additional subadult was seen moving along the ground in the same area. We observed traces of rodents in and around these buildings, suggesting a good prey resource for this species. On the islet of Prasso, we found a large *D. caspius* basking in the sun, and later we collected a shed skin of the same species from elsewhere on the island. This species tends to be the only diurnal large-bodied colubrid on the islands where it is present. The Caspian whipsnake was previously recorded on the small islet of Lechousa, north of Peristera (Buchholz and Schultze-Westrum 1964), then on Alonnisos (Cattaneo 1998, 2010), and then Peristera itself (Kalogiannis 2021). The species has not been reported from Skiathos, Skopelos, Kyra Panagia, or Gioura, where *E. quatuorlineata* and/or another large colubrid species exist.

On the Sporades, the Caspian whipsnake reaches up to 160 cm in total length, thus approaching the largest lengths for its species known across Greece (the length-record being 208 cm from Samos; Cattaneo 2003). The species is common in the archipelago and is encountered in a diversity of habitats ranging from agricultural land (wheat fields, olive groves, and vineyards) and areas near human habitation, to pine and juniper forests, as well as maquis and phrygana vegetation. Notably, it even occurs on relatively small islands like Lechousa and Prasso, where extensive exposed bedrock limits the amount of evergreen woody vegetation. The species feeds primarily on rodents but is opportunistic and will pursue birds, invertebrates, and reptiles, including other snakes (Speybroeck et al. 2016; Plettenberg-Laing and Mee 2020). On Alonnisos, we found individuals preying on lizards (e.g., *A. kitaibelii*, *L. trilineata*), snakes (*V. ammodytes* and other *D. caspius*), and large rodents, while Cattaneo (1998) also reports finding rabbit remains in ingesta content. Thus, it is a significant, generalist predator of a broad range of species, including its own, and a strong competitor with other snake species. Melanism is prominent among the populations of Alonnisos and Peristera, with several melanistic individuals encountered by the authors and others (Cattaneo 1998; Broggi 2010; Kalogiannis 2021).

Specimens: Skantzoura (NHMC 80.3.117.58).

Photographic voucher: Prasso (NHMC 80.3.117.70).

Elaphe quatuorlineata, four-lined rat snake (Lacépède, 1789)

We report here on two newly documented populations of this species on the island of Gioura (Fig. 4C) and the nearby islet of Grammeza. One adult male (SVL 126 cm; total length 155 cm) and a sub-adult male (SVL 71 cm; total length 89 cm) were found on Gioura, and another large adult male (SVL approximately 115 cm with a damaged tail) was found on Grammeza. In addition, we also report here an earlier, unpublished discovery of a subadult photographed near the Bay of Agios Petros on Kyra Panagia (Genevieve Leaper 2020 on iNaturalist).

Early publications had previously reported this species only from Skiathos (Buchholz and Schultze-Westrum 1964; Cattaneo 1997) and Skopelos (Buchholz and Schultze-Westrum 1964; Cattaneo 1998). We confirm the presence of the four-lined snake on Skiathos, where we found four juveniles and one sub-adult. We encountered *E. quatuorlineata* in a variety of habitats on Skiathos, such as olive groves, riparian forests, shrubland, and often near settlements, where it seems to be common. On the eastern islands, we found this species in rocky habitats with phrygana and maquis. This is the largest rat snake in Greece and can reach total lengths up to 200 cm on the mainland, though island populations usually tend to be smaller. On the Cyclades, the species reaches sexual maturity at substantially shorter total lengths (60–80 cm) (Speybroeck et al. 2016). For the Sporades, Cattaneo (1997, 1998) reported specimens with total lengths up to 180 cm on Skiathos and Skopelos. The four-lined snake primarily feeds on rodents and birds, though juveniles feed on lizards as well. On Grammeza, large adults likely also prey on rabbits that have been released and are now numerous on the islet. This was evident in the single adult we found, who exhibited several healed injuries throughout its body, a phenomenon usually attributed to theriophagy (Cattaneo 1998). All adult individuals from the Sporades exhibited the typical phenotype with four dark dorsal lines on a light, beige-yellow background (Fig. 4C). Juveniles from Skiathos showed the typical phenotype for their developmental stage, with dark blotches on a light background, similar to mainland populations, and a pink-red ventral area.

Photographic vouchers: Gioura (NHMC 80.3.31.37) and Grammeza (NHMC 80.3.31.38).

Hierophis gemonensis, Balkan whipsnake (Laurenti, 1768)

We confirm the presence of this species on the small island of Tsougria (Fig. 4J), near Skiathos. A juvenile individual was found in the rock ruins of a building in the vicinity of the coastal wetland area. This species was first recorded in the Sporades from Aspronisi (Buchholz and Schultze-Westrum 1964) and then from Tsougria (Grano et al. 2013), but has not been recorded from Skiathos itself. We also report on a previously unpublished record from Skopelos, confirmed by a specimen deposited in the Alexander Koenig Museum of Natural History in Bonn by T. Schuze-Westrum and W. Weigand on August 25th, 1957. However, we were not able to confirm this species during our own field work, neither on Skopelos nor on Aspronisi, perhaps due to inappropriate timing or low densities. Ultimately, *H. gemonensis* could be present on Skiathos, as the conditions seem suitable, and it is present on a nearby smaller island, though this remains speculative at present. The *H. gemonensis* documented in the Sporades does not exhibit markedly different phenotypes from the mainland populations.

Specimens: Tsougria (NHMC 80.3.25.134), Skopelos (ZMFK 003606)

Malpolon insignitus, Eastern Montpellier snake (Geoffroy Saint-Hillaire, 1827)

Eastern Montpellier snakes have a broad distribution in the lowlands of the Aegean-Anatolian region but do not survive well in island environments. In the Aegean archipelago, their distribution is restricted to the largest, young, near-shore landbridge islands. This conspicuous snake is common and well documented, both from Skiathos (Buchholz and Schultze-Westrum 1964; Cattaneo 1997) and Skopelos (Werner 1930, 1938a; Buresch and Zonkow 1934; Wettstein 1953; Mertens and Wermuth 1960; Buchholz and Schultze-Westrum 1964; Cattaneo 1998). We confirm this species on Skiathos (n=4), as well as Skopelos (n=20), but on no other islands, despite past speculation about its existence on Gioura and Skantzoura (Legakis 2004). Furthermore, a report of the species from Alonnisos (Crucitti and Tringali 1987; Legakis 2004) almost certainly refers to a misidentified *D. caspius* (Cattaneo 1998).

The Eastern Montpellier snake is a large-bodied species (record length in Greece is 160 cm; Kalogiannis and Stefanopoulos 2023) and a very active, diurnal hunter. We often encountered this snake in human-modified environments, including agricultural fields, olive groves, and near settlements, as well as in forested areas and maquis. It is a significant terrestrial predator with a catholic diet and a likely competitor to a number of other snake taxa. We found individuals preying on *P. erhardii*, while Cattaneo (1998) reports mammal and bird remains in stomach content, thus indicating that the species is largely euryphagous on the islands. Adults on Skopelos ranged from 80 cm to 140 cm in total length.

***Platyceps najadum*, Dahl's whipsnake (Eichwald, 1831)**

This species is only documented in the Sporades on the island of Skiathos, where it is common (Bergman 1985, 1995; Cattaneo 1997). We found 13 individuals throughout the island and across a diversity of habitats, including agricultural land, settlements, mesic forested areas, and xeric sites such as phrygana and maquis. The largest individual had a total length of 125 cm. This is a very thin, quick, and agile snake that is also common on mainland Greece. It primarily feeds on small lizards and invertebrates (Speybroeck et al. 2016). Specimens from Skiathos exhibited the same typical phenotype as those from the nearby mainland, with about 25–30 ocelli across the front half of the body.

***Telescopus fallax*, cat-eyed snake (Fleischmann, 1831)**

While this is a relatively widespread species on the Aegean islands, in the Sporades it has previously only been reported from Alonnisos (Broggi 2010). We report here for the first time two new photographic records from Peristera (Vasiliko bay area, August 2020; Vassilis Malamatenios) and the eastern part of Skopelos (August 2018; Luca di Cianni) (Appendix 1: Table A1). We also confirm the Broggi (2010) record (based on an unpublished photograph) with 12 individuals found in various locations around Alonnisos (Fig. 4G). This species is thinly distributed on Alonnisos and is more common at higher elevations in the central and eastern parts of the island, where the habitat is more xeric and rockier. Cat-eyed snakes are nocturnal, and we found most of them on Alonnisos while they were actively crossing roads or moving across rocky terrain at night (n=9), as well as under metal lids that cover concrete wells (n=3). This taxon is rear-fanged and venomous, though it is not considered a threat to humans as it rarely injects venom (Kochva 1965). The snake feeds mainly on geckos and lacertid lizards, which are abundant in the arid, sparsely vegetated areas this snake prefers.

Photographic voucher: Peristera (NHMC 80.3.38.147), Skopelos (NHMC 80.3.38.148).

***Zamenis situla*, leopard snake (Linnaeus, 1758)**

We report here the presence of this species on Alonnisos, as supported by a photograph deposited in the NHMC (November 2020; Konstantina Malamateniou, see Appendix 1: Table A1). Thus, we confirm Broggi (2010), who mentions having seen pictures of the species taken by a local on Alonnisos but did not include them in his publication. We did not encounter any *Z. situla* during our field observations. The species has previously been recorded on Skiathos (Bergman 1995; Cattaneo 1997) and Skopelos multiple times, where both the striped and blotched phenotypes occur (Werner 1929, 1930, 1938a; Buresch and Zonkow 1934; Wettstein 1953; Buchholz and Schultz-Westrum 1964; Bruno 1969; Sigg 1984; Cattaneo 1998).

Zamenis situla is a colorful, slender, medium-sized snake that occurs in a blotched and striped form across its range (Speybroeck et al. 2016). On the Sporades, most individuals have red dorsal blotches outlined in black on a light-grey background with lateral spots. Additionally, there exists a striped form with a dorsal pattern of red stripes, or where the dorsal pattern is lacking any red and has more of a ladder-like black/dark gray bars. The species is typically more common near human habitations, giving it its Greek name, '*Spitofido*' (house snake). It appears to be uncommon on the Sporades.

Photographic voucher: Alonnisos (NHMC 80.3.30.82).

***Vipera ammodytes*, nose-horned viper (Linnaeus, 1758)**

We report this viper for the first time from the island of Peristera (Fig. 4B). The specimen was a female about 40 cm in total length and had a light brown dorsal zig-zag band on a light beige background, resembling the phenotype of females from adjacent Alonnisos. The animal was found in one of the few open areas of the island, at the margins of a wet meadow and abandoned agricultural habitat invaded by patchy maquis.

This species had previously been recorded from Skiathos, Alonnisos, Megalos Adelphos, and Mikros Adelphos (Buchholz and Schultze-Westrum 1964; Cattaneo 1997, 1998). It is absent from all of the islands to the north-east of Alonnisos (e.g., Gioura, Legakis 2004). It also has not been documented yet in Skopelos, where the locals, however, claim it is present. On Skiathos, it is uncommon, and we did not observe any there during our surveys. Others have reported that individuals on Skiathos are relatively large (e.g., a male specimen of 53.2 cm reported by Buchholz and Schultze-Westrum 1964). Female *V. ammodytes* from Skiathos have the same phenotype as conspecifics from the adjacent mainland, with a dark brown dorsal zigzag pattern on a light brown background (Cattaneo 2021). In contrast, females from the islands of Alonnisos and Peristera exhibit a distinct phenotype that is very "washed out" or "blonde," which blends in well with the light-grey limestone bedrock of the island

(Fig. 4B and see fig. 8 in Cattaneo 2021). This female phenotype appears to be unique to Alonnisos and Peristera, according to extensive comparisons across numerous Aegean Sea islands (Roussos 2015; Cattaneo 2021). Males from Alonnisos have faint (or lack) lateral spots along the body, and the tail tip is black instead of green or yellow as found on the mainland. *Vipera* from Skiathos and Alonnisos also differ in scalation, with snakes from Skiathos possessing more dorsal scale rows (Cattaneo 2021). In contrast to Skiathos, the species is common on Alonnisos and can be found in a variety of habitats and at all elevations. A total of 25 observations have been made by the authors across the island near stone walls, settlements, phrygana, olive groves, maquis, and pine forest. Several individuals we found were victims of road mortality, especially during the mating period (May–June), as snakes tend to move during post-copulation dispersion. The largest individual we found on Alonnisos was a male with a total length of 75 cm, and others have recently reported individuals > 50 cm SVL (Cattaneo 2021).

Vipers are typically not able to survive on smaller islands. In the Sporades, the two small islands of Mikros and Megalos Adelphos are exceptional in that they retain viper populations. Vipers on the Adelphia cluster exhibit dwarfism (total adult lengths of 20–30 cm), similar to some Cycladic islands (e.g., Koufonissi, Roussos 2015; Itescu et al. 2018). The only size-appropriate prey items available on these islands are geckos and *Podarcis* lizards, *Scolopendra* centipedes, and, according to Buchholz and Schultze-Westrum (1964), Orthoptera, especially during the summer months. The islands are covered by a mixture of phrygana with some maquis bushes, as well as a few olive and juniper trees. Goats are also present and keep the shrubs heavily manicured. Vipers were found active during the day and near these bushes. The viper populations on these two small islands appear to be relatively dense, and this is the only snake species occurring there. While previous researchers (Buchholz and Schultze-Westrum 1964) have suggested that the vipers may prey on migratory birds, this is, in our opinion, unlikely, as the extremely small viper body size all but precludes the consumption of birds, except perhaps small songbirds by the largest individuals (>30 cm). What is more likely is that snakes benefit indirectly from the marine nutrient subsidies delivered by the strong gull colonies. Indeed, data from other islands in the Aegean Sea suggests that the presence of significant marine subsidies allows for the existence of predators on islands that would normally be considered too small to support them.

Photographic voucher: Peristera (NHMC 80.3.40.61).

Testudines

Only two Testudine species, each from a different family (Testudinidae and Geoemydidae), are present in the Sporades. We report here on one new island record for *Testudo marginata*. Testudines are the rarest group of native herpetofauna on these islands and appear to be continually declining. A variety of threats to their habitat make the long-term future of Testudines uncertain outside of protected areas (Hailey and Willemsen 2003).

***Testudo marginata*, marginated tortoise (Schoepff, 1789)**

The marginated tortoise is known from several islands in the Northern Sporades archipelago, where it has been documented on Skiathos (Cattaneo 1997), Alonnisos (Broggi 2010), and Kyra Panagia (Kock and Storch 1979). This species is also known from the neighboring Skyros cluster, including Skyros proper (Watson 1962; Arnold and Burton 1978; Kock and Storch 1979; Bringsoe 1985; Broggi 2006), and offshore Valaxa island (Gruber and Fuchs 1977). The marginated tortoise appears to be rare on the Sporades, with only occasional recorded encounters.

Here we document for the first time the presence of the marginated tortoise on Skopelos, therefore completing the known occurrence of the species across all major islands of the Sporades chain. On Skopelos, we encountered one adult male (carapace length 25 cm) crossing a dirt road in an agricultural area in the eastern part of the island. We also confirm the presence of *T. marginata* on Kyra Panagia, where we found two alive and two deceased adult specimens. All animals were encountered either in areas with maquis or near agricultural land. All four individuals found on Kyra Panagia were large, full-grown adults, and one of the live specimens had a healed injury to its carapace.

Photographic voucher: Skopelos (NHMC 80.3.22.18).

***Mauremys rivulata*, Balkan terrapin (Valenciennes, 1833)**

The Balkan terrapin has been reported from 31 Aegean and Ionian islands (Broggi 2023), where many of their populations have significantly declined over the last decades and, on some islands, are expected to go extinct (Broggi 2012). This species seems to only be doing well on larger islands with a substantial number of wetlands, such as on Evia (Euboea), Lesvos (Mytilene), Limnos, Ikaria, and Naxos (Broggi 2012; Strachinis and Roussos 2016). On the Sporades, the Balkan terrapin was first reported by Cattaneo (1997) from Skiathos, where small populations currently occur in the stream estuaries of Troulos and Agia Paraskevi. We observed 10 individuals in the streams of Troulos during the summer, where locals have put up signs to dissuade the taking of turtles. Here, the terrapins are regularly fed by tourists and are quite tame. This species is also very likely present in Vromolimnos marsh, but the dense vegetation has made observing them difficult. The Balkan terrapin is also present on Skopelos, where Cattaneo (1998) found a few specimens in the small pond of Ditropo. Furthermore, single individuals of this species have been observed in the coastal marsh of Milia (Broggi pers. comm.). Therefore, similarly to other regions of the Aegean Sea, terrapins in the Sporades survive only in a few isolated, relict wetland sites, and are likely threatened with extinction with the removal of these habitats (Cattaneo 1997; Broggi 2023).

Discussion

The herpetofauna of the Sporades archipelago has been investigated by herpetologists, both foreign and domestic, for almost a century, creating an intriguing but incomplete picture of the biodiversity in this region (Werner 1930; Cyrén 1935). Our 7+ year survey documents 26 new island records of lizards, snakes, and tortoises, confirms 5 unvalidated lizard and snake records, and finds 3 records in the literature erroneous. This is the first review and most comprehensive species account of the Sporades herpetofauna to date.

Biogeography of the islands: Patterns of island diversity and underlying processes

Despite the relatively small extent of the Sporades archipelago, the islands vary significantly in species richness and herptile community structure (Figs 2, 3, Tables 1, 2). At the most basic level, the species assemblages of the Sporades are derivative of those from the Thessaly mainland. Island species richness declines with decreasing island size but also with increasing island age (age of insularity), with the oldest islands in the east having much more depauperate herptile species communities relative to the younger, larger islands in the west (Fig. 2). Thus, Lechousa, a small, young island with a recent landbridge connection to Alonnisos and the Thessallian mainland, has as many herptile species (3) as the almost 20× larger Gioura located off the mainland shelf. Given that the landbridge islands most likely shared the same species assemblages during the last ice age when they were connected (Foufopoulos et al. 2011), present-day differences in species richness are best interpreted as the outcome of differential extinctions.

Recent research has shown that Holocene reptile extinctions in the Aegean are highly predictable and are determined by both species identity and island characteristics (Kalb 2021). Thus, larger-bodied species (e.g., lizards like *P. apodus* or snakes like *M. insignitus*), as well as habitat specialists (such as the moisture-loving species *L. trilineata* and *M. rivulata*), tend to have smaller populations and are more likely to go extinct first following isolation by rising sea levels (Foufopoulos and Ives 1999). Because the particular species' life history traits determining extinction sensitivity are the same across an archipelago, species tend to go extinct in a predictable sequence across all islands, reflecting each taxon's relative susceptibility to underlying extinction drivers. What varies is the extent to which island characteristics cause the original species community to progress down the general sequence of species loss. Thus, islands that are smaller, older, more rugged, and have less water, (e.g., Skantzoura, Piperi, and Gioura) tend to lose a higher fraction

Table 1. Distribution of reptiles across the study islands of the Sporades, together with supporting sources. The table contains the bigger islands in an approximate west-to-east direction. Bolded text indicates records newly reported in this paper.

Species	Skiathos	Skopelos	Alonnisos	Peristera	Skantzoura	Kyra Panagia	Gioura	Piperi
M. rivulata	+ 2, 16, 17	+ 3						
T. marginata	+ 2*	**+ 1**	+ 15			+ 7		
M. kotschyi		+ 8	+ 3, 8	+ 8	+ 8	+ 8, 17	+ 8	**+ 1**
H. turcicus	+ 2	+ 9	+ 3, 10	**+ 1**		**+ 1**		+ 12
P. apodus	+ 13	+ 25						
L. t. trilineata	+ 3, 11	+ 3, 11	+ 3, 10, 14, 15					
P. erhardii	+ 13	+ 3, 14, 20	+ 3, 10, 14	+ 18	+ 18	+ 18	+ 14	+ 10 (*P. gaigeae weigandi*)
A. kitaibelii	+ 2	+ 3, 14	+ 3, 10, 15					
D. caspius			+ 3, 4	+ 5	**+ 1**			
H. gemonensis		+ 26						
P. najadum	+ 2, 13							
E. quatuorlineata	+ 19	+ 3, 19				+ 6, 25	**+ 1**	
Z. situla	+ 2, 13	+ 3, 16, 17, 19	+ 15, 25					
M. insignitus	+ 2, 13, 19	+ 3, 16, 19						
T. fallax		+ 25	+ 15	+ 25				
V. ammodytes	+ 2, 19		+ 3	**+ 1**				
P. kurtmuelleri	+ 2	+ 3, 14	+ 10, 15**					
B. viridis		+ 3, 14						
H. arborea	+ 2							

1 – New record by authors; 10 – Grillitsch and Tiedemann (1984); 19 – Buchholz and Schultze-Westrum (1964)
2 – Cattaneo (1997); 11 – Sagonas (2019); 20 – Wettstein (1957a)
3 – Cattaneo (1998); 12 – Daftsios et al. (2024) ; 21 – Crucitti and Tringali (1987)
4 – Cattaneo (2010); 13 – Bergman (1995); 22 – Grano et al. (2013)
5 – Kalogiannis (2021); 14 – Cyren (1935); 23 – Froer (1979)
6 – Legakis et al. (2004); 15 – Broggi (2010); 24 – Chondropoulos (1986)
7 – Kock and Storch (1979); 16 – Werner (1930); 25 – Record by citizen photographers or from iNaturalist (Appendix Table A1).
8 – Beutler and Gruber (1977); 17 – Werner (1938a); 26 - Alexander Koenig Museum of Natural History in Bonn
9 – Gruber (1974); 18 – Gruber and Schultze-Westrum (1971);
*Cattaneo (1997) identifies this individual as *T. hermanii*, it was likely a misidentified juvenile *T. marginata*
***P. kurtmuelleri* is considered likely extinct on Alonnisos, see species accounts section

Table 2. Distribution of reptiles across the study islands of the Sporades, together with supporting sources. The table contains the smaller islands in an approximate west-to-east direction. See Table 1 for supporting sources.

Island Code	Island Cluster	Island Name	*Mediodactylus*	*Podarcis*	*Hemidactylus*	*Lacerta*	Other
TSA	Skiath	Tsougria			+ 22	+ 22	+ *H. gemonensis* – 22
TSI	Skiath	Tsougriaki	**+ 1**	+ 18			
ARK	Skiath	Arkos	**+ 1**	+ 18	**+ 1**	+ 17, 23, 24	
MAR	Skiath	Maragos		+ 18			
ASP	Skiath	Aspronisi		+ 18			+ *H. gemonensis* – 19
REP	Skiath	Repi	+ 8	+ 18			
DAK	Skiath	Daskalonisi	**+ 1**				
PAX	Skop	Paximadi (Skop)	**+ 1**				
STR	Skop	Strongyllo (Skop)		+ 26			
DAS	Skop	Dasia (Skop)		+ 18			
KAS	Skop	Kassidis (Skop)	+ 8				
PLE	Skop	Plevro (Skop)		+ 18			
AGG	Skop	Agios Georgios	**+ 1**	+ 18			Snake confirmed***
MIK	Skop	Mikronisi	**+ 1**	+ 18			
KOK	Alon	Kokkinokastro		+ 18			
MAN	Alon	Manolas	+ 26	+ 18			
LEO	Alon	Lechousa	+ 8	+ 18			+ *D. caspius* - 19
MEA	Alon	Megalos Adelphos	+ 8	+ 18			+ *V. ammodytes* – 19
MIA	Alon	Mikros Adelphos	+ 8	+ 18			+ *V. ammodytes* – 19
GAI	Alon	Gaidaronisi	+ 8	+ 18			
POL	Skantz	Polemika	**+ 1**	+ 18			
LAC	Skantz	Lachanou		+ 18			
KSD	Skantz	Kassidis		+ 18			
KYR	Skantz	Kyriagos	**+ 1**	+ 18			
PRA	Skantz	Prasso	**+ 1**	+ 18			**+ *D. caspius* - 1**
SKA	Skantz	Skandili	**+ 1**	+ 18			
MKS	Skantz	Mikroskandili		+ 18			
KOR	Skantz	Korakas	**+ 1**	+ 18			
AGP	KyrPan	Agios Petros		+ 17, 18			
PEL	KyrPan	Pelerissa	**+ 1**	+ 18			
MEL	KyrPan	Melissa	+ 8	+ 18			
SFI	KyrPan	Sfika		+ 18			
PAP	KyrPan	Pappous		+ 18	+ 9, 24		+ *H. turcicus* – 9
KOU	KyrPan	Koumbi	+ 8	+ 18			
GRA	KyrPan	Grammeza	+ 8	+ 18			**+ *E. quatuorlineata* – 1**
MYG	Gioura	Myga		+ 18			
PSA	Gioura	Psathoura	+ 14,8	+ 18			

*** Observation of a snake confirmed, possibly *D. caspius, H. gemonensis,* or *M. insignitus.*

of their original species communities (Kalb 2021). Ultimately, this results in a distinct pattern of nested species occurrences where smaller and older islands support only species found on larger ones. The only exception to this pattern is species that likely inhabit similar ecological niches, such as *E. quatuorlineata* and *D. caspius*, which do not co-occur on the same island in the Sporades (though we note these species do co-occur in the Cyclades islands), but instead show characteristic ‘checkerboard’ distributions (Diamond 1975). Furthermore, some species, such as *Mediodactylus* and *Podarcis*, display a phenomenon called density compensation, occurring in more dense populations and having broader habitat utilization on smaller, species-poor islands relative to large islands (Rodda and Dean-Bradley 2002). This means that they are sometimes rare and very hard to detect on larger islands, such as *Mediodactylus* on the larger Sporades.

Skiathos is the island closest to the species-rich Thessaly mainland and was last connected to it until fairly recently (~8.5 kya). Because extinction, which is a time-dependent process, has had less time to act on the resident species communities, the island retains a greater fraction of the original mainland species communities, being the Sporades island with the most snake species and the only island that retains both *P. najadum* and *H. arborea*. Skiathos has more significant wetland areas compared to other larger islands to the east, with 1% of its total surface covered by freshwater (Catsadorakis and Paragamian 2007). Interestingly, *M. kotschyi*, one of the most widely distributed species in the archipelago, has not yet been recorded from the island, even though it occurs on the surrounding satellite islets (Tsougkriaki, Arkos, and Daskalonisi). Similarly, *H. gemonensis* has been recorded from two islets off Skiathos (Tsougria and Aspronisi) but not yet from Skiathos itself.

The island pair of Skopelos and Alonnisos have been isolated almost twice as long as Skiathos from the mainland (15 kya, see Fig. 1, 3) yet harbor similar numbers of taxa. Because Skopelos is more densely forested and retains more wetland areas than Alonnisos, it still sustains several hydrophilic species (*P. kurtmuelleri, B. viridis*, and *M. rivulata*), the latter of which is likely lost to extinction. Snake communities between the two islands also differ somewhat, with only Skopelos harboring *M. insignitus, H. gemonensis*, and *E. quatuorlineata*, while Alonnisos harbors *V. ammodytes* and *D. caspius* instead. This pattern is likely caused by a combination of inter-island differences in habitat in conjunction with interspecific competition between the various snake species.

Skantzoura, Kyra Panagia, and Gioura have all been isolated for longer periods of time, are smaller by land area, and have lower habitat diversity than the main three islands to the west. These smaller islands and their satellite islets are surrounded by very deep waters (> 120 m), suggesting a very long period of isolation (> 200 kya and possibly up to 5.3 million years coinciding with the Zanclean flood; Rohling et al. 2014, assuming depths have not changed significantly due to tectonic movement).

Relatively few exotic reptile introductions appear to have occurred to date on the Sporades, and most species appear to be native to the islands. Three possible exceptions to this are the tortoise *T. marginata*, the gecko *H. turcicus*, and the skink *C. ocellatus* (assuming the Skopelos record is not erroneous). The former is often kept as a pet (Valakos et al. 2008) and may have been introduced in this manner onto the larger, human-inhabited islands of the archipelago. The latter two lizards are often transported by virtue of their small body size, peri-domestic occurrence, and their tendency to hide in building materials and livestock feed. Genetic analyses indicate that for both species, lizard populations across the Mediterranean lack genetic structure, consistent with recent anthropogenic transportation (Kornilios et al. 2010; Rato et al. 2011; Ergül Kalayci et al. 2023). In accordance, we encountered *H. turcicus* only on islands regularly visited by humans.

Reptile and amphibian habitat use in the Sporades

While reptiles occupy most natural habitats in the Sporades, some habitats are clearly more important than others and require particular management attention (see Table 3). In general, both amphibian and reptile taxa prefer wetter and more productive habitats, especially if they provide enough insolation for thermoregulation. Wetland areas are the most important habitats because, at least during the summer season, they serve as oases, allowing species to persist in an otherwise hostile habitat.

While conservation emphasis often centers on the actual water bodies, the surrounding habitats can be equally important. We found that the wet meadows surrounding many coastal wetlands were not only used by most of the local reptile and amphibian species, but that they were, when not degraded, extremely species-rich for many other organisms, including plants and invertebrates. We identified such wet meadows in Koukounaries, Aselinos, and Platanias (Skiathos), but also in Tsougria, in Livadakia (Peristera), and in Agios Petros (Kyra Panagia). Wet meadows face immense pressure not only from touristic development but also from overgrazing and undergrazing, which can result in the encroachment of shrubs and exotic invasives.

On the other hand, extensively shaded areas like pine forests are avoided by most taxa, presumably because they lack opportunities for thermoregulation. Our field observations also suggest that structurally and thermally diverse habitats, such as traditional agricultural landscapes, are disproportionately significant because they provide abundant thermoregulatory opportunities for resident species (Zakkak et al. 2015). For example, dry stone wall terraces, a common feature of agriculture fields on sloped island landscapes, not only create a varied set of thermal environments, but also offer valuable refugia from predation since they are challenging for snakes to ascend (Nossan 2023). Overall, most species of reptiles on the Sporades, with the exception of aquatic taxa, appear to be behaviorally quite flexible and can be found in numerous native and human-modified habitats.

Anthropogenic changes in the landscape of the archipelago

Over the last century, human communities on the Sporades have experienced significant demographic and economic shifts with important ramifications for the ecology and herpetofauna of the islands. Extensive agricultural areas were abandoned, first because of a lack of economic incentives and emigration to urban centers, and again more recently with the emergence of tourism as a more lucrative income alternative. As a result of these changes, all of the smaller island settlements were completely abandoned in the last 50 years (Tsougria, Megalos Adelphos, Skantzoura, Grammeza, Pappous, Gioura, Psathoura, and Piperi) (Wikipedia n.d., National Statistical Service of Greece 2011). On the larger islands, and over the last 80 years, abandonment of traditional agricultural activities has progressively led to the incursion of tall maquis and pine forests. Vegetation modulates microclimates (Osorio et al. 2024), and changes to land cover seriously alter thermal landscapes that ectotherms like herpetofauna use to behaviorally maintain homeostasis. In general, shifts to a more closed vegetation structure are associated with a narrower, cooler thermal gradient that results in a greater mismatch to body temperatures preferred by herpetofauna and therefore decreased thermoregulatory efficiency (Muñoz and Losos 2018). Thus, the widespread shift to closed-canopy pine forest in the Sporades has likely decreased opportunities for efficient herptile thermoregulation, as evidenced by their general dearth in such habitats (Table 3).

Table 3. Habitat use of reptiles and amphibians on the Sporades as determined by our observations.

Species	Abb rev	Settlements	Grain fields	Streams/ wetlands	Gardens/ vineyards	Olives	Phrygana	Pine forest	Maquis	Rocks	Habitats Used
Mauremys rivulata	MR	0	0	1	0	0	0	0	0	0	**1**
Testudo marginata	TM	0	0	0	1	1	1	0	1	0	**4**
Mediodactylus kotschyi	MK	0	1	0	1	1	1	1	1	1	**7**
Hemidactylus turcicus	HT	1	1	1	1	1	1	1	1	1	**9**
Pseudopus apodus	PA	0	0	1	1	1	1	0	1	1	**6**
Lacerta trilineata	LT	1	0	1	1	1	1	1	1	1	**8**
Podarcis erhardii	PE	1	1	1	1	1	1	1	1	1	**9**
Podarcis gaigeae	PG	1	1	1	1	1	1	1	1	1	**9**
Ablepharus kitaibelii	AK	1	1	1	1	1	1	1	1	0	**8**
Dolichophis caspius	DC	1	1	1	1	1	1	1	1	1	**9**
Hierophis gemonensis	HG	0	0	1	1	1	1	0	1	1	**6**
Platyceps najadum	PN	0	0	1	1	1	1	1	1	1	**7**
Elaphe quatuorlineata	EQ	1	1	1	1	1	1	1	1	1	**9**
Zamenis situla	ZS	1	1	1	1	1	0	0	1	1	**7**
Malpolon insignitus	MI	1	0	1	1	1	1	1	1	1	**8**
Telescopus fallax	TF	0	0	0	1	1	1	0	1	1	**5**
Vipera ammodytes	VA	1	0	0	1	1	1	1	1	1	**7**
Pelophylax kurtmuelleri	PK	0	0	1	0	0	0	0	0	0	**1**
Bufotes viridis	BV	0	0	1	0	1	0	0	0	0	**2**
Hyla arborea	HA	1	0	1	1	0	0	0	0	0	**3**
Total Species		**10**	**8**	**16**	**17**	**17**	**15**	**11**	**16**	**12**	

Livestock husbandry and reptiles

Livestock husbandry and the associated vegetation changes appear to have a complex relationship with the resident herpetofauna. In the relatively humid Sporades climate, browsing by goats is an important process for keeping vegetation open and preventing the establishment of a closed canopy. Research in other parts of the Aegean Sea region has demonstrated that the impacts on vegetation cover rise with an increasing stocking rate (Cheng 2015). In the Sporades, similar to other parts of the region (Gizicki et al. 2018), island size determines both the type of livestock husbandry, as well as the ultimate impacts on vegetation structure and the resident reptile community. On the larger islands with permanent human populations, individual families have traditionally kept small numbers of goats and sheep for personal use. In addition, a limited number of larger flocks of livestock were typically kept by shepherds who carefully managed grazing patterns to avoid crop damage. In these open and carefully managed agropastoral systems, our personal observations indicate a particularly high reptile presence. Thus, on the main islands, most species of reptiles prefer habitats moderately disturbed by grazing, and avoid (with the apparent exceptions of *D. caspius* and *A. kitaibelii*) undisturbed, closed-canopy pine forests. Even on medium-sized islands like Peristera and Skantzoura, reptiles are concentrated in open field areas maintained by goats and appear to avoid the expanding dense maquis.

In contrast to the controlled animal husbandry of large islands, on small islands and islets, goats are typically released unguarded for extended periods of time and left to forage freely. Given the limited resources there, goat releases on islets typically result in severe soil erosion and large-scale vegetation degradation (Gizicki et al. 2018). A formal study from the nearby Skyros cluster demonstrated that goat grazing on small islands has serious negative impacts on both plant cover and the resident herpetofauna (Pafilis et al. 2013). Our own field observations confirm that on islets smaller than a few hectares, livestock grazing results in negative outcomes for local species communities. Based on our field observations, many, if not most, of the smaller islands in the Sporades appear to have suffered from past overgrazing, which has shifted plant communities and severely damaged the vegetation cover. Pelerissa Isl., near Kyra Panagia, still bears the scars of past overgrazing, with the evident trunks of many kermes oaks killed by goats, and the ground covered by a degraded graminoid monoculture that is inhabited only by *Mediodactylus* and *Podarcis* (Fig. 6A).

While this practice has nowadays been largely discontinued on the smallest islets, several of the somewhat bigger, yet uninhabited islands (Gioura, Piperi, Grammeza, Peristera, Lechousa, Skantzoura, Megalos Adelphos, Mikros Adelphos, and Agios Georgios) still harbor populations of goats in different stages of abandonment and only thin *Podarcis* and *Mediodactylus* populations. Other islands (Repi, Grammeza) suffer similarly, but from released rabbits that dig out top soil, creating another form of erosion detrimental to the sustainability of vegetation (Kossoff 2023).

Habitat use and status of hydrophilic herpetofauna

Wetland ecosystems, both in the Aegean generally and on the Sporades specifically, are typically scarce, vulnerable, and disproportionately important for reptiles and amphibians (Catsadorakis and Paragamian 2007). From the whole archipelago, Skiathos has the most notable wetlands and streams, which are nonetheless few and under human pressure. The marsh of Vromolimnos on Skiathos is in good condition, although some hotels and beach bars exist nearby. The wetland of Koukounaries, although modified, is also in relatively good condition and is now strictly protected, despite the presence of hotels constructed in the surrounding area before the lake's declaration as a nature reserve. The area of Koukounaries, including the lake, coastline, and *Pinus pinea* forest, is currently a Natura 2000 site (GR1430003). Skopelos possesses only three wetlands of significance, two of which are seasonal. The permanent lagoon of Milia is in seemingly good condition and hosts populations of frogs and terrapins. On Alonnisos, there is a single seasonal, coastal lagoon located at Agios Dimitrios. Additional small, typically seasonal wetlands exist on Tsougria, Peristera, Kyra Panagia, and Psathoura. Unfortunately, some of the natural wetlands of the archipelago have been reduced or completely destroyed by human activities during the last decades. On Skiathos, Skopelos, and Alonnisos, many wetlands, lagoons, and streams have been drained and built over for touristic development (e.g., hotels and airport infrastructure) or are heavily exploited for irrigation.

From the sites mentioned above, only the wetlands of Skiathos and Skopelos currently host hydrophilic reptiles and amphibians (*M. rivulata*, *B. viridis*, *P. kurtmuelleri*, and *H. arborea*). Green toads, *B. viridis* (Laurenti, 1768), are widespread throughout insular Greece, often inhabiting surprisingly small islets (e.g., Stille and Stille 2015; Strachinis 2022), where they commonly reproduce in artificial water bodies. Despite this, they can be severely affected by human activities and are known to have had significant population declines on some islands (e.g., Ithaki, Strachinis and Artavanis 2017). Similarly, marsh frogs of the genus *Pelophylax* are also well distributed across insular Greece (Valakos et al. 2008), occupying streams, marshes, lagoons, and artificial water bodies such as reservoirs and wells, where they greatly depend on these open water resources (Broggi 2021). In the Sporades, marsh frogs have vanished from Alonnisos, where, according to the locals, they used to thrive until the 1980's. Specific changes in irrigation, along with heavy exploitation and destruction of streams, caused frogs on Alonnisos to steadily decline, eventually dying out during the 2000's. Tree-frogs in the genus *Hyla* are less widespread in the Greek islands, occurring on Thassos, Skiathos, Evia, Corfu, Lefkada, Kefalonia, Zakynthos, Lesvos, Chios, Samos, Kos, Rhodes, and Crete (Valakos et al. 2008), islands known to have high freshwater availability (Catsadorakis and Paragamian 2007). On Skiathos, they seem to occur in abundance around settlements where they breed in anthropogenic structures such as swimming pools. Although they exist in seemingly good populations on the island, the aforementioned breeding sites are unreliable in the long term and readily impacted even by small changes in human activity. For the Balkan terrapins, both in the Sporades and in the rest of the Aegean, the main threats concerning the viability of island populations are habitat destruction and, in the long term, climate change (Broggi 2012). The populations of *M. rivulata* on Skopelos have significantly declined and have been limited to isolated waterbodies that are particularly susceptible to human activities and climate change.

Overall, habitat loss constitutes the main risk to amphibian and terrapin populations on the islands, since the remaining wetland habitats are now in small and isolated, relict sites. In addition to natural habitats, artificial water bodies (e.g., wells and canals) used for agriculture serve as important breeding sites for green toads and marsh frogs on Aegean islands. Nonetheless, these breeding sites are disappearing as traditional cisterns are being replaced by the modern irrigation system. Consequently, the conservation of wetland habitats, as well as of traditional agriculture, is crucial for the survival of amphibians and terrapins in the Sporades archipelago.

Management and conservation recommendations

The maintenance of healthy reptile and amphibian communities is directly tied to appropriate habitat management. On the western, more developed islands, the main threats to the native communities are road construction, touristic development, and surface water diversion. On the smaller, eastern islands, the main problems center on the abandonment of the traditional agricultural activities, as well as the overgrazing of small islets. As a result, management recommendations differ among the islands. Given that many of the eastern islands are within the MPA and least threatened by human development, the terrestrial aspect of the reserve could be one of promising restoration and management. This would not only promote suitable habitat for the herptile community but also for all biota native to these islands. Another significant threat to reptile populations on human-inhabited Aegean islands are feral cats (Case and Bolger 1911). While spaying and neutering cats can help control their population numbers, it will not stop their killing of birds, small mammals, and reptiles. Outdoor cats are significant predators of snakes and lizards and have caused island extinctions the world over (Medina et al. 2011). We recommend that cats be kept indoors for their own health and safety and the preservation of biodiversity. Fortunately, smaller islands that do not have cats are refuges for snakes and lizards from this invasive predator.

Recommendations for large, human-inhabited islands

Our field research suggests that the continuation of traditional agricultural activities, including some goat grazing, which create a mosaic of thermally variable microhabitats, is important for the persistence of native reptiles and amphibians (Papanastasis et al. 2009). This includes the preservation of the diverse stone structures such as drystone walls and terraces, stables, and cisterns, all of which provide cover and refugia for resident reptiles. In addition, maintenance of any sources of surface water, whether in the form of springs, seasonal streams, or cisterns, is essential for many species. The practice of illegally drawing water from springs and streams with small pumps for irrigation desiccates the landscape and is particularly damaging, and its prohibition should be enforced. On Skopelos and Skiathos, exurban development for tourism and associated roads is increasingly leading to the fragmentation of the natural habitats and represents a growing problem for native reptiles and amphibians.

Wetlands and wet meadows harbor many unique species and require urgent priority protection and management. On Skiathos and Skopelos, perhaps small ponds could be constructed in order to enhance the reproductive success of amphibian species. Wet meadows, such as the wetlands of Koukounaries and the mouths of the Aselinos stream (Skiathos), are tremendously important and require special management. This requires the absence of water extraction to maintain high water tables, the absence of plowing, and the removal of encroaching bushes, ideally through activities such as scything. Grazing, if it occurs, must happen only briefly, perhaps late in the summer, with a focus on controlling encroaching bushes.

We observed significant seasonal road mortality in certain areas, suggesting that the creation of under road tunnels would be an excellent way to allow for safer passages. These can be used as general wildlife crossings for other species as well. Road signage is a cost-efficient and educational strategy to warn drivers to slow down and be alert to species' crossings.

Recommendations for medium-sized, uninhabited islands

Overgrazing by unmanaged, feral goats constitutes the biggest problem for the resident herpetofauna on medium-sized, uninhabited islands. Overstocking on such islands can lead to dramatic habitat degradation through vegetation loss and subsequent soil erosion, as seen on Kyra Panagia and Pelerissa islands (Fig. 6A). Nonetheless, lower levels of grazing on some larger uninhabited islands can open up forest and dense maquis thickets and provide basking opportunities for local reptiles. On some islands where grazing pressure has declined significantly in the last few years (esp. on Peristera and Skantzoura), habitat quality for resident reptiles may be declining, as has been the case in other parts of Greece (Zakak et al. 2015).

Some of the medium-sized islands of the archipelago also include key wet meadows that exist today in severely overgrazed and degraded form: Livadakia (on Peristera) and Agios Petros (on Kyra Panagia). Given the importance of these habitats for reptiles, it would be important to eliminate grazing and plowing on these specific sites, something that on Agios Petros is particularly easy given that the property is already fenced in. In the medium term, additional restoration measures may be needed, including the re-introduction of some sensitive plant species as well as potential scything to prevent invasion of the bushes.

Recommendations for very small, seabird islets

While small islets harbor only a few reptiles and no amphibians, they are often disproportionately important for conservation because they tend to be free of human interference, act as important stop-over points for migrating birds, and often showcase unusual reptile populations with often extreme phenotypes. Lizards from seabird islands occur in extremely high population densities, possess unusually large body sizes, and often display exceptional territorial, cannibalism, or fearlessness behaviors (Brock et al. 2014, 2015; Donihue et al. 2016). Islet species communities are primarily dependent on the existence of large seabird colonies to provide marine nutrient subsidies (Stadler et al. 2023) and tend to be severely impacted by invasive goats, rabbits, and rats (Weber 2014; Gizicki et al. 2018). As a result, the removal of invasive species and the promotion of seabird colonies are the most important management activities for small island reptile populations .

Conclusion

Based on our surveys, we find that the herpetofauna of the Sporades is rich, and contains relatively few exotic taxa. Species communities are a subset of the neighboring mainland, and decline as mostly nested subsets with decreasing island size and increasing age of isolation, especially for the off-the -shelf islands. Endemic elements include the unique subspecies *Podarcis erhardii ruthveni* and *Mediodactylus kotschyi fuchsi*, with wide distributions across most of the archipelago. While none of the local non-aquatic populations appear outright endangered, many populations face pressures stemming primarily from habitat conversion and degradation. These occur under the combined effects of touristic development, including wetland destruction and abandonment of traditional agricultural activities. However, most species associated with wetlands and humid ecosystems (terrapins, amphibians) are in decline and require urgent protection of their habitats. By extension, wetlands and wet meadows harbor many unique species and require priority protection and management. Both overgrazing and undergrazing represent problems for the resident reptiles and amphibians, the former on small desert islets, and the latter on the larger, wetter islands.

Figure 6. Some of the habitats important for reptiles and amphibians across the Sporades. **A.** Impacts of overgrazing and habitat recovery on Pelerissa. Note the remnants of Kermes oak trunks killed by goats in the center and the expanding margins of recovered *Pistacia* bushes on the left; **B.** *Q. coccifera* forest and open area on the slopes of Gioura; **C.** Dry stone walls on one of the last open fields on Peristera; **D.** Recovering maquis vegetation on Grammeza, with Gioura in the background; **E.** Wetland on Tsougria; **F.** Expanding *Juniperus* forest on Skantzoura; **G.** Stream estuary on Skiathos; **H.** Human-created species-rich wet meadow on Skiathos.

Acknowledgements

This work has benefited from the support and contributions of numerous individuals. Dr. Petros Lymberakis (Natural History Museum of Crete) provided insight and logistical support in regard to collection permits and museum specimens. We are also greatly indebted to the Thalassa NGO, whose crew (Antonis Voutsinos, Josephina Zoulias, and Efi Miskedaki) selflessly transported us on their boat NIRIIS to many of the study areas. Special thanks also go to Valia Stefanoudaki (director of Sea Shepherd Greece) and the crew of MV Emannuel Bronner for helping with further transportation to the uninhabited islands. We also thank MoM NGO and, in particular, Panos Dendrinos for advice and on-the-ground assistance. We would also like to acknowledge the support of the management body of Alonnisos Marine Park, especially Spyros Iosifidis and Theodoros Mouratidis, for their tireless support. This work also benefited from the input of the Hellenic Society for the Study and Protection of the Monk Seal (MOm). This work was conducted under permits from the Greek Government (Ω6ΛΝ4653Π8-ΤΨΑ, ΔΔΔ/41002/1289, ΔΔΔ/26742/981, ΔΔΔ/29761/985).

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Appendix 1

Table A1. Photographic records from iNaturalist or citizen photographers provided to the authors.

Photographer	Date	Species	Island	Additional Information (URL, coordinates, location description etc.)
Theodora Tsimpo	7/2009	*P. apodus*	Skopelos	N/A
Genevieve Leaper	5/29/2020	*E. quatuorlineata*	Kyra Panagia	https://www.inaturalist.org/observations/49866458
Luca di Cianni	3/8/2018	*T. fallax*	Skopelos	39.126064°N, 23.746339°E
Vassilis Malamatenios	8/25/2020	*T. fallax*	Peristera	Bay of Vasiliko
Konstantina Malamateniou	11/29/2020	*Z. situla*	Alonnisos	39.233056°N, 23.953889°E

Table A2. Local names of some reptile species in the Sporades.

Binomial name	Greek common name	Local name(s)
Ablepharus kitaibelii	Αβλέφαρος	Ήλιος (*Ìlios*) (Alonnisos) - meaning 'sun'
Lacerta trilineata	Τρανόσαυρα	Αγκουστέρα (*Angustéra*) (Skiathos)
Podarcis erhardii	Αιγαιόσαυρα	Ακουστέρα (*Akustéra*) (Alonnisos)-'ου' not audible
Dolichophis caspius	Έφιος	Δεντρογαλιά (*Thendrogaliá*) (Alonnisos)
Elaphe quatuorlineata	Λαφιάτης	Δενδρίτης (*Thendreétis*) (Skiathos), Τυφλίτης (*Tifleétis*)(Alonnisos)
Malpolon insignitus	Σαπίτης	Δεντρογαλιά, Σαπίτης (*Dendrogaliá, Sapeétis*)
Platyceps najadum	Σαΐτα	Σαϊτάρι (*Saitári*)
Telescopus fallax	Αγιόφιδο	Αστρίτης (*Astreétis*)
Zamenis situla	Σπιτόφιδο	Αστρίτης (*Astreétis*)
Vipera ammodytes	Οχιά	Οχιά or Αστρίτης (*Ochiá*) - used to differentiate between color morphs
Testudo marginata	Κρασπεδωτή χελώνα	Αχελώνα (*Achelóna*) (Alonnisos)

Table A3. List of islands/islets and their alternative names, with abbreviations as used in Fig. 1. and Tables 1, 2.

Name used here	Other names	Abbreviation	Coordinates
Skiathos	–	–	39.178667°N, 23.469917°E
Kastronisia	–	KST	39.212611°N, 23.461528°E
Aspronisi	Aspro	ASP	39.171389°N, 23.520944°E
Arkos	Arko, Arkaki	ARK	39.15075°N, 23.517806°E
Repi	Repio, Repion, Trypiti	REP	39.147222°N, 23.528167°E
Maragos	Marangos, Maranko	MAR	39.151278°N, 23.500361°E
Daskalonisi	Daskalio, Daskalos	DAK	39.160917°N, 23.494944°E
Tsougria	Tsougrias, Sugria	TSA	39.122667°N, 23.499472°E
Tsougkriaki	Mikrosugria	TSI	39.125083°N, 23.4815°E
Paximadi	Paximada	PAX	39.14725°N, 23.591861°E
Plevro	Plero, Klima	PLE	39.135694°N, 23.619639°E
Kassidis (Skopelos)	Kassida	KAS	39.129833°N, 23.614333°E
Strongylo (Skop)	Stroggylo	STR	39.113778°N, 23.626028°E
Dasia	Dhasia, Dasa	DAS	39.116639°N, 23.637639°E
Skopelos	–	–	39.131833°N, 23.683806°E
Agios Georgios	Agios Giorgos	AGG	39.135139°N, 23.80175°E
Mikronisi	Mikro	MIK	39.14125°N, 23.809778°E
Alonnisos	Liadromia, Chiliodromia, Ikos	–	39.198389°N, 23.902167°E
Manolas	Manola	MAN	39.201556°N, 23.862611°E
Kokkinokastro	Kokkinonisi	KOK	39.161111°N, 23.904361°E
Peristera	Xero, Xeronisi, Peristeri	PER	39.186°N, 23.973611°E
Lechousa	Lykoremma, Lykourina	LEO	39.228556°N, 23.998917°E
Megalos Adelphos	Adelfi, Megalo Adelfi	MEA	39.111694°N, 23.979361°E
Mikros Adelphos	Adelfopoula, Mikro Adelfi	MIA	39.125639°N, 23.988111°E
Gaidaronisi	Gaidaros, Gaidares	GAI	39.067611°N, 23.951139°E
Prasso	Prassonisi, Paraos, Parausa	PRA	39.072306°N, 24.095972°E
Strongylo	Stroggylo, Kyriagos	KYR	39.082833°N, 24.086306°E
Skantzoura	–	–	39.080028°N, 24.110222°E
Kassidis (Skantzoura)	Kassida	KSD	39.106528°N, 24.091722°E
Lachanou	–	LAC	39.104611°N, 24.096222°E
Polemika	Polemiko	POL	39.101389°N, 24.100167°E
Skandili	Skantili, Korakas	SKA	39.048056°N, 24.08125°E
Mikroskandili	Kyriakos	MKS	39.054111°N, 24.082861°E
Korakas	Korakonisi, Prasouda	KOR	39.035°N, 24.061361°E
Kyra Panagia	Pelagonisi, Pelagos, Ephthyros	–	39.330222°N, 24.073056°E
Agios Petros	–	AGP	39.321722°N, 24.055028°E
Pelerissa	Sphagru, Fagrou	PEL	39.313361°N, 24.037722°E
Melissa	–	MEL	39.292694°N, 24.090139°E
Sfika	Sphiga	SFI	39.359861°N, 24.078833°E
Pappous	Papous, Papu	PAP	39.353972°N, 24.121°E
Koumbi	Strongyli	KOY	39.350278°N, 24.127889°E
Grammeza	Gramsa, Prasso	GRA	39.342306°N, 24.139139°E
Gioura	Gioura, Yioura, Gerontia	–	39.388528°N, 24.170611°E
Psathoura	Psathura	PSA	39.498472°N, 24.179778°E
Mikropsathoura	Psathonisi, Myga	MPS	39.482583°N, 24.181444°E
Piperi	–	–	39.348333°N, 24.324333°E

ÖGH Herpetozoa
Austrian Herpetological Society

Herpetozoa 37: 257–267 (2024)
DOI 10.3897/herpetozoa.37.e125163

Assessing the vulnerability of a sky island lizard to climate and land-use change

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https://zoobank.org/AFD1B440-2542-44F8-906E-C03560963D65

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Academic editor: Lukas Landler ♦ **Received** 10 April 2024 ♦ **Accepted** 30 July 2024 ♦ **Published** 16 August 2024

Abstract

Under climate change, species are expected to migrate along with their climate envelope. However, many species' distribution models do not include the human footprint, thus overestimating distributional zones with high probabilities of occurrence. Species inhabiting sky islands (high-elevation landscapes that differ from landscapes in intermediate valleys) are particularly sensitive to climate and land-use change, given their limited ability to migrate. We aimed to assess the suitability of the climatic conditions for a sky island lizard under different climate scenarios and how that could affect its distribution based on (i) its climate envelope and (ii) the human footprint (croplands and buildings). Using climatic variables to develop a species distribution model and the indicator Human Footprint, we predicted the presence probabilities of *Liolaemus nigroviridis* Müller & Hellmich, 1932 populations under climate change scenarios (current, year 2040, and year 2080). We analyzed the relevant variables for *L. nigroviridis*'s climate envelope, which we projected to decrease and shift southward by 2080. The species could track its climate envelope in the Andes, but not in the Coastal mountains, given the strong human footprint. We propose assisted migration as a possible adaptive strategy, and show that conservation of sky islands species can be enhanced by integrating climatic and anthropogenic factors.

Key Words

assisted migration, climate envelope, conservation, human footprint, Liolaemidae, management, Random Forest, species distribution model

Introduction

Currently, biodiversity is rapidly declining due to climate change and habitat modification such as land-use change (Gardner et al. 2007). Biological conservation in the face of climate and land-use change is a key challenge, given that these phenomena will increase in future scenarios (Kaky and Gilbert 2017; Newbold 2018). Studying the effects of future climate and land-use change on species distribution is fundamental to managing informative activities for conservation of biodiversity (Kaky and Gilbert 2017; Baker et al. 2021). One way in which conservation biologists have addressed this challenge is by developing species distribution models (SDMs) to understand changes in distribution that might occur due to climate change (Hijmans and Graham 2006; Seo et al. 2009; Sunny et al. 2019). The climatic conditions that define a species' distribution at a given time form its climate envelope (Chardon et al. 2015). There are studies aimed to determine changes in a species' climate envelope with a view to future climatic scenarios (Iverson and McKenzie 2013; Laspiur et al. 2021; Shadloo et al. 2021). However, incorporating land-use indicators in these studies, such as the Human Footprint (Rosas et al. 2021; Sun et al. 2021), allows us not to overestimate the possible areas of species occupancy in current and future scenarios (e.g. Sofi et al. 2023).

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Species with dispersal ability tend to follow their climate envelope as long as there are no land-use changes preventing their movement (Sun et al. 2021). For example, most taxa would move latitudinally and altitudinally in South America with increases in temperature and drier climatic conditions (Freeman et al. 2018; Laspiur et al. 2021), seeking out zones of higher humidity, lower temperatures, and lower anthropic pressure (Sáenz-Romero et al. 2015). Dispersal can occur on its own (in highly vagile species) or can be achieved through assisted migration (i.e. human intervention to assist a species in moving to a new location that is more suitable for its biological fitness; Butt et al. 2021) in low-vagility species that are not capable of moving great distances, overcoming geographical barriers, or adapting to habitats modified by anthropic pressure (Vitt et al. 2009; Butler 2019).

Sky island species (i.e. species inhabiting patches in elevated zones that differ notably from patches in intermediate valleys; Shepard and Burbrink 2008; Cianferoni et al. 2013) are particularly sensitive to climate and land-use change, given that they would have increasingly restricted space due to the reduction of available area (Shepard and Burbrink 2008; Sáenz-Romero et al. 2015). As the temperature and precipitation patterns shift toward a more arid climate, the climate envelope for a species will decrease in size or shift toward southern latitudes (Deutsch et al. 2008; Fuentes-Castillo et al. 2019, 2020). In addition, it is expected that species inhabiting sky islands will undergo altitudinal displacements as they track their climate envelope and respond to the altitudinal advance of the urban border (forced altitudinal displacements due to land-use change; Sáenz-Romero et al. 2015).

Herpetozoans are one of the groups most affected by climate and land-use change (Gardner et al. 2007; Cordier et al. 2021), and are considered the most threatened group of vertebrates worldwide (Gibbons et al. 2000; Cordier et al. 2021). Reptiles have been affected by climate and land-use change, resulting in population declines, range shifts, and local extinctions (Gardner et al. 2007; Bellard et al. 2012; Winter et al. 2016). This conservation problem is present in areas of the southern cone of South America (Cordier et al. 2021). One of these areas is central Chile, which is one of the priority zones for conservation worldwide (Brooks et al. 2006) and is considered a nucleus of high risk in terms of future climate and land-use change (Marquet et al. 2010; Benavidez-Silva et al. 2021). Further, Chilean reptiles are characterized by their high degree of endemism (60%), and 45.9% are currently in some category of threat (Ruiz de Gamboa 2020). The species that inhabit the Chilean central zone are at risk due to future population declines and/or local extinctions (Marquet et al. 2010). Few reptile species from the central zone inhabit the sky islands (Fuentes and Jaksic 1979; Carothers et al. 2001; Mella and Mella-Romero 2020). Among them, *Liolaemus nigroviridis* Müller & Hellmich, 1932 is an endemic Chilean lizard inhabiting the sky islands of both Coastal and Andean mountain ranges, in altitudinal floors preferably between 2,000 and 2,800 m a.s.l. (Mella-Romero et al. 2023). *Liolaemus nigroviridis* is distributed from the southern Coquimbo Region (30°S) to the northern O'Higgins Region (34°S) (Cianferoni et al. 2013; Mella-Romero et al. 2023). Although *Liolaemus nigroviridis* is considered by the IUCN as Least Concern (Mella-Romero et al. 2023), this species would be facing threats derived from climate change (Mella-Romero et al. 2024) and anthropization (Moya et al. 2024) and is thus of conservation concern.

Within this context, we assessed the suitability of the climatic conditions for *L. nigroviridis* under different climate scenarios and how that may affect its distribution based on (i) its climate envelope and (ii) the human footprint. We hypothesized that *L. nigroviridis* will show a southward distribution shift and a reduced geographic range (limited to higher altitudes compared to the current distribution), assuming that the human footprint does not affect this movement in its climate envelope.

Materials and methods

To determine potential distributional changes based on the species' climate envelope, we developed an SDM to identify areas with the most suitable climatic conditions for the species and to provide presence probabilities for the future scenarios. Regarding the development of SDMs, studies have demonstrated the effectiveness of machine learning algorithms compared to other methods, such as logistic regression (Prasad et al. 2006; Cutler et al. 2007; Benito et al. 2013; Laspiur et al. 2021). Therefore, we selected Random Forest (RF) for obtaining the models, since it is a widely used algorithm in SDM studies (e.g. Laspiur et al. 2021; Shadloo et al. 2021).

Presences and pseudoabsences

We used a total of 199 georeferenced records from 53 different localities gathered from literature reviews, online museum collection catalogues, and web platforms (GBIF and iNaturalist) (Mella-Romero et al. 2023). These 199 points were obtained after a rigorous process of filtering the raw data (cleaning and validation; for details, see table 1 in Mella-Romero et al. 2023). The 199 occurrences cover the known range of the species in both the Coastal and Andean mountain ranges. Non-georeferenced records were not considered, as suggested by Zermoglio et al. (2020).

Pseudoabsences (n = 597) were generated using the BIOMOD2 package (version 4.2.5; Thuillier et al. 2023) in R software (version 4.3.2), during the data-formatting process with the BIOMOD_FormatingData function. We performed a preliminary sensitivity analysis to assess the impact of the number of pseudoabsences on model performance. This analysis was performed according to the recommendations of Barbet-Massin et al. (2012) and the BIOMOD team (2023) for RF models, which confirmed that 597 was an adequate number for maintaining accuracy without overfitting the model. This was carried out with TSS (True Skill Statistic) validation ($TSS = 0.86$).

Climatic variables

For the inclusion of climatic variables in the model, we relied on: (i) the information on the biology/ecology of the study species (thermoregulation in the context of climatic variables); (ii) the background literature on *Liolaemus* species with similar habitat requirements in terms of variables used for SDMs; (iii) multicollinearity among the climatic variables; and (iv) RF Importance index. These selection criteria were applied to the 19 climatic variables of the WorldClim 2 dataset (Schmidt et al. 2006; Fick and Hijmans 2017) (Table 1).

Liolaemus nigroviridis is a reptile species that, like other members of the genus *Liolaemus*, depends on ambient temperature for thermoregulation (Labra et al. 2008). These reptiles use the warm months for such activity, while they markedly decrease their activity or remain inactive during the cold months (Labra et al. 2008; Llanqui et al. 2022). Therefore, a relevant *a priori* variable that we should consider is the seasonality of temperature. From our review of existing works that have developed climate envelope models for *Liolaemus* species (e.g. Winck et al. 2014; Demangel et al. 2015; Laspiur et al. 2021; Vera et al. 2023), we found that the seasonality of temperature is indeed a relevant variable used for modeling (Bio4). In these reviewed works, we noticed that two precipitation variables were frequently used: precipitation of driest quarter (Bio17) and precipitation of warmest quarter (Bio18).

Then, to refine the selection of variables, we evaluated the multicollinearity and importance of the climatic variables in two stages. In the first step, we used the Variance Inflation Factor (VIF) from R package USDM (Naimi and Araújo 2016) to identify and quantify the multicollinearity; VIF quantifies how much of a variable's variance is explained by its correlation with other variables in a model (Craney and Surles 2002). Using this approach, we excluded all the highly correlated variables from the model (VIF greater than 10; see e.g. Jara et al. 2019). We took into consideration the remaining uncorrelated variables: mean diurnal range (Bio2), seasonality of temperature (Bio4), minimum temperature of coldest month (Bio6), annual precipitation (Bio12), and precipitation of warmest quarter (Bio18). In a second stage, we applied an RF regression with R package RANDOMFOREST (Liaw and Wiener 2002), configuring with 500 trees to obtain the RF Importance index (and thus evaluate which variables would be the most relevant). The higher the value of this index, the greater the relevance of the variable (Kamusoko et al. 2014; Laspiur et al. 2021). We considered variables with an RF Importance index greater than 30 (especially if that variable was relevant according to the criteria previously described): mean diurnal range (Bio2), annual precipitation (Bio12), precipitation of driest quarter (Bio17), and precipitation of warmest quarter (Bio18). These last 2 variables had the highest RF Importance index values (77.04 and 71.04, respectively). This approach, combining VIF analysis with RF, offered a nuanced understanding of each variable's contribution to the species' ecological model (see Laspiur et al. 2021).

Therefore, according to the previously mentioned criteria, we decided to model with the variables mean diurnal range (Bio2), seasonality of temperature (Bio4), minimum temperature of coldest month (Bio6), annual precipitation (Bio12), precipitation of driest quarter (Bio17), and precipitation of warmest quarter (Bio18).

To describe the change in the climatic variables, we calculated the rate of change (expressed in %) between the current model and the 2080 (RCP 8.5) scenario for each variable obtained from the WorldClim 2 dataset (Schmidt et al. 2006; Fick and Hijmans 2017), using the mean values of the variables (Table 1). To do this, from the clipping of each layer (by area of interest), we extracted the values using R software (SUMMARY() function). This allowed us to identify the magnitude of change in each of the 19 WorldClim variables and thus obtain complementary information to analyze how these changes could affect the biology/ecology of the species.

Estimating species distribution models (SDMs) and climate envelope

To determine habitat suitability of the climate envelope under current and future conditions, we used the six raster layers (Bio2, Bio4, Bio6, Bio12, Bio17, Bio18) out of the 19 climatic variables at a 30-second (1 km) resolution from the WorldClim 2 dataset (Schmidt et al. 2006; Fick and Hijmans 2017). For all variables, we included future climatic projections for CMIP6 data at 30-second spatial resolution (model MPI-ESM1-2-HR) for RCP (representative concentration pathways) 4.5 and 8.5 experiments, projected for years 2040 and 2080 (also available at https://www.worldclim.org/) (Eyring et al. 2016). Scenario 8.5 is the most catastrophic in terms of greenhouse gas emissions (Babaeian et al. 2021). The use of CMIP6 has been validated in studies of montane species due to its high accuracy (e.g. Laspiur et al. 2021).

All SDM visualizations for present and future projections were performed using R modeling package BIOMOD2. We employed a five-repeat scheme (Run1 to Run5), focusing on an RF algorithm with 2,000 trees (see e.g. Laspiur et al. 2021). The dataset was divided into 80% for training and 20% for testing in each run (Pham and Tran 2022). This approach allowed for a more reliable and accurate evaluation of the model, ensuring that our results more accurately reflected the model's performance under various data divisions (Breiman 2001; Liu et al. 2023). After the evaluation, the best-performing model was selected based on the highest scores in TSS and ROC (Area Under the Receiver Operating Characteristic Curve) metrics, ensuring the most accurate and reliable representation of the data (Phillips et al. 2006; Rather et al. 2020; Shadloo et al. 2021).

With the best model (according to metrics), we developed projected habitat suitability maps for *L. nigroviridis* under present and future climatic conditions in RCP 4.5 and RCP 8.5 scenarios. Then, these projections were visualized as distribution maps to show the geographical zones of high and low suitability probability of species presence (Fig. 1). Scenarios 4.5 and 8.5 have been used over other models in studies on herpetozoans of the South American southern cone, which makes these studies comparable (see e.g. Jara et al. 2019; Laspiur et al. 2021).

To analyze potential changes in suitable habitat distribution for *L. nigroviridis*, we used the BIOMOD_RANGESIZE function from BIOMOD2 (following the method described by Liu et al. 2023). The BIOMOD_RANGESIZE function allowed us to identify areas predicted to be lost, remain stable, or gain suitability, as well as to calculate their percentages of total expected change (Table 2). Subsequently, the distributions of high suitability (> 0.6) were processed in the open-source Geographic Information Systems software QGIS (version 3.22) to determine the surface area in square kilometers. Although we show the values for all scenarios in Table 2 [i.e. Current; 2040 (4.5, 8.5); 2080 (4.5, 8.5)], in the results and discussion sections we generally use only the current and the most catastrophic greenhouse gas emission (2080; 8.5) scenarios, for purposes of establishing a more contrasting comparison and synthesizing the obtained results.

Human footprint

While an SDM based solely on climatic variables can effectively depict a species' distribution on a broad scale, it may overestimate its regional distribution by including areas with unsuitable habitats due to land use. Many studies that model habitat suitability do not consider this factor (Santini et al. 2021). To minimize overestimation and obtain a qualitative visualization, we use the land-cover layer HUMAN FOOTPRINT available through worldwide land-cover mapping (WorldCover; Zanaga et al. 2022). This layer was included in the model *a posteriori*. Thus, present and future scenarios incorporated the information obtained from this layer (cropland and built environments, Zanaga et al. 2022) through QGIS, thereby increasing their realism (in the most conservative scenario regarding land use).

To produce the maps, habitat projections for *L. nigroviridis* generated under an RF algorithm at current and future scenarios (GCM: MPI-ESM1-2-HR, https://www.worldclim.org/), with a human footprint layer (WorldCover, http://https://viewer.esa-worldcover.org/worldcover/), Chile regional divisions (Biblioteca del Congreso Nacional de Chile, https://www.bcn.cl/), and 199 occurrences (Mella-Romero et al. 2023), were used.

Results

Climatic variables

When analyzing the climatic variables (Table 1) numerically, all variables related to temperature (Bio1 to Bio11; °C) increased between the current scenario and 2080 (8.5), and all the variables related to rainfall and humidity (Bio12 to Bio19; mm) decreased in the mentioned time range (except the variables associated with rainfall in warm months: Bio14, Bio17, and Bio18) (Table 1). Regarding the variables we selected for the model, (i) minimum temperature of coldest month (Bio6) was the variable that showed the greatest change, increasing 70.97% by 2080 (8.5); (ii) annual precipitation (Bio12) decreased 28.90% toward the 2080 (8.5) scenario; and (iii) precipitation of driest quarter (Bio17) and precipitation of warmest quarter (Bio18) remained constant at all times analyzed (Table 1).

Table 1. Percentage of change between the current and the 2080 (RCP 8.5) scenarios for each variable. We developed this table using the mean values of the variables, obtained from Worldclim 2 dataset (Fick and Hijmans 2017). From the clipping of each layer (by area of interest), we extracted the values using the SUMMARY() function of R software. ED: Standard deviation; Coef.: Coefficient of variation.

Variable	ID	2080 (8.5)/ Current	% of change 2080 (8.5)
Annual mean temperature	Bio1 (°C)	1.21	21.39
Mean diurnal range	Bio2 (°C)	1.03	2.71
Isothermality	Bio3 (%)	1.01	0.70
Temperature seasonality	Bio4 (ED)	1.02	1.81
Max. temperature of warmest month	Bio5 (°C)	1.11	11.44
Min. temperature of coldest month	Bio6 (°C)	1.71	70.97
Temperature annual range	Bio7 (°C)	1.03	2.88
Mean Temperature of wettest quarter	Bio8 (°C)	1.25	25.24
Mean temperature of driest quarter	Bio9 (°C)	1.20	19.53
Mean temperature of warmest quarter	Bio10 (°C)	1.16	16.28
Mean temperature of coldest quarter	Bio11 (°C)	1.30	30.23
Annual precipitation	Bio12 (mm)	0.71	-28.90
Precipitation of wettest month	Bio13 (mm)	0.64	-35.62
Precipitation of driest month	Bio14 (mm)	1.00	0.00
Precipitation seasonality	Bio15 (Coef.)	0.92	-8.48
Precipitation of wettest quarter	Bio16 (mm)	0.67	-33.15
Precipitation of driest quarter	Bio17 (mm)	1.00	0.00
Precipitation of warmest quarter	Bio18 (mm)	1.00	0.00
Precipitation of coldest quarter	Bio19 (mm)	0.67	-33.33

Estimating species distribution models (SDMs) and climate envelope

The best-performing model obtained a *TSS* of 0.86 and a *ROC* of 0.98. For this type of algorithm (i.e. machine learning: RF), a value of *TSS* and *ROC* > 0.85 is considered indicative of good performance (Rather et al. 2020).

The BIOMOD_RANGESIZE function indicated that under the 2080 (8.5) scenario, the areas with probable presence of the species (i.e. > 0.6) would decrease by 42.5% compared to the current scenario (3,577 km^2 to 2,058 km^2) (Table 2).

Table 2. Percentage change of the *Liolaemus nigroviridis* climate envelope in different scenarios (areas with probability of occurrence > 0.6). Calculations based on surface area (km^2) using the function BIOMOD_RANGESIZE of R software.

Scenario	Area (km^2)	%Area Gain	%Area Loss	%Total Change
Current	3,576.9	-	-	-
2040 RCP 4.5	3,538.2	26.4	27.5	-1.1
2040 RCP 8.5	3,465.7	33.2	36.3	-3.1
2080 RCP 4.5	3,336.3	29.4	36.1	-6.7
2080 RCP 8.5	2,058.0	16.6	59.1	-42.5

There was no climate envelope for the species in the Coquimbo Region under the 2080 (8.5) scenario (regional extinction, purple rectangle of Fig. 1D). A small zone of occupation remains present in the mountains of the Coastal range (near to Coltauco, current location inhabited by *L. nigroviridis*) (pink rectangle of Fig. 1G and Fig. 2B) in the future. As for the Andean range, under the 2040 (4.5) scenario, the climate envelope was projected to move a maximum of 33.8 km to the south (zones of high probability of presence; > 0.6) (Fig. 2A). Under the 2080 (8.5) scenario, the *L. nigroviridis* distribution was projected to

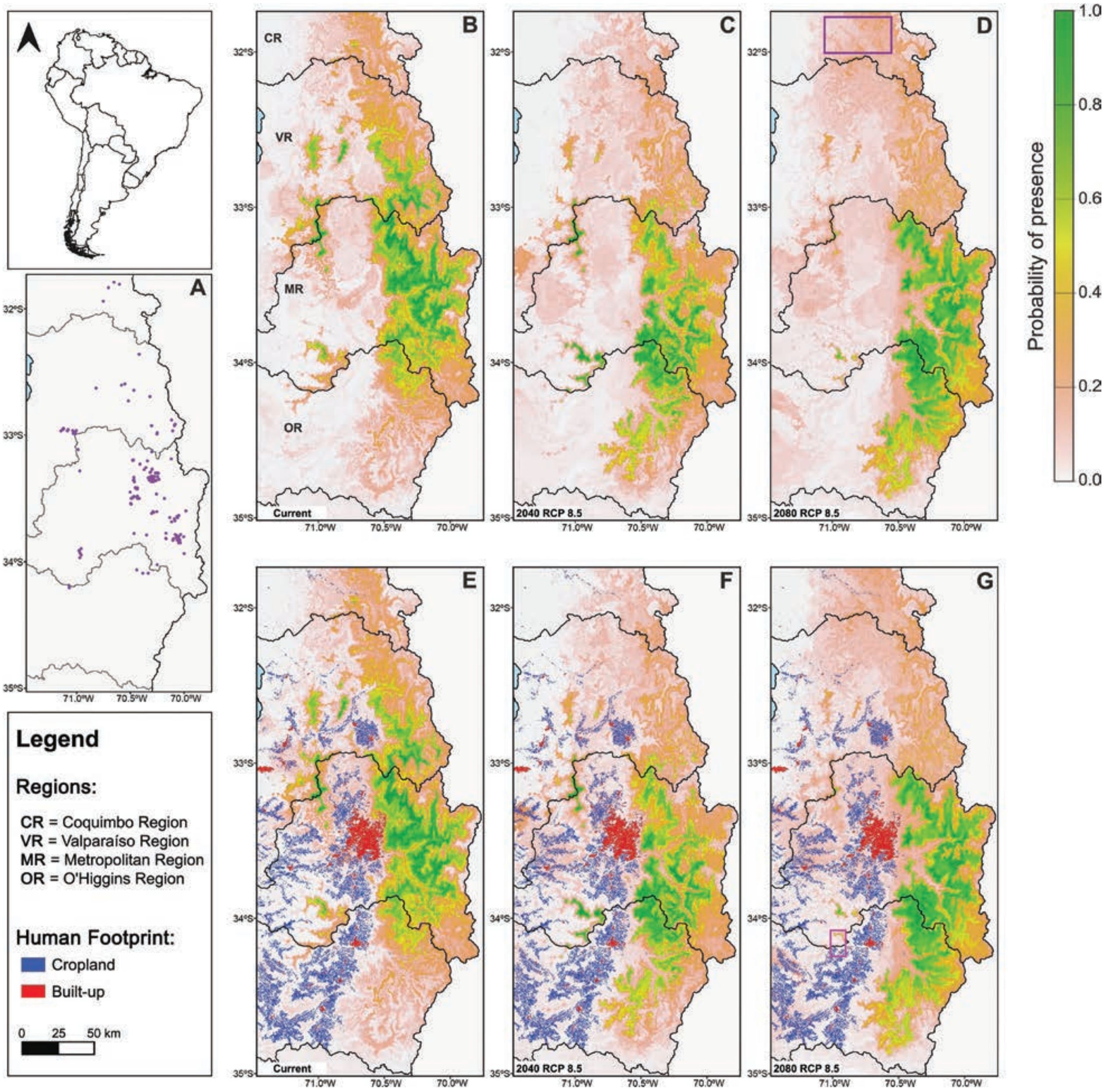

Figure 1. SDMs for *Liolaemus nigroviridis*. Habitat projections for *L. nigroviridis* generated under climatic layers (GCM: MPI-ESM1-2-HR); human footprint layer (WorldCover); Chile regional division (Biblioteca del Congreso Nacional de Chile); 199 presence points (panel **A**, Mella-Romero et al. 2023); and Random Forest algorithm at current (panels **B** and **E**), 2040 RCP 8.5 (panels **C** and **F**), and 2080 RCP 8.5 (panels **D** and **G**) projections. The purple rectangle (panel **D**) indicates the regional extinction in the Coquimbo Region. The pink rectangle (panel **G**) indicates a zone of hills adjacent to Coltauco. These maps were produced in the R environment (R Core Team, version 4.3.2) using the packages: BIOMOD2 version 4.2.4 (Thuiller et al. 2023) and TERRA version 1.7.65 (Hijmans et al. 2023). Spatial integration of the layers was performed using QGIS software version 3.32. Coordinate system: WGS84.

reach Chimbarongo-Las Peñas (north of Santuario de la Naturaleza Alto Huemul) (Fig. 2B; end of the vertical red line). Under 2080 (8.5), the climate envelope was projected to move a maximum of 60.6 km to the south (zones of high probability of presence; > 0.6) (Fig. 2B). The model indicated that the climate envelope of the species would move latitudinally (toward the south) but not longitudinally (in altitude) in the future (Figs 1, 2).

Human footprint

The human footprint (croplands and buildings) in the Coastal mountain range was greater than that in the Andean mountain range (Fig. 1E–G). Coastal mountain range presents a strong human footprint (including Coltauco to the south, Fig. 1G; pink rectangle).

Discussion

In this study, we assessed the suitability of the climatic conditions for a sky island lizard under different climate scenarios and how that may affect its distribution based on its climate envelope and human footprint (croplands and buildings). For this purpose, we hypothesized that *L. nigroviridis* will undergo a distributional shift toward the south, with a smaller geographical range limited to higher altitudes compared to its current distribution, given the pressures of climate and land-use change. We corroborated our hypothesis regarding the latitudinal (southward) movement of the species' climate envelope into the future and the decrease of the same, but not regarding the longitudinal (altitudinal) movement and the human footprint, which would affect a potential future migration of the species in the Coastal mountain range, but not in the Andean mountain range.

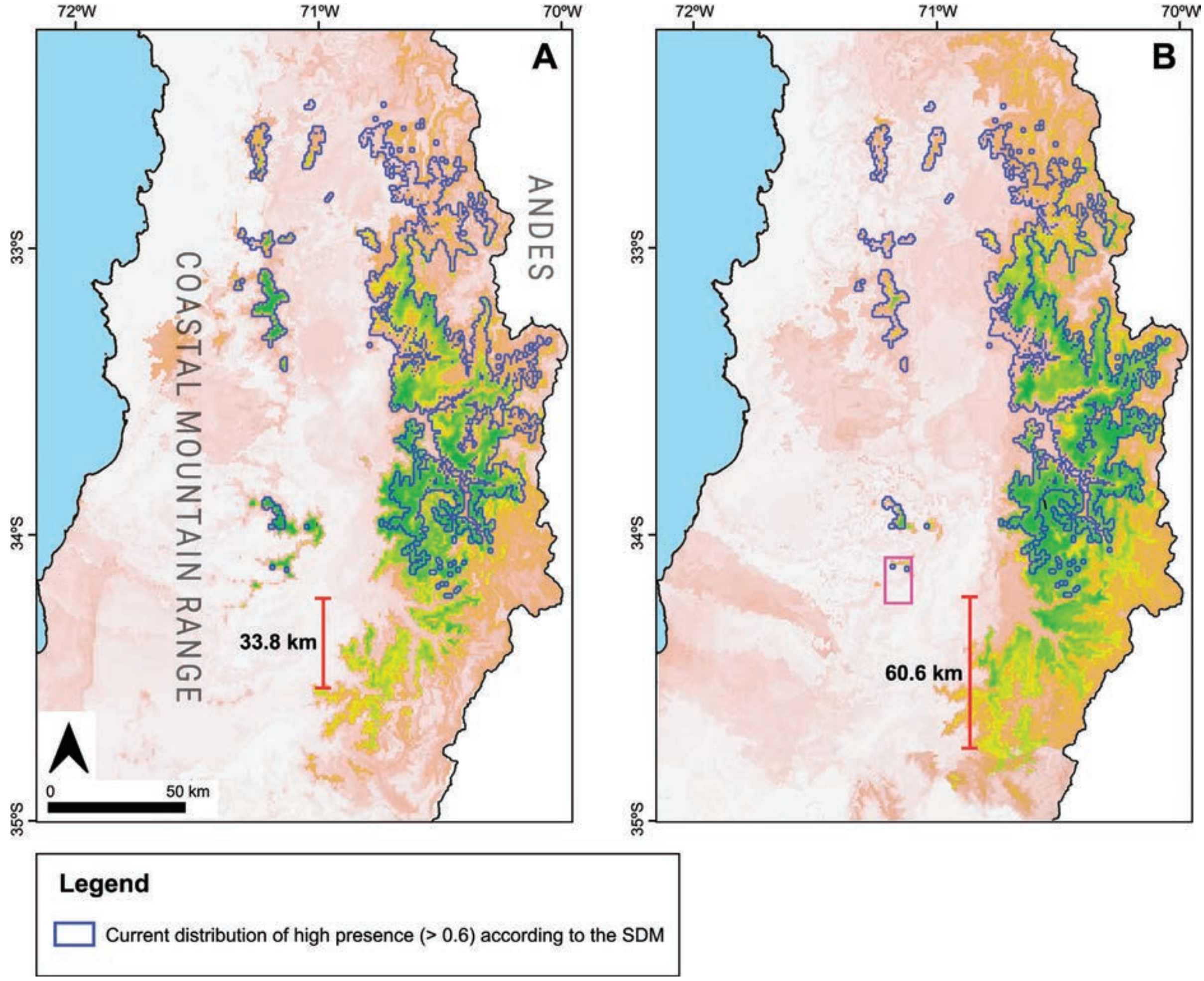

Figure 2. Southward movement of the climate envelope for *Liolaemus nigroviridis*. Habitat projections for *L. nigroviridis* generated under climatic layers (GCM: MPI-ESM1-2-HR) for the years 2040 (panel **A**) and 2080 (panel **B**) under RCP 8.5 conditions. In both panels, the blue outline indicates the current distribution with a probability presence > 0.6, as generated by the model (based on the Random Forest algorithm). The red vertical lines represent the climate envelope expansion towards the south in the Andean mountain range. The pink rectangle indicates the area that remains towards the future in the Coastal mountain range (near Coltauco). These maps were produced in the R environment (R Core Team, version 4.3.2) using the packages: BIOMOD2 version 4.2.4 (Thuiller et al. 2023) and TERRA version 1.7.65 (Hijmans et al. 2023). The filtering based on the presence probability threshold > 0.6, shown and represented by the blue outline, was performed using the THRESHOLD COLOR function of the ImageJ software (Schneider et al. 2012), applied to the BIOMOD2 current projection. The spatial integration of the layers, the blue outline, measurements, and integration of red vertical lines was performed using QGIS software version 3.32. Coordinate system: WGS84.

Habitat suitability for *L. nigroviridis* (in terms of high probability of occurrence: > 0.6) decreased by 42.5% in the most catastrophic future scenario (2080; 8.5). The reduction of the species' climate envelope in the future was remarkable, with no high probability of the species' presence in the Coquimbo Region (Fig. 1D), where *L. nigroviridis* is currently present (Cianferoni et al. 2013; Mella-Romero et al. 2023). Regarding the latitudinal movement of the future climate envelope, the new habitat suitable for *L. nigroviridis* for year 2080 was located up to 60.6 km to the south of its current range in the Andes (in the mountains to the north of Alto Huemul; end of the red vertical line in Fig. 2B). This southward movement was notable in the Andes range, but it was practically nil in the Coastal range, with the mountains in the northern zone of Coltauco being the limit of its climate envelope even by 2080. This, together with the existence of a strong human footprint in the vicinity and to the south of Coltauco, shows limitations for the dispersal of populations that inhabit the Coastal range in future scenarios after 2080.

The decrease and latitudinal movement of the species' climate envelope in the future can be explained by the most relevant climatic variables (according to the biology of *L. nigroviridis* and RF Importance index): temperature seasonality, annual precipitation, precipitation of the driest quarter, and precipitation of the wettest month. These variables are closely associated with precipitation/humidity and its seasonality. All the variables related to temperature showed an increase between the current scenario and 2080 (8.5), while the variables related to rainfall and humidity showed a decrease in the same time range (except those associated with rainfall in warm months) (Table 1). This implies that rainfall in the warm months was equally scarce in all temporal scenarios, but in the cold months, it decreased considerably with increasing time (Table 1). This implies a consequent decrease in available humidity in the Chilean central zone. For instance, the minimum temperature of the coldest month increased considerably toward the 2080 scenario (Table 1), which would also affect the decrease in humidity and indicated that the central zone would indeed be warming up. These findings are consistent with those reported by other research groups analyzing the zone in the context of climate change, both at the species and ecosystem levels (Marquet et al. 2010; Pliscoff and Uribe-Rivera 2020; Torres et al. 2022).

Precipitation and humidity directly influence the existence of high Andean shrubs, which play a significant role in the thermal ecology of *Liolaemus* species (Labra et al. 2008; Laspiur et al. 2021; Cruz et al. 2022). A decrease in precipitation during cold months could affect the recruitment and growth of plants, thereby reducing the number of shrubs available as refuges (see e.g. Sinervo et al. 2010; Laspiur et al. 2021). Likewise, rainfall and humidity in the warm months also play a significant role in maintaining these shelters, which provide the lizards the possibility of avoiding dehydration through behavioral thermoregulation (Labra et al. 2008; Brizio et al. 2021; Laspiur et al. 2021; Cruz et al. 2022). This is supported by field observations and literature; the general pattern observed involves individuals sunning themselves directly in the morning, and retreating to shaded patches of shrubs to avoid overheating (which leads to dehydration) before noon (Laspiur et al. 2021). As a result, the number of active lizards outside the shelters would progressively decrease during the hottest hours, being forced to retire to shaded microhabitats, seeking optimal thermal quality (Laspiur et al. 2021). This would imply an increase in the hours of activity restriction (Sinervo et al. 2010; Cruz et al. 2022; Mella-Romero et al. 2024); that is, the lizards wait until suitable conditions of heat and humidity occur to resume their active behavior. This phenomenon may increase the local extinction risk of a population (Sinervo et al. 2010; Huey et al. 2012).

Additionally, humidity can significantly impact insect abundance (*L. nigroviridis* is mainly insectivorous; Labra et al. 2008), as it affects several aspects of insect life history traits, including reproduction and survival (He et al. 2021). In general, high humidity is favorable for the survival and reproduction of insects (He et al. 2021), while low humidity can reduce the survival of some insect species and limit their capacity to reproduce (Miedaner and Juroszek 2021). In dry environments, such as those predicted for central Chile by 2080, certain insect species might have difficulty finding water, leading to death by dehydration (Thorat and Nath 2018). Furthermore, humidity can affect insect migration (He et al. 2021; Miedaner and Juroszek 2021) due to some insect species migrating in response to drought patterns and possibly being attracted to wet regions or repelled by dry regions (Miedaner and Juroszek 2021). The aforementioned factors could lead to a decrease in the primary food resource of *L. nigroviridis*, thereby affecting its biological fitness.

Despite the hypothesis that biota in South America's southern cone may need to seek higher altitudes to escape high temperatures caused by climate change (see e.g. Sáenz-Romero et al. 2015), our analysis suggests that *L. nigroviridis* will not follow this pattern. Instead, our model showed only decreases in the high probability of its presence at the same altitude in the future (as shown in Figs 1D, 2). Although this hypothesis has been suggested for the biota of the zone, our results show that the species-specific climate envelope does not shift in altitude at least until 2080 under the RCP 8.5 scenario (there is no high probability of the species' presence at altitudes higher than those it currently occupies). In the Coastal mountain range, the species cannot climb since it already occupies the highest altitudes available (2,281 m a.s.l.; Mella-Romero et al. 2023). On the other hand, in the Andes there is an extensive network of peaks above 4,000 m a.s.l. that would not be occupied by the species according to our model. This could be explained by the magnitude of change in temperature and precipitation variables (Table 1) within the analyzed time range (from the present time to year 2080), which may be insufficient for visualizing any significant change in altitudinal displacement. The extent of this displacement varies depending on the taxa analyzed and their specific context, and may take from decades to centuries (Kwon et al. 2016; Guaraldo et al. 2022). In some cases, this event can occur within a few decades in response to extreme weather phenomena (White 2016). Generally, a shift in altitude over short periods of time is more easily observable in organisms with high vagility, such as birds (Kwon et al. 2016; White 2016). Our results coincide with what was proposed by Marquet et al. (2010) for *Liolaemus bellii*, in terms of a decrease in the distribution range of the species in future scenarios, although that study did not refer to latitudinal or longitudinal movement.

Thus, our analysis suggests that *L. nigroviridis* will have a more restricted climate envelope in future scenarios and, therefore, would seek to track the current climatic conditions to the south, especially in the Andes, where there is a low human footprint. The populations of these mountains have geographical accidents as dispersal barriers, such as river basins (Lamborot and Eaton 1997), but not croplands and buildings. The populations in the Coastal mountain range would also have to navigate a vast network of human footprints (even considering the most conservative scenario of land-use change, as in this study) if the climate envelope does indeed shift southward in a future beyond 2080. Therefore, assisted migration appears as a plausible alternative for *L. nigroviridis* (especially the populations of the Coastal mountain range) and other species with the described future limitations. Assisted migrations mainly apply to species with low vagility (Vitt et al. 2009), but they could also apply to species that, despite having a dispersal capacity, are restricted from following their climate envelope due to the presence of anthropogenic barriers, such as the potential case of *L. nigroviridis* in the Coastal range.

Assisted migrations have recently been proposed as adaptive strategies to climate change (Müller and Eriksson 2013; Dunlop et al. 2021; Lavrik 2021). In Chile, environmental public policies have focused on implementing climate change law in recent years. Nevertheless, the topic of biodiversity and climate and land-use change is absent from the current discussion, despite (i) the clear evidence that without biodiversity we cannot make adjustment plans to face climate change (Butt et al. 2021), and (ii) the dramatic reports from the IPBES (Intergovernmental Science-Policy Platform on Biodiversity and Ecosystem Services) and the IPCC (Intergovernmental Panel on Climate Change). Studies like ours may foreground the discussion about the usefulness of assisted migrations in the current context of climate and land-use change at the national level (especially with the future implementation of the Biodiversity and Protected Areas Service), a discussion that is already taking place globally (Müller and Eriksson 2013; Dunlop et al. 2021; Lavrik 2021).

Our work has the scope to suggest the vulnerability to climate change of other reptile species present in the area (e.g. *Liolaemus bellii*, *Liolaemus leopardinus*, and *Pristidactylus volcanensis*; Mella-Romero and Mella 2023), given their similar habitat requirements (Marquet et al. 2010; Santoyo-Brito et al. 2020). However, to obtain more specific information on how climate change would affect the thermoregulatory behavior of these species, additional work is needed, with ecophysiological sampling during all seasons of the year (e.g. measurements of body and critical temperatures, see Laspiur et al. 2021; Cruz et al. 2022). Our research demonstrates how *L. nigroviridis* could be affected in a relatively short time and, therefore, helps to assess the vulnerability of its populations to climate and land-use change. SDMs that incorporate information on the human footprint are thus valuable tools for developing biological conservation initiatives. We conclude that it is necessary to incorporate the human footprint into species distribution analyses because it can have heterogeneous effects on the future habitat availability on which the species will depend.

Acknowledgements

JM-R thanks to ANID; CONICYT-PCHA, Doctorado Nacional/2019-21190472 for financing his postgraduate studies. The authors thank two reviewers, who improved the manuscript with their comments.

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ÖGH Herpetozoa
Austrian Herpetological Society

Herpetozoa 37: 269–280 (2024)
DOI 10.3897/herpetozoa.37.e130731

Phenological, ecological, and demographic data of the slow worm (*Anguis fragilis*) population from southern Catalonia (Spain)

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Academic editor: Lukas Landler ◆ **Received** 28 June 2024 ◆ **Accepted** 11 August 2024 ◆ **Published** 29 August 2024

Abstract

Studies on the slow worm (*Anguis fragilis*) in the Iberian Peninsula have primarily focused on northern and north-western populations, with limited data, based mainly on distribution short notes, on the north-eastern populations in Spain. This study examines the ecology, demography, and phenology of a slow worm population located at a suboptimal site near the southern edge of its distribution range on the Iberian Peninsula, characterized by a Mediterranean climate, in southwestern Europe. The study area encompasses 2.9 hectares in Tarragona Province, Spain, at an altitude of 990 m. Sixteen sampling stations were established to cover all available habitats. In this population, adults comprised 73.73% (n = 87) of individuals, with a sex ratio (♂♂/♂♂+♀♀) of 0.44. Of the captured individuals, 46.61% (n = 55) exhibited non-intact tails. The estimated apparent survival probabilities during the study period (1.5 years) were 0.76 (0.54, 0.94) for immatures, 0.79 (0.29, 0.95) for males, and 0.83 (0.68, 0.96) for females. Population density was estimated at 16.11 ind./ha (7.78, 45.42) for immatures, 44.39 ind./ha (30.38, 72.57) for males, and 64.08 ind./ha (35.35, 129.93) for females. The activity period extends from March to October, with males emerging from hibernation earlier than females, peaking in May. Female emergence occurs one month later, peaking in June. A total of 41.18% of captured males had recent scars and wounds, indicative of fighting during the mating season from mid-April to mid-June. Pregnant females were observed from mid-May to early September, with a peak incidence in June, which is an extended period compared to other European populations at similar altitudes and latitudes.

Key Words

activity cycle, Anguidae, biometry, demography, phenology, population size

Introduction

The slow worm (*Anguis fragilis*) is a lizard with a wide distribution throughout Europe (Strzała et al. 2017; Jablonski et al. 2021). The genus *Anguis* comprises five species: *Anguis fragilis*, *A. veronensis*, *A. cephallonica*, *A. colchica*, and *A. graeca* (Jablonski et al. 2021), previously considered *A. fragilis* (Salvador 1997).

In the Iberian Peninsula, *A. fragilis* is distributed in the northern half, with high abundance in the Cantabrian-Pyrenean strip. It becomes increasingly rare in more southern latitudes, with the populations of the lower Tajo basin in Portugal being the most southern known (Galán 2008; Jablonski et al. 2021). In the northeastern Iberian Peninsula, *A. fragilis* is restricted to Catalonia, where it is abundant in the northern third. From there, the distribution of the species decreases towards the south and west, where it is mainly confined to humid or mountain habitats. The southernmost known populations are located in the Montsant massif in the province of Tarragona (Soler-Massana et al. 2006). However, records of the species in the Ebro Delta confirm the existence of isolated populations further south along the Ebro River, specifically restricted to hygrophilous vegetation habitats within riparian forests (Roig 2008).

Studies on its spatial distribution and reproductive ecology have been published in several European countries outside the Iberian Peninsula (Stumpel 1985; Smith 1990; Platenberg 1999; Ceirans 2004; Brown and Roberts 2008; Haley 2014). Published studies about *A. fragilis* from northeastern Italy (Capula et al. 1992, 1998; Luiselli 1992; Capula and Luiselli 1993) could really be *A. veronensis*, according to Jablonski et al. (2016, 2021) and Gvoždík et al. (2013). According to these authors, the distribution limits of *A. veronensis* and the areas where it overlaps with *A. fragilis* remain poorly understood in the western part of their range (Gvoždík et al. 2013; Renet et al. 2018; Jablonski et al. 2021). Despite this, due to the lack of conclusive evidence, the slow worm from northeastern Italy is included in studies as *A. fragilis* (Mezzasalma et al. 2013).

Several studies have been published on the slow worm in Spain, including works by Braña (1982), Cabido et al. (2004), Ferreiro et al. (2007), Ferreiro and Galán (2004), and Galán and Fernández (1993). These studies have focused on populations in the north and northwest of the Iberian Peninsula. However, there is a lack of published data on the biology, demography, and ecology of *A. fragilis* from the northeastern populations of the Iberian Peninsula. This is probably due to the lack of optimal study areas, populations with generally low numbers of individuals, the scarcity of optimally known populations, and the difficulty of monitoring species with fossorial habits. The climatic factor may also be significant, as the Mediterranean climate has much lower humidity levels, making the area generally sub-optimal for the species.

This study aims to describe the ecology, demography, and phenology of one population of slow worms located in the province of Tarragona, in the southern distribution of the northeastern Iberian Peninsula.

Methods

Study area

The study area is 2.9 ha of plain located within the "Muntanyes ENP de Prades" (Tarragona, Spain) at an altitude of 990 m. The climate in the study area is oro-mediterranean, with cold winters and dry summers. The average annual rainfall in the area is 600–800 mm. It has a high diversity of mosaic habitats (Fig. 1), characterized by the dominance of a wet grassland of mosaic grasses with water points, numerous ground-level shelters, stone walls, and shrubby margins. The surrounding forest is dominated by *Pinus sylvestris*, *Pinus nigra*, and *Cedrus atlantica*, the latter two being

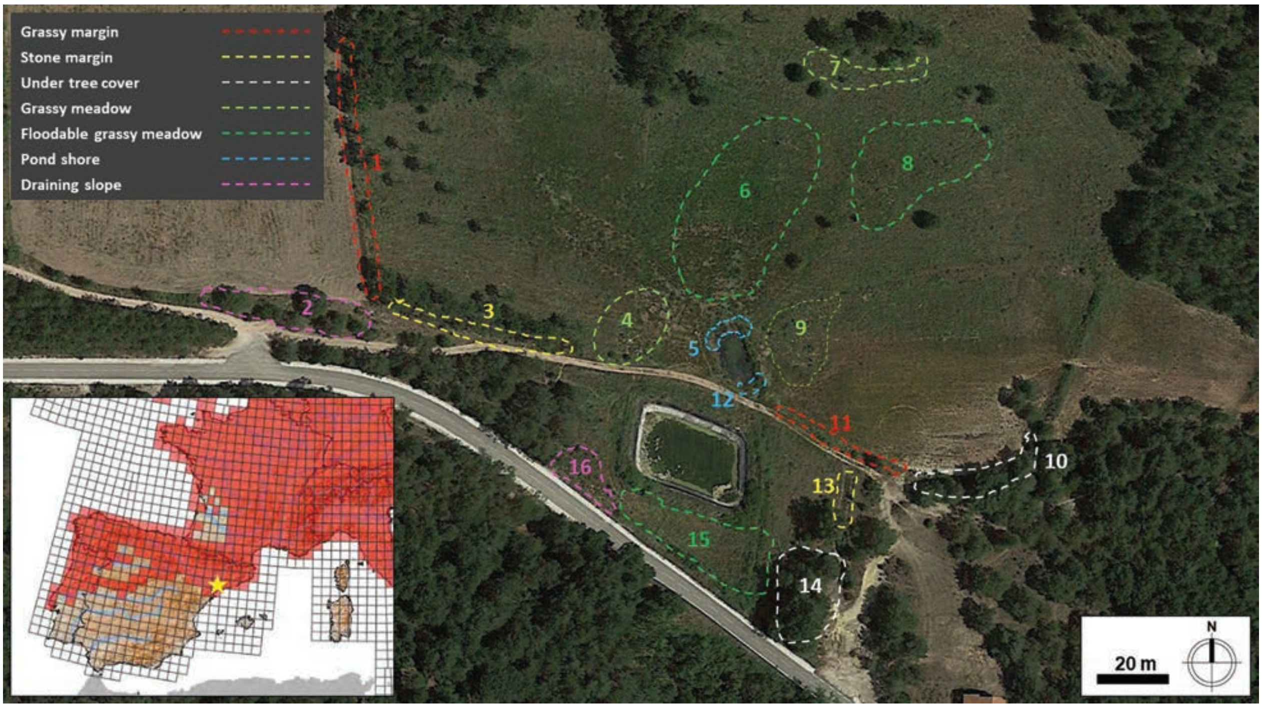

Figure 1. Study area and location of sampling stations: (1, and 11): grassy margin; (2, and 16): draining slope; (3, and 13): stony margin; (4, 7, and 9): grassy meadow; (6, 8, and 15): flooded grassy meadow; (10, and 14): stony margin under tree cover and *Pinus nigra* pine forest; (5, and 12): Pond shore. Low-left map, western distribution in Europe of *Anguis fragilis* (Sillero et al. 2014) with the studied population (yellow star).

non-autochthonous species introduced as a result of various reforestation actions carried out in the area in the 1980s. The central zone consists mainly of meadows that are prone to flooding. Two sub-zones can be distinguished: a floodable zone with a majority presence of *Brachypodium sp.* and *Juncus acutus*, and another one with isolated low and medium-sized shrubs of *Prunus spinosa*, *Crataegus monogyna*, *Juniperus oxycedrus*, *Buxus sempervirens*, and *Rubus caseius*.

The herpetological community includes the following species: *Salamandra salamandra* (Linnaeus, 1758), *Alytes obstetricans* (Laurenti, 1768), *Bufo spinosus* Daudin, 1803, *Epidalea calamita* (Laurenti, 1768), *Pelodytes punctatus* (Daudin, 1802), *Pelophylax perezi* (Seoane, 1885), *Psammodromus algirus* (Linnaeus, 1758), *Podarcis liolepis* (Boulenger, 1905), *Timon lepidus* (Daudin, 1802), *Chalcides striatus* (Cuvier, 1829), *Malpolon monspessulanus* (Hermann, 1804), *Coronella girondica* (Daudin, 1803), *Zamenis scalaris* (Schinz, 1822), *Natrix maura* (Linnaeus, 1758), *Natrix astreptophora* (Seoane, 1884) and *Vipera latastei* (Boscá, 1878) (García-Salmeron et al. 2023; Montori et al. 2024). The grasslands were grazed until recently. Without livestock control, the vegetation has become luxuriant. The human presence in this area is minimal.

In the study area, 16 sampling stations were determined to cover all available habitats and microhabitats (Fig. 1). These stations were situated in areas with concentrations of refugees, primarily characterized by scattered or stacked stones and logs naturally occurring within the study zone. During each visit, a total of 182 shelters were checked, with varying numbers at each station.

Sampling methodology

A systematic sampling of the delimited area was conducted, checking all the shelters at each sampling station. Sampling occurred fortnightly from March 2, 2015, to October 28, 2016, under favorable weather conditions. Sampling was interrupted from November 2015 to February 2016 due to the winter rest period of the species in the area (Roig, Giner, and Gómez, own data). Individuals were captured manually during the period when the animals were concluding their daily activity and settling in their shelters, approximately one hour before and after sunset. The sampling stations and the spatial locations (UTM 31N - ETRS89) of each individual were georeferenced using the HandyMobile GPS App with an accuracy of one meter. The animals were handled using gloves.

The following variables were recorded for each captured individual: sex (when showing secondary sexual characters such as body size, head shape, and coloration), stage (size classes: adult, subadult, and juvenile), weight (with a precision of 0.01 g), snout-vent length (SVL, with a precision of 1.00 mm), and tail length (TL, with a precision of 1.00 mm), recording whether the tail was regenerated or autotomized. Captured individuals with non-intact tails were considered invalid for general biometric studies, except for SVL. Females showing any signs of pregnancy were excluded from the average weight calculation, as they do not represent the average condition of the species.

The size classes were determined according to the criteria of Platenberg (1999), which are based on a stage of growth and maturation that is necessary for reproductive success. Individuals are considered adults when their SVL exceeds 130.00 mm. Those with a SVL below 100.00 mm were classified as juveniles, and those with a SVL between 100 and 130 mm were classified as subadults. In some cases, sexual maturity could not be clearly determined due to inconclusive coloration patterns or the absence of hemipene evagination.

The individuals were initially examined visually for combat marks in males, which could present characteristic markings, with particular attention paid to the size and shape of the jaws of *A. fragilis* on any part of the body. Additionally, pregnancy or copulation marks in females were identified. Copulation marks consist of scale scratches on the neck and head caused by the biting of males during copulation (Rollinat 1934). Finally, the identification of recaptures was also carried out. They were marked using a low-temperature electric cautery (model FIAB7255), commonly used in medical and veterinary practice and previously employed in studies by Ferreiro et al. (2004, 2007). A mark (individual identification code) was assigned to different body marks and dorsal body positions during the marking process, following techniques described by Ferreiro et al. (2007), Ferreiro and Galán (2004), and Stumpel (1985). Subsequently, the marked area was treated with an antiseptic iodine solution. After confirming the animal's condition, it was released back into the same shelter where it was initially captured. Neonates or individuals in very early stages were not marked due to their small size, resulting in most of them (n=18) being excluded from this aspect of the study. No complications arising from the marking method were observed in any of the recaptured animals. The marks remained visible throughout the follow-up period, even up to one year later. The sex ratio was estimated as the proportion of mature males to total adults (Wilson and Hardy 2009).

Data analysis

The population parameters were estimated using the POPAN model estimator, which is an adaptation of the Jolly-Seber model in terms of a superpopulation. The MARK^{TM} software was used (Schwarz and Arnason 1996; White et al. 1999). The POPAN model assumes that the catchability of marked and unmarked individuals is the same, which we accept as true. Once all the models were obtained, the model with the lowest Akaike value (AICc) was selected as it is more robust and has a better fit to the data. The general model is as follows: POPAN - $p(g*t)$, ϕ

(g*t), *pent*(g*t) (where *p* is the capture probability, which depends on the group (immatures, males and females) and assuming the animal is alive and in the study area, ɸ is the apparent survival, varying with the group and time, and *pent* is the probability of entry into the population per occasion *t* concerning *t-1*, and it depends on group and time). Model selection was performed using Akaike's information criterion corrected for small sample size (AICc) (Akaike 1973), and models differing by ≤2 ΔAICc were considered as potential alternatives (Burnham and Anderson 2002).

We used chi-square (χ^2) tests to compare sex ratios (observed and estimated frequencies of males and females) against an expected 1:1 ratio. To analyze differences in biometric variables across different size and sex groups, an ANOVA test is employed. The Kruskal-Wallis test was used to evaluate whether the survival rates for the size and sex groups were statistically significant. All statistical analyses were performed with R software (Development R Core Team 2017), with α set at 0.05 to evaluate statistical significance.

Results

During 2015–2016, 32 sampling campaigns were conducted, totaling approximately 60 hours of effort with an average survey yield of 2.55 captures/hour. During this period, 151 captures and recaptures were made, identifying 118 different individuals (Table 1). Adults comprised 73.73% (n=87) of the individuals in the studied population, with a sex ratio (♂♂/♂♂+♀♀) of 0.44 ($n_{♂♂}$=39; $n_{♀♀}$=48) that did not differ significantly from parity ($\chi^2_1 = 0.9310$, $P = 0.3346$), suggesting a relatively balanced distribution of males and females. In addition, 46.61% (n=55) of the captured individuals were found with tails that were not-intact (cut, regenerated, or in the process of regeneration). It is unclear whether the tailings were fully regenerated or are still in the process of regeneration, as this is not within the scope of the present study. Additionally, the limited number of recaptures has not yielded sufficient data to make a determination. A total of 21.85% (n=33) of the captures were recaptures. The percentage of recaptures by size class was as follows: juveniles, 4.55% (n=1); subadults, 9.09% (n=1); and adults, 26.27% (n=31). The proportion of recaptures by sex was 25.00% (n=13) for males and 27.27% (n=18) for females.

Table 1. Total captures (including recaptures) and individuals by age class and sex of *Anguis fragilis* from the population studied.

		Individuals		Captures	
		n	**%**	**n**	**%**
Juveniles		21	17.80	22	14.57
Subadults		10	8.47	11	7.28
Adults	Males	39	33.05	52	34.44
	Females	48	40.68	66	43.71
Total		**118**	**100.00**	**151**	**100.00**

The population exhibited a snout-vent length (SVL) of 149.50 mm (56.00, 204.00) (Table 2), without significant differences between adult males and adult females ($F_{(1,116)} = 0.0600$, $P = 0.8021$). The tail length (TL), excluding individuals with non-intact tails, was 163.09 mm (57.00, 249.00) (Table 2), also showing no significant differences between males and females ($F_{(1,45)} = 3.6831$, $P = 0.0613$). For weight, excluding individuals with amputated or regenerated tails and gravid females, the mean was 13.72 g (1.05, 32.30) (Table 2), with significant differences between the four size classes and sexes ($F_{(3,56)} = 69.0023$, $P < 0.0001$), but not between males and females ($P = 0.6543$).

Table 2. Biometric data of the size and sex classes considered. TotL: total length. SVL: snout-vent length. TL: tail length (from cloaca to end). All lengths are expressed in mm. Weight is expressed in grams. M: maximum. m: minimum. SD: standard deviation. *: Only individuals with the tail intact.

		n	Mean	m	M	SD
SVL	**Juveniles**	21	76.24	56.00	89.00	9.82
	Subadults	11	121.91	111.00	132.00	6.88
	Males	52	164.69	133.00	204.00	16.62
	Females	66	165.44	121.00	196.00	15.58
TL*	**Juveniles**	18	87.78	57.00	112.00	15.58
	Subadults	10	157.90	141.00	171.00	9.97
	Males	19	199.32	159.00	249.00	21.29
	Females	28	188.79	146.00	210.00	16.30
TotL*	**Juveniles**	18	163.67	113.00	202.00	26.01
	Subadults	10	280.60	252.00	303.00	15.90
	Males	19	359.16	296.00	414.00	32.74
	Females	28	348.36	267.00	393.00	32.71
Weight	**Juveniles**	18	2.50	1.05	5.00	1.09
	Subadults	10	9.16	6.36	12.24	1.98
	Males	19	22.32	10.34	32.30	5.66
	Females	13	20.19	9.23	29.34	6.86

Of the 151 captured individuals, 49.67% had their tails cut, regenerated, or regenerating. For size classes and sexes groups, they were 13.63% of juveniles, 10.00% of subadults, 57.14% of males, and 59.61% of females. The ratio (TL/TotL) obtained for all analyzed individuals was 0.56 ± 0,01 SD. Males have a slightly longer tail (TL) about the total length (TotL) than females (♂♂: 0.5544 ± 0.0147; ♀♀: 0.5425 ± 0.0115), with significant differences ($F_{(1,45)} = 9.6708$, $P = 0.0032$).

For the slow worm studied population, 16 possible models were tested to examine the influence of the variables considered on survival, including the interaction model including groups and time. The model focusing on Φ depending on time and sex was the most parsimonious, with a lower AICc value. The 15 remaining models were uncompetitive (>2.00 ΔAICc) (Table 3).

This model for the *A. fragilis* population considers the apparent survival (Φ) variable according to the group (males, females, or immatures) and over time, the probability of capture (*p*) variable with the group and constant over time, and the probability of entry to the population per occasion (*pent*) also remains variable by group and throughout the study period.

Table 3. Tested models in MARK, where p: capture probability; ϕ: apparent survival during the study period; pent: probability of entry into the population per occasion t; g: size class and sex groups (immatures, females, and males); and t: time. The selected model is in bold.

Model	AICc	ΔAICc	AICc Weights	Model Likelihood	Num. Par	Deviance	-2log(L)
{p(g*.), ϕ (g*t), pent(g*t)}	**-645.23**	**0.00**	**1.0000**	**1.0000**	**174**	**-186.8625**	**423.0466**
{p (.*t), ϕ (g*t), pent (g*t)}	-367.25	277.98	0.0281	0.0295	200	-211.9403	397.9688
{p(g*t), ϕ (g*t), pent(g*t)}	-190.27	454.96	0.0097	0.0099	258	-263.8636	346.0455
{p (g*.), ϕ (g*.), pent (g*t)}	2.03	647.26	0.0032	0.0033	1	0.0000	0.0000
{p (.*.), ϕ (.*.), pent (g*t)}	552.19	1197.42	0.0000	0.0000	20	-105.2897	504.6194
{p (g*t), ϕ (.*t), pent (g*t)}	662.81	1308.04	0.0000	0.0000	20	-105.2897	504.6194
{p (.*.), ϕ (g*t), pent (g*t)}	679.50	1324.73	0.0000	0.0000	70	-250.0525	359.8567
{p (g*t), ϕ (g*.), pent (g*t)}	710.19	1355.42	0.0000	0.0000	70	-250.0525	359.8567
{p (g*.), ϕ (.*.), pent (g*t)}	1102.23	1747.46	0.0000	0.0000	91	-108.2834	501.6257
{p (.*.), ϕ (g*.), pent (g*t)}	1102.23	1747.46	0.0000	0.0000	91	-108.2834	501.6257
{p (.*t), ϕ (g*.), pent (g*t)}	1755.50	2400.73	0.0000	0.0000	109	-162.4087	447.5004
{p (.*t), ϕ (g*.), pent (g*t)}	1755.50	2400.73	0.0000	0.0000	109	-162.4087	447.5004
{p (.*.), ϕ (.*t), pent (g*t)}	2118.43	2763.66	0.0000	0.0000	113	-148.8100	461.0991
{p (.*t), ϕ (.*t), pent (g*t)}	2497.37	3142.61	0.0000	0.0000	113	-148.8100	461.0991
{p (g*.), ϕ (.*t), pent (g*t)}	2851.29	3496.53	0.0000	0.0000	118	-154.9237	454.9854
{p (g*t), ϕ (.*.), pent (g*t)}	34698.98	35344.21	0.0000	0.0000	130	-230.9317	378.9774

The estimated apparent survival probabilities (Φ) during the study period (557 days or around 1.5 years) were variable according to the group (males, females, or immatures) and over time. The estimated mean apparent survival probability for the study period was 0.76 (0.54, 0.94) for immatures, 0.79 (0.29, 0.95) for males, and 0.83 (0.68, 0.96) for females (Table 4). No significant differences were found between size class and sexes groups ($H_{(2, 87)} = 3.4763$, $P = 0.1758$).

The probability of capture (p) variable with the group is constant over time. For immatures, it was 0.13 (0.05, 0.32), 0.15 (0.09, 0.24) for males, and 0.05 (0.03, 0.10) for females, without significant differences between size class and sexes groups.

The population size estimated (n) for the three classes considered was 46.69 ind. (22.56, 131.73) for immatures, 128.73 ind. (88.12, 210.46) for males, and 185.84 ind. (102.52, 376.79) for females (Table 4). The estimated sex ratio (♂♂/♂♂+♀♀) was 0.41, which significantly deviated from parity ($\chi^2_1 = 10.368$, $P = 0.0012$). The lower bound sex ratio of 0.36 ($\chi^2_1 = 1.0964$, $P = 0.2950$) is not significantly different from 0.5, while the upper bound sex ratio of 0.66 significantly deviates from parity ($\chi^2_1 = 47.0910$, $P < 0.0001$). The estimated density of population is 16.11 ind./ha (7.78, 45.42), 44.39 ind./ha (30.38, 72.57), and 64.08 ind./ha (35.35, 129.93) for immatures, males, and females, respectively.

The species' activity period extends from March to October, although isolated instances of activity have been observed as late as February. The species exhibits its highest level of activity between the months of May and June (Fig. 2). The males emerge from hibernation earlier than the females, with a peak in May. Female emergence occurs one month later, with a peak in June (Fig. 3). The activity patterns of juveniles and subadults were found to be more similar to those of females for the former and to those of males for the latter (Fig. 3). The species' activity is significantly diminished during the summer months, with males exhibiting almost no activity at all. Female, juvenile, and subadult individuals exhibit minimal activity from the end of summer until the month of October (Fig. 3).

A total of 41.18% of captured males exhibited recent scars and wounds, indicative of fighting during the mating season from mid-April to mid-June, with the majority (61.90%) of these injuries occurring in May (Fig. 4). Of the 66 females captured, 41 (62.12%) were pregnant. In 2015, this percentage was 60.87% (n=28), while in 2016 it was 65.00% (n=13). Pregnant females were observed between mid-May and early September, with the greatest incidence occurring in June (69.79%). During the period from May to August, 78.48% of the females were pregnant (Fig. 4). No gravid females were found with a snout-vent length (SVL) of less than 149 mm. Regarding female size, 100% of females with an SVL greater than 180 mm were pregnant between May and July, compared to only 69.40% of females of smaller sizes.

Table 4. Estimated population and survival parameters for three groups of *Anguis fragilis* population. SE: Standard error. CI: confidence interval. Φ: survival rate**.**

Group	Population size				Survival estimators			
	n	SE	95% Lower CI bound	95% Upper CI bound	ϕ	SE	95% Lower CI bound	95% Upper CI bound
Immatures	46.69	24.09	22.56	131.73	0.7522	0.0602	0.6287	0.8758
Males	128.73	29.74	88.12	210.46	0.7803	0.0615	0.6541	0.9965
Females	185.84	65.46	102.52	376.79	0.8335	0.0644	0.6913	0.9557

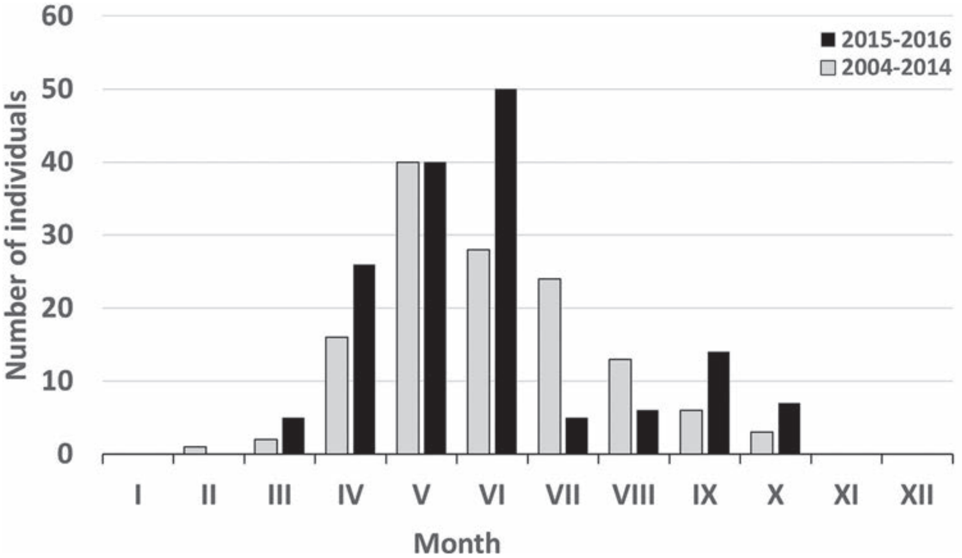

Figure 2. Capture frequency by month during the study period (2015–2016) and during sporadic sampling from 2004–2014 (J. Roig and G. Giner in SARE monitoring, Montori et al. 2014).

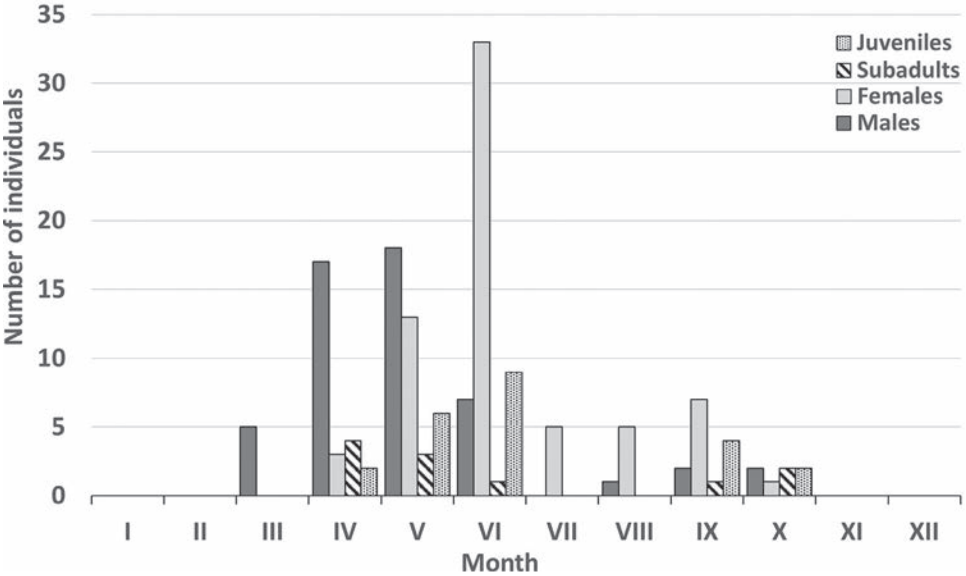

Figure 3. Frequency of monthly captures by size, class, and sex during the present study (2015–2016).

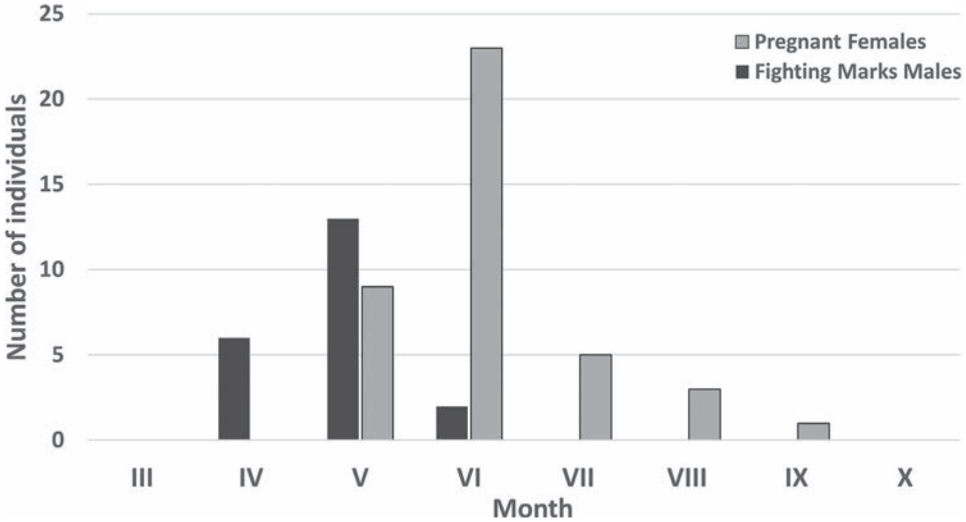

Figure 4. Monthly distribution of recently combat-marked males and pregnant females (2015–2016).

Discussion

Adults constitute 89.58% of the population, indicating either a low number or low detectability of subadults. Additionally, the low catchability of juveniles has prevented the collection of sufficient data on this age class.

Dely (1981) reports a maximum snout-vent length (SVL) of 291 mm and a maximum tail length (TL) of 280 mm for the species. The maximum sizes obtained in the present study (204 mm SVL and 249 mm TL) are smaller but within the described ranges for the species (Table 5). Males in this population tend to be slightly longer and heavier than females, although the differences are not significant. There is considerable variability in the average lengths of individuals across different populations, with either males or females reaching greater lengths depending on the study (Table 5), which often vary in time, season, and sampling methodology.

Most studies have not focused on collecting biometric parameters, resulting in limited data on the species' biometry across different populations, often based on small sample sizes (Galán 2003). While most studies report the maximum and minimum lengths of the specimens found, the average total lengths of adults are practically non-existent. Additionally, averages are often calculated together with the entire juvenile and subadult population (Welsch and Schlüpmann 2022), leading to a general lack of detailed information. Smith (1990) attempted to estimate the maximum lengths of males and females in his studied populations using various statistical methods to compare sexes, but these were not definitive in establishing actual lengths (Table 5).

In specimens with complete tails, the tail length (TL) was always greater than the snout-vent length (SVL). The TL/SVL ratios were as follows: males (average = 1.25, SD = 0.08) and females (average = 1.18, SD = 0.05). Dely (1981) reports a ratio of 1.02–1.36, Malkmus (1987) of 1.05–1.26, Vences (1993) of 1.27 on average, and Blosat (1997) of 1.19 on average. Ratios below 1 are indicative of tail autotomy. The percentage of individuals with broken or regenerated tails is similar to other studies, at around 50% of adult specimens (Stumpel 1985; Smith 1990; Blosat 1997; Ferreiro and Galán 2004). There is no significant difference between sexes in the incidence of broken tails, with males and females showing similar percentages (males: 59.61%; females: 57.14%), although the incidence is slightly higher in males. As observed in other populations (Welsch and Schlüpmann 2017), 100% of large individuals have regenerated or cut tails. In our population, this occurs in males with SVL greater than 180 mm, while in females, 100% of regenerated or cut tails are observed at 190 mm SVL.

In males, tail injuries are often interpreted as resulting from combat behavior between males during the mating period or from antipredator encounters. Capula et al. (1998) found that, in an alpine *A. fragilis* (*sensu lato*) population, males defeated in combats were always smaller than the victors, similar to other reptile species (e.g., Luiselli 1993). The mean length of males courting and successfully mating with females was significantly greater than the mean length of males in the study area. As suggested by Capula et al. (1998), access to reproductive females is likely monopolized by the larger males in the population. During mating, males bite females (usually on the head or neck) and try to align their vents, apparently forcing copulation. This could explain the high number of cut or regenerated tails found in females.

The population estimation results indicate an average density of 120.57 individuals per hectare (16.10 immatures/juveniles, 44.39 females, and 60.08 males), resulting in an estimated mean population of 349.65 individuals in our study area. Capula et al. (1998) reported densities higher than 80 individuals per hectare. Vences (1993) estimated a total population of 150 individuals in an isolated area of 1500 m², corresponding to a density of 1000 individuals per hectare. Hubble and Hurst (2006) captured 577 individuals in 2.5 hectares, representing 230.8 individuals per hectare. These values are higher than those obtained in the present study but are much lower than those reported by Ferreiro and Galán (2002), who estimated a density of 1700 individuals per hectare in the Visma population (Coruña A, Spain) using mark-capture-recapture methodology.

Table 5. Biometric data for different countries and regions in the natural populations of the species. TotL: average of total length. SVL: average of snout-vent length. TL: average of tail length. Lengths in mm. Weight in grams. MM: males, and FF: females.

Region (Country)	TotL		SVL		TL		Weight		References
	MM	FF	MM	FF	MM	FF	MM	FF	
Asturias (ES)			168.00	160.00					Braña (1983)
Utrecht (NL)	282.10	330.20	142.70	156.40			8.50	19.40	Stumpel (1995)
Dorset (UK)			151.70–156.30	153.50–157.70					Smith (1990)
Coruña A (ES)			151.00	154.00					Vences (1993)
Islas Cíes (ES)			151.00	170.00	192.20	236.30	17.00	20.20	Galán (2003)
Coruña A (ES)			172.00	153.00					Cabido (2004)
Galicia (ES)				168.30	183.90	184.00		13.76	Ferreiro and Galán (2004)
Coruña A (ES)			159.8	156.4					Ferreiro et al. (2007)
North Rhine (Westfalia)	308.60	334.20	143.60	158.60	171.60	185.10	16.30	22.40	Blosat (1997)
North Rhine (Westfalia)	293.00	291.00	173.00	166.00	120.00	125.00	22.30	20.30	Schlüpmann (2020)
Westfalia (D)			131.00						Welsch and Schlüpmann (2022)
Tarragona (ES)	358.40	344.80	165.10	162.90	198.90	187.40	22.05	19.31	Present study (2015–2016)

In the study area, the species begins to be active at the end of February or the beginning of March and remains active until the end of October, with activity potentially extending into November in years with favorable weather conditions. This activity period aligns with that described for many European populations inhabiting temperate areas or low altitudes, such as in Coruña A (Ferreiro and Galán 2004). In these areas, individuals maintain a much longer period of activity. Males can be active from early February until mid-October, with peak activity from March to June, while females are active from mid-March until mid-December, with peak activity from May to August. Subadults are active from February until mid-December, with peak activity in May and June. In contrast, populations at higher altitudes or latitudes, where temperatures are colder, have a reduced activity period. For instance, in the Italian Alps (Tarvisio Forest, Capula et al. 1998), activity starts after the snow melts, usually in mid-April. In southern England, activity may be delayed until May or June (Smith 1990; Platenberg 1999). However, in more temperate regions such as North Rhine-Westphalia, Germany, activity starts at the end of February and ends in early October (Schlüpmann 2020).

The reproductive period for males in the study area is estimated to start approximately two to three weeks after they emerge from their winter shelters, in early April. From then until the end of June (the end of the mating season for males in the area), males have been found with recent wounds and scars, typically matching the shape and size of the mouth of *A. fragilis*. This method of assessing recent wounds is effective for determining the reproductive period of males in this species. The period of male combat starts earlier in our population compared to Alpine populations (May, Capula et al. 1998) in northeastern Italy. Despite similar altitudes (990 m and 1100 m), the greater influence of the Mediterranean climate on our population may explain this earlier onset of male-male combat. Male-male combats occur earlier than copulations (Fig. 4), but both fighting and copulation behaviors peak in May, as described by Capula et al. (2008). These authors indicate that this peak coincides with the maximum of copulations. In our study, we did not observe copulations directly but noted a peak in the presence of pregnant females in June (Fig. 4).

The reproductive pattern of *A. fragilis*, with 62.12% of females being gravid, is consistent with findings from other European studies, indicating that not all females breed annually. In southern England, gravid rates ranged from 55% to 70%, indicating that a significant proportion of females may skip reproduction each year (Reading 1997). Such variability may be influenced by several ecological and physiological factors, including the availability of resources, health status, and age (Platenberg 1999). Similar trends are observed in Germany, with annual pregnancy rates varying between 50% and 65% due to environmental conditions (Strijbosch et al. 1989). In the Czech Republic, approximately 60% of females were found to be gravid, thereby confirming that while *A. fragilis* is capable of adapting to a variety of habitats, not all females reproduce each season (Gvoždík et al. 2007). This strategy may confer an evolutionary advantage by allowing females to prioritize growth and survival in unfavorable conditions, thereby enhancing long-term reproductive success.

Females were observed in a clear state of gestation between April and September. Many of them exhibited various wounds and excoriations on the scales of the cervical area, caused by male bites during copulation. These marks were much lighter than those resulting from fights between males. The presence of these marks, along with the ventral palpation of suspected gravid females, was decisive in most cases where pregnancy was not visually apparent. However, some females with breeding bites did not show signs of pregnancy upon subsequent recapture. This may suggest that sperm storage occurs in *A. fragilis*, as observed in lacertids, with evidence of sperm storage in *Acanthodactylus schreiberi* (Zotos et al. 2012). The role of stored sperm in determining breeding dates in female sand lizards (*Lacerta agilis*) has been documented, indicating the importance of this mechanism in reptilian reproductive strategies (Olsson et al. 1997). In the study area, gravid females are found from May to September (Fig. 5), which is a particularly long period. In Coruña A (Ferreiro and Galán 2004; Ferreiro et al. 2007), gravid females were found from mid-July to early August, a shorter time interval compared to our population.

The smallest female observed in the field showing evidence of being pregnant was 149 mm SVL, a size that coincides with findings by Capula et al. (1992) in Italy NE and by Alfermann and Völkl (2004) in Augsburg, Germany. However, this is considerably larger than the 120–129 mm size class in which Patterson (1983) found pregnant females on the island of Portland in southern Great Britain. In the Northwestern Iberian Peninsula, the minimum size of pregnant females found was 137.0 mm in Asturias (Braña 1983) and 135 mm in Coruña A (Ferreiro and Galán 2004). Ferreiro and Galán (2004) suggest that the minimum maturity size of slow worm females varies between 135 and 150 mm.

In the study area, the reproductive cycle extends from March to September, closely aligning with the findings of other researchers (Braña 1983; Ferreiro and Galán 2004). The percentage of gravid females in our population (Fig. 5) is high compared to other populations. This data indicates that most females are gravid each year, in contrast to the results obtained in northern Europe (Patterson 1983; Stumpel 1985; Smith 1990) or in alpine populations (Capula et al. 1992, 1998), where gestation is predominantly biennial. In Asturias, Braña (1983) reports a percentage of 65% of gravid females during the reproductive period. In Coruña A, 88.8% of the females reproduce annually, likely due to the temperate and humid coastal climate (Ferreiro and Galán 2004). However, in the Netherlands, the percentage of gravid females varies between 31% and 81% in different years (Stumpel 1985).

Several hypotheses are proposed to explain these variations. One suggests that only gravid females engage in superficial thermoregulatory activity in warm

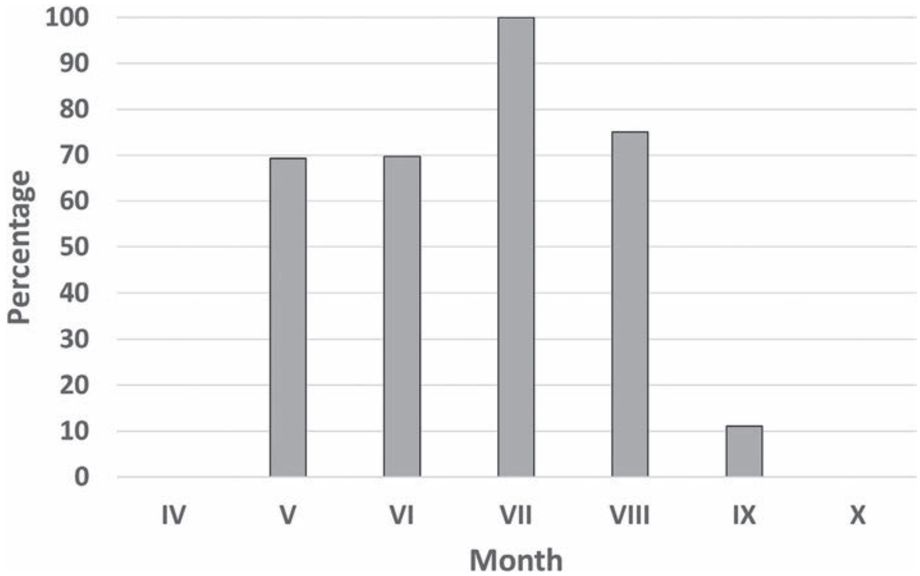

Figure 5. Percentage of gravid females found throughout the year (n=41).

environments, as observed in our population (Patterson 1983; Platenberg 1999; Graitson 2003), while non-gravid females remain active in deeper, cooler, and more humid areas. Another hypothesis posits that the high Mediterranean nature of the population allows all females to accumulate sufficient reserves during the activity period to reproduce annually. Under this assumption, annual or biennial reproduction could depend on climatic conditions or the abundance of trophic resources rather than following a fixed pattern. Further studies are required to validate these hypotheses.

Throughout July and August, soil moisture decreases and temperatures increase in the study area, resulting in fewer encounters under the surveyed shelters. Meek (2005) demonstrates that the species continues underground activity if the surrounding substrate temperature is within its preferred range, thus reducing the need to bask and seek warmth. This behavior is of particular importance during the warmer months, as very few individuals are detected in the shelters, with gravid females representing the exception. Similarly, Riddell (1996) observed a comparable pattern during an especially hot and dry summer in his study area, noting the presence of only a few individuals. Graitson (2004) obtained analogous results. This evidence suggests an interaction between thermoregulation and hydroregulation, with a potential shift in priorities among pregnant females (Gregory 1980). Patterson (1983) states that drought conditions hinder foraging for food, such as slugs and worms, preventing many females from fully recovering from the energy investment of previous pregnancies. This is one reason why the species exhibits biennial reproduction in females, due to the limited optimal feeding time available to some populations. However, in Hampshire, UK, peak activity occurs in late August and early September due to the milder and more humid climate.

Low numbers of juveniles (SVL<100 mm) were observed throughout the study period, with none detected in July and August. This absence is likely due to high temperatures and low humidity conditions, which force juveniles to seek deeper shelters to avoid dehydration (Sannolo and Carretero 2019). Similar results were obtained by Graitson (2004) in Belgium, where mainly gravid females were found during the summer period. No reliable evidence was found for the timing of parturition and recruitment. Hatchling size has been shown to be directly proportional to maternal size (Ferreiro and Galán 2004), making it difficult to determine with certainty whether the smallest juveniles found were hatchlings. The smallest juveniles found were two specimens measuring 56 mm, likely considered neonates, located in mid-September and late October, respectively. This suggests a normal calving period for the species, although it is longer in the studied area than in other European populations. Ferreiro and Galán (2004) suggest that, in years with milder weather, this gestation period is reduced. The normal breeding period in England is between mid-August and mid-September (Beebee and Griffiths 2000), similar to Galicia (Ferreiro and Galán 2004).

The sex ratio fluctuates significantly throughout the year, which is common for the species according to other long-term studies (Smith 1990; Ferreiro and Galán 2004; Thiesmeier et al. 2013). Males are typically found first in spring, followed by an increase in females throughout the year, while males almost completely disappear in summer. The sex ratio can vary greatly between populations. For instance, some populations have a higher proportion of males (♂♂/total): 0.6 in two populations in Coruña A and 0.65 in Dorset (Smith 1990; Vences 1993; Ferreiro and Galán 2004). In contrast, many populations have a female-biased sex ratio, as observed in our study, with values such as 0.22 in Utrecht, 0.38 in Dorset, 0.30 in Hampshire, 0.35 in two populations in Germany, and 0.10 in Ireland (Stumpel 1985; Smith 1990; Greven et al. 2006; Hubble and Hurst 2006; Thiesmeier et al. 2013; Parry 2020). The sex ratio of the studied population (♂♂/total = 0.44) suggests a slightly higher proportion of females compared to males, although this difference was not statistically significant and remains balanced between both sexes, fitting within the species' variability. Studies based on short-term monitoring (Stumpel 1985) or visual detection of active individuals (Parry 2020) may present a biased view of sex ratios.

A very low number of subadults was detected in the studied population. In long-lived species such as the Slow Worm, it is typical for the number of adults to be much higher than the number of juveniles (Beebee and Griffiths 2000). However, other studies (Riddell 1996; Hubble and Hurst 2006; Thiesmeier 2013) have reported populations with a much higher percentage of both subadults and juveniles. For instance, in Petersfield, UK, 310 immatures (51.7%) were recorded compared to 267 adults. In our population, both juveniles and subadults were detected in low percentages, with no significant differences in their capture rates throughout the monitoring period. This could indicate a very low turnover rate, but consistent with previous research, it may also be due to the need for more appropriate methodologies, such as installing artificial refuges (Hubble and Hurst 2006). The presence of other refuges, such as ground cracks, which are more suitable for juveniles, likely hinders their detection and is the most probable cause of this underestimation.

Acknowledgements

We want to thank all the people who helped in the field surveys: J. Roig, D. Gomez, A. Simó, G. Mochales, and J. Bisbal. In addition, the authors would like to sincerely thank the two anonymous reviewers for their comments and suggestions, which have contributed to a significant improvement of the manuscript. The study and its preparation for publication were not financially supported in any way and were entirely funded by the authors. Permits for capturing and handling individuals were granted by the Departament d'Agricultura, Ramaderia i Pesca de la Generalitat de Catalunya; references: SF/553/2015 and SF/401/2016.

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ÖGH Herpetozoa
Austrian Herpetological Society

Herpetozoa 37: 281–286 (2024)
DOI 10.3897/herpetozoa.37.e118175

New data on the diversity and distribution of lineages of the *Acanthodactylus erythrurus* species complex in North Africa derived from mitochondrial DNA markers

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Academic editor: Peter Mikulíček ♦ **Received** 3 January 2024 ♦ **Accepted** 19 June 2024 ♦ **Published** 4 September 2024

Abstract

Patterns of morphological and genetic diversity within the fringe-toed lizards of the genus *Acanthodactylus* have puzzled systematists since the first assessments, and none more so than the *Acanthodactylus erythrurus* complex. A recent study combining multi-locus sequence data and morphological characters partially resolved the situation, identifying two new species in the southern part of the range in Morocco, but leaving an unresolved "Ibero-Moroccan" clade containing much of the genetic and morphological diversity. Here we sequenced a mitochondrial marker for new samples from across much of the distribution. Our data notably increase the known ranges of various species and lineages found in Morocco, and indicate a divergent genetic lineage within one of the newly described species. While far greater numbers of genetic markers will be needed to resolve taxonomic questions, greater geographic sampling is also still needed both to delimit the species, and to identify regions where potential genetic admixture may occur.

Key Words

Acanthodactylus lacrymae, *Acanthodactylus montanus*, NADH dehydrogenase subunit 4, phylogeography

Introduction

Acanthodactylus, or fringe-toed lizards, comprise the most specious genus within the family Lacertidae, with 44 recognized species (Uetz 2023). The genus is a member of the Eremiadini tribe within the subfamily Lacertini, which along with its phylogenetically closest members (including *Mesalina*; Garcia-Porta et al. 2019) primarily inhabits xeric habitats in North Africa and Asia (Sindaco and Jeremčenko 2008). *Acanthodactylus* ranges from the Iberian Peninsula, across North Africa and the Arabian Peninsula into western India, and northward to Turkey.

Acanthodactylus erythrurus (Schinz, 1838) is distributed across Morocco and Algeria and is the only species of the genus occurring in the Iberian Peninsula. It is usually found in habitats with a moderate supply of moisture in shrubland, mesic forests and rocky areas (Schleich et al. 1996). The Iberian Peninsula was colonized from North Africa (Harris et al. 2004), and the respective populations have shown high levels of admixture in their mitochondrial DNA (mtDNA), especially in the northern part of their distribution (Harris et al. 2019). Concerning the North African populations, *Acanthodactylus erythrurus* group is of great interest due to its unclear taxonomy and complex intraspecific relationships (Harris et al. 2004; Fonseca et al. 2009; Tamar et al. 2016; Beddek et al. 2018).

Intraspecific variation within *Acanthodactylus erythrurus* is extensive, and has led to notable differences in how

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studies have treated this. Salvador (1982) and Arnold (1983) accepted *A. e. lineomaculatus* and *A. e. belli* as the two subspecies occurring in North Africa, while Bons and Geniez (1995) added the subspecies *A. e. atlanticus* from the Atlas Mountains and suggested that *A. e. lineomaculatus* deserved specific status. On the other hand, Squalli-Houssani (1991) proposed that all Moroccan subspecies should be considered as one highly variable species with morphological differences reflecting adaptation to local habitats. Studies employing mtDNA sequences revealed that both the Tunisian *A. blanci* and *A.* (*e.*) *lineomaculatus* are nested within the *A. erythrurus* complex (Harris et al. 2004; Fonsesca et al. 2009; Tamar et al. 2016). A comparative phylogeographic study based on mtDNA data, including samples of *A. erythrurus* and *A. blanci* from Algeria, revealed considerable mtDNA diversity within the species, with one species-delimitation approach (Automatic Barcode Gap Discovery, ABGD; Puillandre et al. 2012) indicating potentially 15 taxonomic units, the most of any of the species examined (Beddek et al. 2018). At the same time, a strong East-West divergence pattern across the Maghreb was identified, with two main clades showing this motif with a third basal clade reported only from the southern Atlas Mountains.

Some taxonomic issues regarding the *A. erythrurus* complex were resolved by Miralles et al. (2020), employing multilocus sequence data and a morphological assessment. They recovered five major lineages, that were supported by both mtDNA and nuclear DNA (nDNA). They continued to find a complex pattern across most of an Ibero-Moroccan (IM) clade, with highly diverse mtDNA lineages that did not fully coincide either with the three subspecies accepted in Morocco, or with nuclear markers. However, in the Atlas Mountains of Morocco two divergent lineages were identified in both mtDNA and the nuclear markers, which also showed minimal differences with some morphological characters, and these were recognized as distinct species, *Acanthodactylus montanus* from the High Atlas Mountain region and *Acanthodactylus lacrymae* from the Middle Atlas region. These were more closely related to Algerian and Tunisian forms rather than the Ibero-Moroccan clade (Miralles et al. 2020). These authors also highlighted the need for further sampling to better assess the distribution of these forms. Rancilhac et al. (2023) attempted to resolve the situation within the IM clade of *A. erythrurus* using mtDNA and nine nuclear gene fragments. However, even with this enlarged dataset they were only able to distinguish four major groups of populations, separated by barriers to gene flow. A recent genome-wide RADseq approach to investigate a contact zone within the IM clade indicated that there was spatial restricted gene flow highlighting high levels of reproductive isolation, consistent with even more species-level diversity within the complex (Doniol-Valcroze et al. 2024).

To obtain new insights into the distribution of the different lineages and species of the *A. erythrurus* complex, here we sequenced a partial ND4 mitochondrial gene region for 42 individuals, primarily from Morocco. Since mitochondrial and nuclear DNA were completely congruent for *A. montanus*, *A. lacrymae*, and the major lineages within the *A. erythrurus* complex (Miralles et al. 2020), this should give additional information regarding the distribution of these species, and also help delimit the ranges of mtDNA lineages within the rest of the *A. erythrurus* complex.

Material and methods

We analyzed 42 samples of *A. erythrurus* (Table 1). Animals were caught in the field over a 20-year period, and a small piece of tail tissue was removed before releasing them at the collection site. Locations and codes of the samples are represented in Fig. 1. Since lineages

Table 1. List of samples sequenced for this study.

Figure Code	Specimen number	Coordinates (Latitude, Longitude)
1	DB20115	31.4417, -9.7178
2	DB11946	31.4934, -9.7683
4	DB3661	32.6030, -9.1916
5	DB365	34.2044, -6.5619
6	DB1605	35.1659, -6.1209
7	DB3386	35.0225, -5.2044
8	DB3641	35.0626, -5.1950
9	DB3642	35.0626, -5.1950
11	DB3640	35.0626, -5.1950
14	DB4832	33.9447, -5.0279
15	DB15522	33.6521, -5.0226
16	DB15524	33.4085, -5.1082
17	DB15525	33.4085, -5.1082
18	DB14962	33.4085, -5.1082
19	DB14507	33.4085, -5.1082
20	DB25360	33.4056, -5.1030
21	DB1533	33.6218, -4.9034
22	DB949	33.1590, -5.0638
23	DB23755	33.1124, -5.0277
24	DB1015	31.8018, -5.4669
25	DB78	32.2164, -5.5497
26	DB81	32.1964, -5.6429
27	DB91	32.1964, -5.6429
28	DB95	32.1964, -5.6429
29	DB134	32.1964, -5.6429
30	DB137	32.2164, -5.5497
31	DB3628	32.1964, -5.6429
32	DB1512	31.9697, -5.4879
33	DB915	31.2908, -7.3814
34	DB1461	30.7880, -7.5935
35	DB24038	32.5694, -3.7186
36	DB24128	32.5694, -3.7186
37	DB24136	32.5694, -3.7186
38	DB24158	32.5694, -3.7186
39	DB24160	32.5694, -3.7186
40	DB14625	33.8653, -3.0323
41	DB3647	33.8724, -3.0387
42	DB3648	33.8724, -3.0387
43	DB3654	33.8724, -3.0387
44	DB3655	33.8724, -3.0387
45	DB14453	33.8653, -3.0323
48	DB11221	35.293, 1.2631

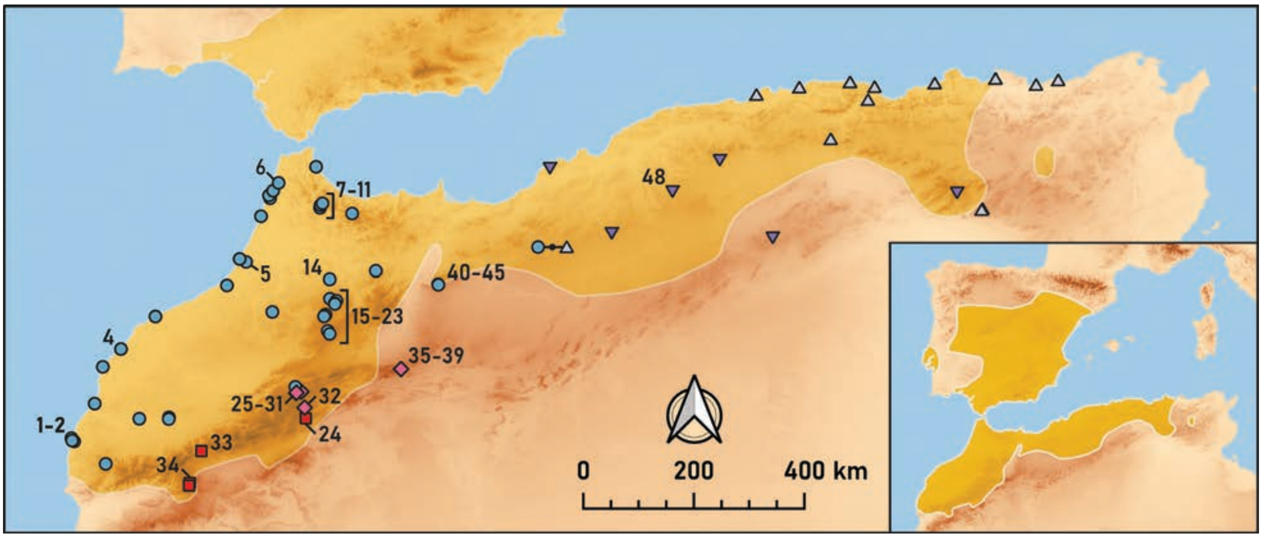

Figure 1. Distribution map of lineages within the *Acanthodactylus erythrurus* complex within North Africa with complete range inset. Distribution outline follows the IUCN. Newly sequenced individuals in this study are numbered, others are from GenBank. Colour codes indicate the different forms – *A. erythrurus* complex IM clade (blue circles), *A. montanus* (red squares), *A. lacrymae* (pink diamonds), *A. erythrurus* complex Central Algeria Clade (purple triangles), *A. erythrurus* complex Algero-Tunisian clade (light blue triangles).

and species cannot always be determined with certainty based on morphological characters of single individuals (see Miralles et al. 2020), all individuals were sequenced. Additional data from previous studies were retrieved from GenBank (Tamar et al. 2016; Beddek et al. 2018; Miralles et al. 2020) covering the distribution range of the species. Sequences of *A. micropholis*, *A. blanfordii*, *A. grandi* and *A. boskianus* (Heidari et al. 2014) were included as outgroups. All new sequences were submitted to GenBank (PQ000925–PQ000966).

Total genomic DNA was extracted from alcohol-preserved tail tissue following standard high-salt protocols (Sambrook et al. 1989). We amplified a mitochondrial gene fragment, NADH dehydrogenase subunit 4 gene with the adjacent tRNAs (ND4+His, Ser, Leu) in order to allow comparison with previous published studies of *A. erythrurus* using this marker (Tamar et al. 2016; Beddek et al. 2018; Miralles et al. 2020). The fragment was amplified performing a Polymerase Chain Reaction (PCR) with primers ND4 and Leu from Arévalo et al. (1994), in a total volume of 25 µl, with 5 µl of 5x reaction Buffer, 3.2 µl of 25 mM $MgCl_2$, 1 µl of 5 mM dNTPs, 0.5 µl of 4.0 M of each primer, and 0.2 µl (1U) of Promega GoTaq DNA polymerase. PCR conditions were: pre-denaturation step of 94 °C (3 min), 33 cycles with 94 °C (30 s) denaturing, 47 °C (40 s) annealing, 72 °C (90 s) extension and with a final extension conducted at 72 °C for 5 min. Positive PCR products were sent to GENEWIZ (Germany) for sequencing.

Sequences were edited and aligned using ClustalW with default parameters in MEGAX (Kumar et al. 2016). Genetic uncorrected *p*-distances were also calculated in MEGAX (Kumar et al. 2016).

We employed two methods of phylogenetic inference based on the ND4 sequences, Maximum Likelihood (ML) and Bayesian Inference (BI). Best-fit partition schemes were selected using Partition Finder v1.1.1 (Lanfear et al. 2012). We used codon partitions and the tRNAs fragment was set as a fourth data block. ML analysis was performed with MEGAX with the built-in tool to choose the most appropriate model under the AIC (GTR+G), while nodal support was assessed by bootstrapping with 5,000 replicates. The BI analysis was carried out using MrBayes v3.2.7 (Ronquist et al. 2012) and separate models were set for the different partitions (in each case GTR+I+G). Two independent runs of 5×10^6 generations were performed, with a sampling frequency of 1,000 and 25% of the trees were discarded as burnin. Trees were imported to FigTree v1.4.4 for visualization.

Results

The dataset consisted of 42 newly sequenced members of the *A. erythrurus* complex, along with 105 previously published sequences from GenBank, with an aligned length of 769 bp. Both Bayesian Inference and Maximum Likelihood analysis for the mitochondrial fragment (ND4) produced almost identical topologies, differing slightly in the deeper nodes, with the BI tree revealing higher support on some nodes (Fig. 2). Our results are generally congruent with previous studies regarding the major clades (Beddek et al. 2018; Miralles et al. 2020). Following the taxonomy of Miralles et al. (2020), all five major clades – the species *A. montanus* (WHA) and *A. lacrymae* (EHA), the highly diverse Ibero-Moroccan (IM) clade, the Algero-Tunisian clade (AT) and the Central Algerian clade (CA) can all be identified (Fig. 2). However, when comparing the distribution of these clades (Fig. 1) with the newly sequenced specimens for this study there are

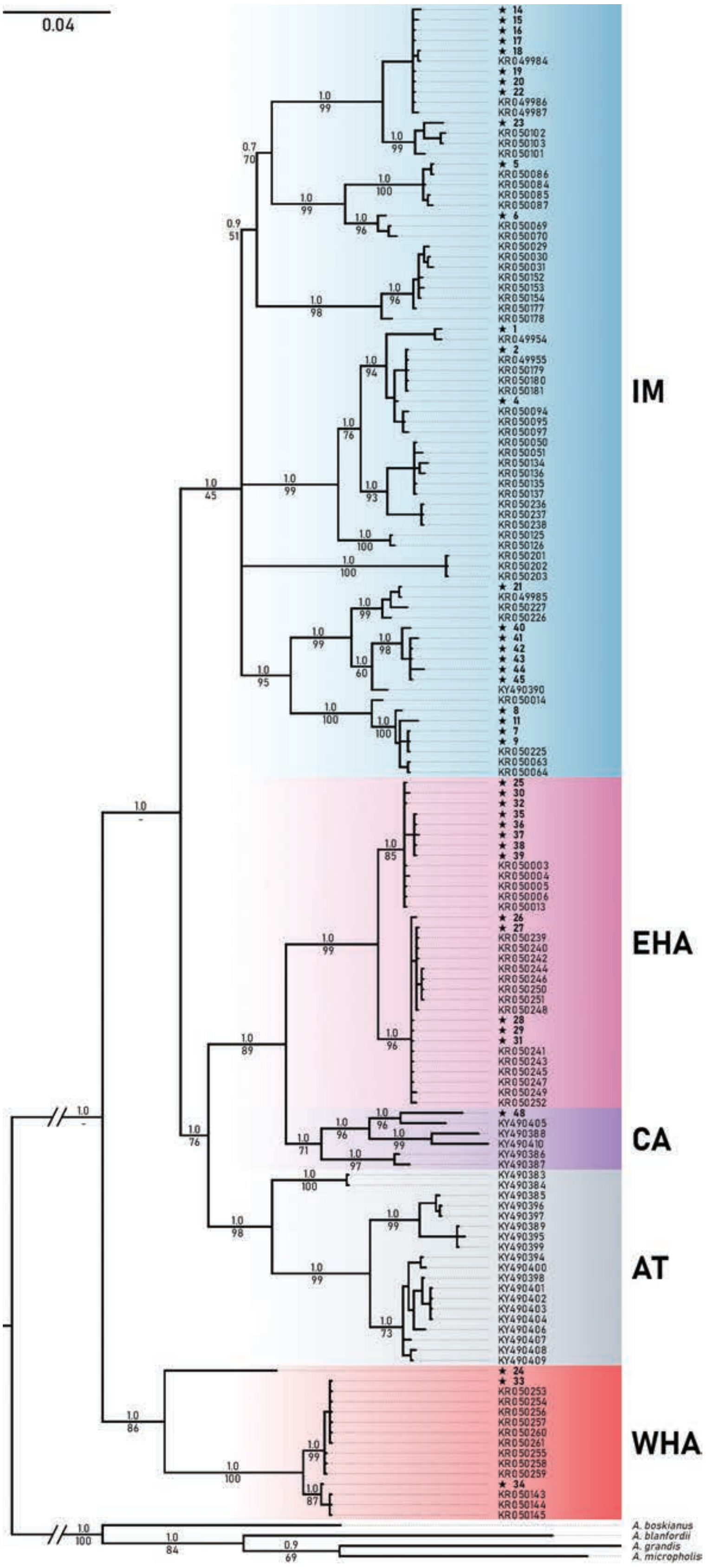

Figure 2. Estimate of relationships within the *A. erythrurus* complex in North Africa derived from a Bayesian analysis. Lineages are labelled following Miralles et al. (2020): *A. erythrurus* complex Ibero-Moroccan clade (IM), *A. montanus* (WHA), *A. lacrymae* (EHA), *A. erythrurus* complex Central Algeria Clade (CA), *A. erythrurus* complex Algero-Tunisian clade (AT). Stars indicate novel samples from this study, and numbers above and below nodes indicate Bayesian Posterior Probabilities and Bootstrap support from a Maximum Likelihood analysis respectively. Missing Bootstrap support values are due to different placement of the WHA clade with Maximum Likelihood, where it appears as sister-clade to EHA, CA, and AT.

some notable modifications. Regarding the IM clade, our new sequences from Debdou (Fig. 1: 40–45) fills a wide gap in sampling for this clade, which had previously been confirmed around the region of Taza and then a single sample over 200 km to the East in Algeria (Fig. 1). For *A. lacrymae*, our samples from just north of Aït Aïssa (Fig. 1: 35–39), extend the range over 100 km to the East of the previously confirmed populations. For *A. montanus* our sample 24 (Fig. 1) not only increases the apparent range of this lineage about 200 km to the northeast, it also means that the ranges of *A. montanus* and *A. lacrymae* can no longer be considered highly allopatric, since they are separated by at most around 10 km. This sample 24 is also interesting genetically, as although it is strongly supported as sister taxa to *A. montanus* (97% BPP), it is highly distinct from the samples from the southern part of the range (samples 33–34, 8±1% SE).

Discussion

Just as early assessments of morphological variation within the *A. erythrurus* complex identified high levels of complexity (Salvador 1982; Arnold 1983), so later assessments of genetic diversity have continued to perplex researchers. Miralles et al. (2020) managed to describe two species from the southeastern edge of the range, *A. lacrymae* and *A. montanus*, while leaving the bulk of the morphological and genetic diversity within an unresolved *A. erythrurus* IM clade. Our additional sampling further supports the distribution of this latter clade, across the Moulouya river valley region in Debdou, with these most closely related to a single published sequence from even further east (KY490390, Beddek et al. 2018), that indicates the IM clade reaches into Algeria (Fig. 1). The Moulouya region is often considered a biogeographical barrier separating herpetofauna into western and eastern forms (reviewed in Salvi et al. 2018), and the samples from the East of the Moulouya valley do form a subgroup within the IM clade, again highlighting the intricacy of the phylogeographic patterns within the *A. erythrurus* complex (Rancilhac et al. 2023).

Regarding the situation in the Atlas Mountains and the southeastern range of the distribution, Miralles et al. (2020) recognized *A. montanus* and *A. lacrymae* for the two highly genetically distinct lineages they recovered in this region. While these two forms can be morphologically separated from the IM clade of the *A. erythrurus* complex, Miralles et al. (2020) noted that *A. montanus* is “very similar to the allopatric *Acanthodactylus lacrymae* and single individuals are not always possible to separate”. However, the situation was simplified by the large distance between the ranges of the species – indeed these authors specifically presented networks of nuclear haplotype sharing between both *A. montanus* and *A. lacrymae* with the IM clade, but not between *A. montanus* and *A. lacrymae* (fig. 5 of Miralles et al. 2020). The finding of a distinct lineage apparently of *A. montanus* very close to the range of *A. lacrymae* (sample 24; Fig. 1) complicates this situation. Since these species cannot be easily separated in the field, extensive genetic sampling of individuals from the region of contact between *A. montanus* and *A. lacrymae* will be needed to confirm if there is genetic admixture, or if the two lineages are found in strict sympatry. Furthermore, the high degree of genetic differentiation between this sample (24) and *A. montanus* from the type locality (8% with this ND4 marker) is higher than between some accepted lacertid lizard species (e.g. *Dinarolacerta mosorensis* and *Dinarolacerta montenegrina*; 6.7%, Mendes et al. 2016). On the other hand, divergence levels were similar (7.8%) within another lacertid, *Timon tangitanus*, in Morocco which showed a lack of lineage sorting with nuclear markers (Abreu et al. 2020). While Rancilhac et al. (2023) were unable to fully delimit sublineages within the IM clade based on multiple nuclear markers, recent analyses of RADseq genome-wide data indicate that some of these at least correspond to cryptic species (Doniol-Valcroze et al. 2024). A similar approach will probably be needed to determine the taxonomic status of this new lineage.

To conclude, phylogeographic patterns within the *A. erythrurus* complex continue to slowly take shape. Our additional geographic sampling extends the ranges of some forms found in Morocco, the *A. erythrurus* IM clade, *A. montanus* and *A. lacrymae*. In particular, a genetically distinct apparent individual of *A. montanus* was found very close to the known range of *A. lacrymae*. Determination of genetic admixture between *A. montanus* and *A. lacrymae* will be necessary to confirm the genetic distinctiveness of these morphologically extremely similar forms. Our data highlights that, as well as the previously identified need for inclusion of greater numbers of genetic markers or even genomic level assessments (Doniol-Valcroze et al. 2024), increased geographic sampling is also needed, especially across the southeastern part of the range in Morocco where the distribution of the different species and lineages is still not fully determined.

Acknowledgements

Lizards were captured under permit of the Haut Commissariat aux Eaux and Forets of Morocco (HCEFLCD/ DLC-DPN/DPRN/DFF No14/2010). We thank our colleagues from CIBIO for assistance during the fieldwork. Part of this work was carried out during an ERASMUS scholarship of DS, supervised by DJH. DJH is funded through the Fundação para a Ciência e Tecnologia (FCT), CEEC-INST/00104/2021/CP2819/CT0003. This work was also supported by the European Union’s Horizon 2020 Research and Innovation Programme under the Grant Agreement Number 857251.

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ÖGH Herpetozoa
Austrian Herpetological Society

Herpetozoa 37: 287–294 (2024)
DOI 10.3897/herpetozoa.37.e120806

Microhabitat preferences in the European green lizard (*Lacerta viridis*): implications for conservation management of isolated populations

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https://zoobank.org/DD93CD41-9142-46C2-A686-088B0189B917

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Academic editor: Yurii Kornilev ♦ **Received** 12 February 2024 ♦ **Accepted** 3 September 2024 ♦ **Published** 7 October 2024

Abstract

The European green lizard (*Lacerta viridis*) populations in Bohemia, Czech Republic, are isolated by more than 150 km from the northern border of the continuous range of the species. These populations are fragmented and further isolated from each other. In this landscape mosaic, they are tied to specific habitats located in deeply incised river valleys (the so-called river phenomenon) and thus may be viewed as stenotopic. The research site is located on the northern edge of the city of Prague. Since 1998, this site has been the subject of long-term conservation management aimed at strengthening and maintaining the abundance of the local *L. viridis* population. To formulate recommendations for the management of other isolated *L. viridis* populations, we performed a spatial analysis of the localities with observed individuals to determine and evaluate the significance of the chosen biotic and abiotic factors for habitat discrimination. We applied principal components and discriminant function analyses and examined the effect of 24 variables on the species' presence. The results revealed the principal role of the presence of rock debris and hiding places for lizard occurrence. The strongest negative predictors were the presence of tall grass and high vegetation coverage. We discuss the applicability of our findings in both the theory and practice of species conservation and population management.

Key Words

climate change, conservation modeling, discriminant function analysis, population characteristics, population ecology, regional stenotopy, spatial analysis

Introduction

Reptile species distribution has been modeled and analyzed on various, but typically large scales – from landscapes to continents (Kaliontzopoulou et al. 2008; Sillero and Carretero 2013; Oraie et al. 2014; Hosseinian Yousefkhani et al. 2015; Wirga and Majtyka 2015; Vargas-Ramírez et al. 2016; Petrosyan et al. 2020; Chmelař et al. 2020, 2023; Srinivasulu et al. 2021). However, due to ecological specifics, reptiles may also show major inter/intraspecific differences in microhabitat preference and usage, which have received less focus. Both micro- and macro-scales need to be taken into account for effective management of their habitats and populations.

Studies based on positive or negative discrimination of environmental factors are common in botanical works but have been applied just recently to predicting the occurrence of reptiles (Sacchi et al. 2011). A similar analysis can be used to separate sympatrically occurring species according to their ecological demands (Melville and

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Swain 1997; Heltai et al. 2015) or to study preference or avoidance of certain environmental factors, such as invasive plant species (Hacking et al. 2014).

The European green lizard, *Lacerta viridis* (Laurenti, 1768), is a robust lizard from the family Lacertidae, characterized by distinct sexual dichromatism with males exhibiting a bright blue throat. The species range covers mostly central and eastern Europe, the Balkan peninsula, and the coast of the Black Sea. Czech populations, located on the fringe of the range, are generally isolated and declining due to habitat degradation, making the species' survival in this location uncertain (Baruš et al. 1989, 1992; Mikátová and Nečas 1997; Mikátová 2002; Moravec 2015; Rehák 2015; Mikátová and Jeřábková 2023). All populations in the Czech Republic belong to the nominotypical subspecies *L. v. viridis* (Böhme et al. 2007b). According to legislative regulations in the Czech Republic (Act no. 114/1992), the European green lizard remains listed among critically endangered species even though the current Czech Red List decreased the category to endangered (Chobot and Němec 2017). The reason for this change is the generally favorable state of the populations in the southeastern Moravia region in contrast to the populations in the Bohemia region. However, the biggest differences are the noticeably lower genetic diversity, heterozygosity rate, and allele richness of the scattered populations in Bohemia (Böhme and Moravec 2011) compared to the populations in the South Moravian region, which are connected to the core area of *L. viridis* (Nemitz-Kliemchen et al. 2020). This is apparently a consequence of the geographic isolation of the Bohemian populations and represents an important aspect for their conservation and management.

Molecular data confirmed genetic affinities of Bohemian populations to those in neighboring parts of their distribution range in NE Germany (Elbe River) and Moravia (Böhme et al. 2006; Böhme and Moravec 2011). No recent records of *L. viridis* presence are known from Poland (Skawiński et al. 2019). Moreover, individual relic Bohemian populations are genetically slightly distinct (Böhme et al. 2006; Böhme et al. 2007a; Böhme et al. 2007b; Böhme and Moravec 2011). These populations are ecologically notable as inhabitants of biotopes retaining ancient characteristics, mainly rocky steppes, and these habitats can differ significantly in ecological parameters from the surrounding landscape matrix (Strödicke 1995; Joger et al. 2010; Fischer and Rehák 2010; Blažek 2013) and are in most cases fragmented (Prieto-Ramirez et al. 2018).

All Bohemian populations are bound to the so-called "river phenomenon" that affects deeply incised river valleys where thermophilous organisms, otherwise absent in the surrounding landscape, inhabit slopes with southern exposure (Ložek 1988). Therefore, the formation of metapopulations is unlikely. This creates the possibility of comparing these individual populations, in terms of morphology, ecology, and ethology. These populations also show regional stenotopy, linked to specific biotopes at the northern limit of the species distribution, and occur sympatrically with other animal and plant species connected with the river phenomenon (Ward 1998; Chmelař et al. 2023). The data on the ecology obtained from these localities are therefore very valuable, as the European green lizards are probably found here at the ecological limits of the species. The Bohemian relict autochthonous populations of *L. viridis* have high scientific and conservation value due to their genetic exceptionality related to isolation, fragmentation, small population size, genetic drift, reduced variability, and the possibility of occurrence of unique genetic variants; therefore, they also require special methods of conservation management (cf. Böhme et al. 2007a; Joger et al. 2010).

The distribution of *L. viridis* in the Czech Republic has already been analyzed, and a model has been developed to identify suitable habitats on a large state-wide (78,870 km^2) scale (Chmelař et al. 2020). The aim of the current work is to identify the key factors influencing microhabitat selection of isolated populations of *L. viridis* at the fringe of their distribution, to evaluate if these factors correspond at both micro- and macro-habitat scales, and to contribute to the practice and theory of conservation of isolated populations in general.

Material and methods

The research was carried out along the Únětický stream, otherwise also called Tiché údolí (the silent valley), located on the border of the Prague and the Central Bohemian regions (coordinates: 50.1472°N, 14.3772°E; Fig. 1). The whole sampling area is part of the "Roztocký háj-Tiché údolí" Nature Reserve.

Geologically, the research site falls into the area of the Barrandien Paleozoic, where sedimentary rocks, especially shale and silicite, predominate. The filling of the valley consists of alluvium deposits on sandy gravels (Fediuk 1997). The rock composition is important for the soil characteristics and vegetation species and coverage, thus affecting the thermal and hydrological properties of the surface and thus for the distribution of the lizards. Parts of the area were inaccessible either due to steep rocky slopes or vegetation too dense to pass through.

The location of the European green lizard population is a south-facing slope with an area of 4.2 ha consisting of two abandoned quarries and the slope itself. It mainly consists of fragments of heaths and rocky steppes with native flora on rocky outcrops. Such diverse terrain provides a considerable number of microclimates with relatively high temperature differences. There are frost basins in the area of the valley floor, with a frequent temperature inversion, especially in spring. In contrast, the exposed rock outcrops showed significantly higher temperatures than would be usual for the given time of year when measured by an infrared thermometer. The part of the site is shown in Fig. 2, and a photograph of an individual from the site is shown in Fig. 3.

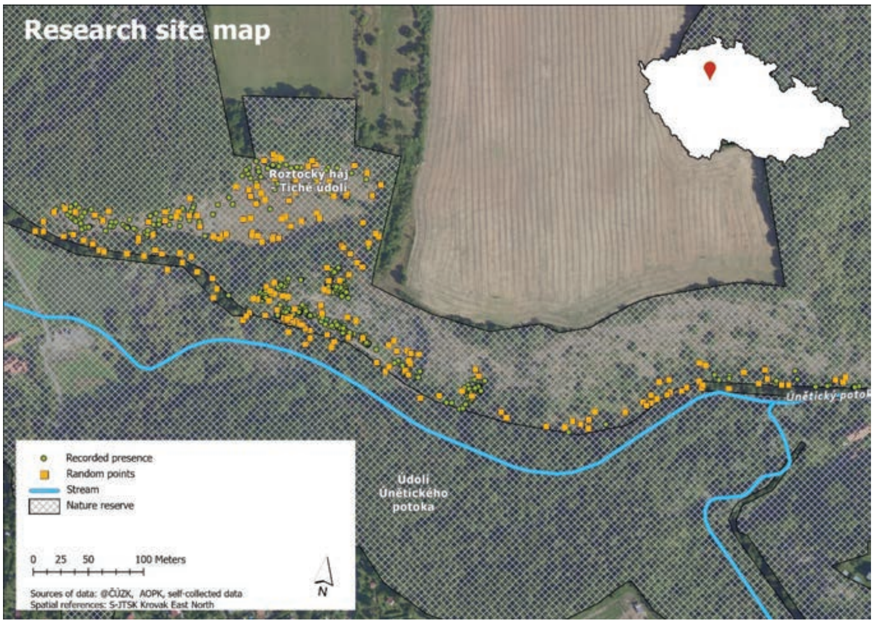

Figure 1. Map of the research site at "Roztocký háj - Tiché údolí" Nature Reserve, Bohemia, Czech Republic.

Figure 2. Typical habitat of *Lacerta viridis* (maintained by active management) at "Roztocký háj-Tiché údolí" Nature Reserve, Bohemia, Czech Republic.

Figure 3. Adult male *Lacerta viridis* from the "Roztocký háj - Tiché údolí" Nature Reserve, Bohemia, Czech Republic.

A linear transect of 1.9 km was laid out through the research site, maximizing the coverage of the area inhabited by lizards and habitat diversity by its effective width. The observer (JC) walked once, alternating starting eastern and western starting points per sampling to avoid double counting of individuals. The location of each lizard visually detected along this transect was recorded by GPS, along with their relative age and sex. Detection was at times aided with binoculars. In case the presence of the observer caused the lizard to move, its original position was recorded. The obtained data were also used for mapping the annual and daily activity, for estimating the size of the population, and as an indicator of the relative composition of the population in terms of gender ratios and age categories (juveniles, subadults, and adults). Presences were recorded between 2011 and 2014 throughout the whole activity period of the lizards by the same observer. The observer visited the site 119 times in total, walking the transect 60 times. Only observed presences during the transect sampling are included in the study since many visits were made in periods with low or no lizard activity for confirmation purposes. The transect was sampled during different times during the years, and depending on seasonal and weather conditions, sampling started between 7:00 and 17:00 h and ended between 11:00 and 21:00 h. The sampling lasted between 2 and 4 hours, depending mainly on the number of recorded individuals. Yearly and monthly distribution is summarized in Fig. 4.

The coordinates of the observations were obtained by a Trimble GeoXT GeoExplorer 2005 hand-held GPS receiver. The accuracy of the positions was further enhanced by geodetical software Leica GNSS SPIDER V4. The post-processed accuracy of the recorded points was within 50 cm in 87.6% of cases, within 1 m in 9%, and only 3.4% of the measurements had a deviation between 1 and 2 m.

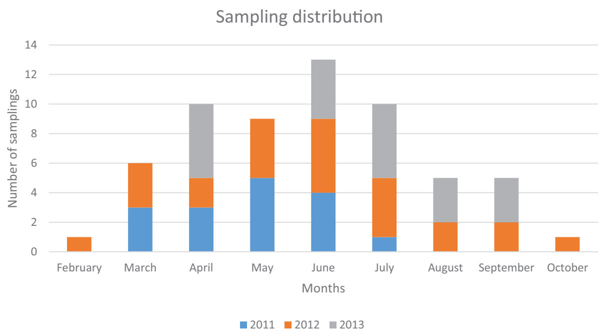

Figure 4. Sampling distribution in consecutive years.

These recorded locations were used to perform the spatial analysis. The mapping was carried out during June and July 2014 in order to minimize seasonal differences in microhabitat layout. We did not notice substantial changes in microhabitat layout between the consecutive years of the study. Also, all the variables were selected with minimizing the effect of seasonal change in mind. Especially in the case of variables related to vegetation, we focused on the percentage of their coverage and/or number rather than the exact height or the degree of shading of the surface. We standardized the environmental variables according to their assumed ecological function, not according to systematics. Variables were visually assessed in the field within a radius of 0.5 and 2.5 m from the location of each lizard's point of presence and are summarized in Table 1. The distance of 2.5 m was chosen as it is the approximate maximum distance that an adult individual was able to run without stopping. In this environment, we assumed discrimination based on the presence of a potential long-term shelter.

For comparison with the presence records, we created 200 random points within the same area along the transect using QGIS software version 2.2.0. Variables were recorded for these points using the same method as above. We calculated the Haversine distance in order to measure the minimum distances between real presence and random points.

Prior to further analyses, environmental variables were screened for spatial correlation using the Mantel test, and variables with a significant correlation of $r > 0.2$ were not included in further analyses. Principal component analysis (PCA) was performed to identify ordination axes in the factor plane. Points of occurrence and random points were then compared using discriminant function analysis (DFA) in the STATISTICA software version 9.0, using the presence as the grouping variable (value of 0 for a random point and 1 for a point of recorded presence). The final model was constructed by a method of backwards stepwise variable elimination. Other DFAs were performed afterwards with the age category (adult, subadult, juvenile) and sex of the adults as a grouping variable in order to identify possible differences in their microhabitat structure. Again, backwards stepwise variable elimination was used.

Results

A total of 403 presences were recorded during 60 samplings. On average, 6.7 individuals were recorded along the transect per visit (=1–16, SD = 3.7). The highest monthly (?) average numbers of recorded individuals were in May (9.7) and June (9.6) and the lowest in September (4.3). Of all the observed individuals, 99 were males, 70 females, 93 unidentified adults, 42 subadults, and 103 juveniles.

The calculated Haversine distance (shortest distances of real presence and random points) was 6.9 m (0.16–21.1 m, median = 6.2 m). The unreduced model shows significant differences between random and recorded presence points (Wilks' Lambda: 0.577, $F_{(24.578)} = 17.69$, $p < 0.0001$).

Using the Mantel test to identify correlating variables, only the percentage of soil (at 0.5 and 2.5 m) from the presence record exceeded the predetermined correlation value of $r > 0.2$ and thus were not included in further analyses. The reduced number of variables in the model was therefore 9: scree percentage (0.5 m), grass percentage

Table 1. Measured variables within 0.5 and 2.5 radius of presence and random points.

Name	Description	Measured as	Removed due to correlation
Scree	Continuous scree coverage	%	Retained
Grass	Herbs shorter than 30 cm	%	Removed
Soil	Exposed soil	%	Retained
Tall vegetation	Herbs higher than 30 cm	%	Retained
Raised rock	Min. length 30 cm, min. elevation 15 cm	%	Retained
Stump	Stump or fallen log	%	Retained
Leaves	Fallen leaves coverage	%	Retained
Branches	Mounds of cut or fallen branches	%	Retained
Bush	Woody plant up to 2 m tall, sprouting close to surface	count	Retained
Thornbush	Woody thorny plant up to 2 m tall, sprouting close to surface	count	Retained
Tree	Woody plants higher than 2 m with branches high above the ground	count	Retained
Raised rock	Min. length 30 cm, min. elevation 15 cm	count	Retained
Stump	Stump or fallen log	count	Retained
Shelter	Subsurface space large enough to hide	count	Retained
Deep shelter	Usable for over-wintering or laying eggs	count	Retained

(0.5 m), high vegetation (0.5 m), branches percentage (0.5 m and 2.5 m), elevated rock/stump percentage (0.5 m), number of trees (0.5 m), number of shelters (2.5 m), number of deep shelters (2.5 m).

Wilks' Lambda of the reduced model (0.598) remained significant ($F_{(9,593)} = 44.24$, $p < 0.0001$). The model was able to classify the random points correctly in 82.5 percent of cases (165 out of 200). The classification success rate of points of presence was 79.9% (322 out of 403).

DFA of age categories: the unreduced model (Wilks' Lambda: 0.75, $F_{(48,754)} = 2.392618$, $p < 0.00001$) shows significant differences in classification between points of presence of adults and juveniles (Table 2) (Squared Mahalanobis distance = 1.25, F = 3.52, $p < 0.00001$) and between points of presence of juveniles and subadults (Squared Mahalanobis distance = 1.60, F = 1,65, p = 0.03). The subsequent canonical analysis of age categories showed no distinguishable clusters.

The PCA scree plot identified 5 factors that explained a significant percentage of variability. The first two factors were selected as determinants. The first factor explained 20.13% of the internal variability, while the second factor explained 13%. Four main vectors of synchronous variables can be identified by projecting the variables onto the plane (Fig. 5).

Table 2. Classification matrix of DFA analysis with age category as grouping variable. Rows: Observed classifications, Columns: Predicted classifications.

	Percent	a	s	j
a	88.4	236	4	27
s	13.5	27	5	5
j	35.4	64	0	35
Total	68.5	327	9	67

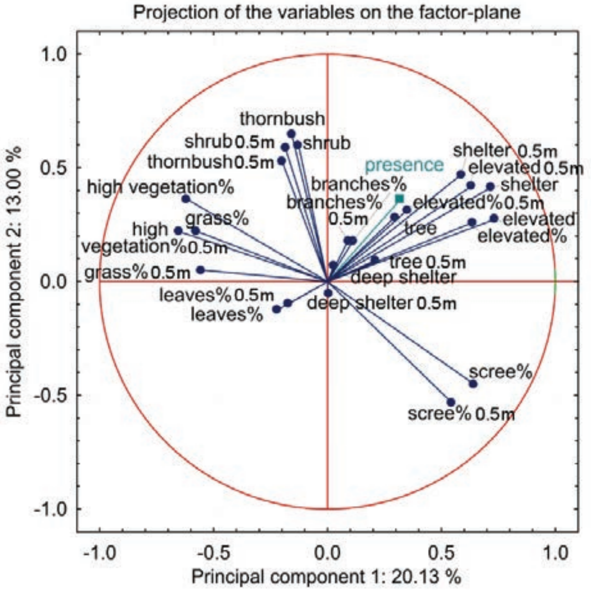

Figure 5. Projection of PCA into factor plane.

Regardless of the chosen statistical method, all the analysis results show that the distribution of individuals in the study site was not random.

The discriminant analysis shows that the created model is reliably able to distinguish a point of presence from a randomly selected point. However, it cannot reliably classify the presence points of individuals into the correct age category. There was a significant discrimination between juveniles and adults and between juveniles and subadults, but the classification success rate was relatively small. The sex of adult individuals was not evaluated in the analysis due to a non-significant difference in the success of the classification of presence points of males and females in preliminary analyses.

Discussion

Previously, we identified four factors that showed positive influence on the species distribution in the Czech Republic: annual precipitation up to 600 mm, slope inclination between 5–25°, mean temperature of the warmest quarter up to 20 °C, and precipitation in the coldest quarter above 150 mm (Chmelař et al. 2020). These factors seem to describe well the preferred habitats that can support the *L. viridis* populations, including our chosen research site.

According to the results of the reduced DFA model, several variables are key to characterizing the localities with lizards present: scree percentage, grass percentage, high vegetation, branches percentage, raised rock/stump percentage, number of trees, proportion of debris, grasses, and tall vegetation within 0.5 m and branches percentage, number of shelters, and number of deep shelters within 2.5 m. It is precisely these variables that are able to distinguish multidimensional groups of objects, in this case localities within the study site. However, these results should be interpreted with great caution. The European green lizard is a relatively large and very mobile species. A circle with a radius of 0.5 m from the place of observation would therefore show which microhabitats are used by individuals. Habitat characteristics at this distance will be important mainly in terms of thermoregulation and passive antipredation. From the point of view of prey accessibility, both these distances are important. The availability of a main shelter is essential in respect to active predation avoidance (escape, temporary cover, vantage point), even at a greater distance.

From the PCA visualization (Fig. 5), four vectors can be recognized.

1. The first vector (corresponding with the direction of recorded presence) contains mainly variables of elevated rock and shelter, both within 0.5 m and 2.5 m from the point of observation, and includes variables directly linked to anti-predatory/thermoregulatory function. An elevated position provides a basking opportunity and a vantage point to see

potential predators or competitors, while the availability of shelter in the immediate vicinity is necessary to avoid predation (Majláth and Majláthová 2009; Fischer and Rehák 2010).

2. The second vector consists of bush and thorny bush variables, which we interpret as mainly anti-predatory. The vector is directed roughly in accordance with the recorded presence, which corresponds with data from similar studies (Heltai et al. 2015). Lizards have been frequently observed running first into a nearby shrub if disturbed and only if pursued further, seeking a refuge in subterranean shelter. This interpretation can also be supported by the fact that 95% of recorded observation points had at least one shrub or shelter within 0.5 m and 99% had at least one shrub or shelter within 2.5 m. We found no difference between the usage of thorny or not thorny shrubs.
3. The third identified vector contains grass and high vegetation variables and does not correspond with the direction of recorded presence. Our preferred explanation is that high vegetation and grass provide shade and thus lower the temperature of surfaces covered by them. Also, these objects limit the lizard's field of view without providing substantial cover from the predators, posing a potential risk. Of course, during periods of supra-optimal temperatures, individuals have been observed seeking lower temperatures in shade, but mostly preferring a shade provided by shrubs or seeking a subterranean shelter.
4. The last identified vector consists only of the scree coverage (both within 0.5 and 2.5 m) and does not correspond to the presence of lizards, but neither goes in the opposite direction. This is interpreted as mainly related to thermoregulatory effects since the screed can be very easily warmed by the sun, but these surface temperatures can easily reach 60 °C in warmer periods of direct sunlight (measured on site with an infrared thermometer), which makes them unusable. The gaps between scree are sufficient for juveniles and most subadults to be used as cover from predators, but larger adults are not able to fit in most of them. The rising percentage of warmer days in the area (Zahradníček et al. 2020) could also lead to a switch in both microhabitat and habitat usage in the future (Rehák et al. 2022). Thus, we consider scree to be the preferred surface only under very specific circumstances.

The nature of the site must also be considered when interpreting the results. The random points were plotted into the polygon covering the site (4.2 ha) and did not include areas inaccessible to lizards. There was therefore no risk that a random point would be placed, for example, in the middle of a stream or beyond the boundary of the site. Due to the relatively small size of the polygon, it was therefore unavoidable that a significant portion of the random points was located in the immediate vicinity of the points of recorded presence, as confirmed by the Haversine distances. Despite this, the analysis was able to distinguish the vast majority of random points from real observation locations.

The observed lizards were not individually identified, and the dataset thus contains some recordings of the same individuals multiple times. This problem could not be avoided but was minimized by using the line transect method, thus significantly lowering the probability of repeated records of the same individuals during the same visit. We were unable to avoid recording the same individual in different visits. The time delay between individual site visits lowered the risk of dependence on subsequent records even more. Nevertheless, we reasonably expected that repeated records of the same individual would be spatially clustered, and thus we tested the dataset for spatial autocorrelation.

The change of habitat preference by *L. viridis* during ontogeny is widely recognized and supported by the published data from many populations (e.g., Fischer and Rehák 2010; Harta et al. 2017). In spite of this, we failed to prove such an ontogenetic change. Concerning differences of microhabitat preferences among adult males and females, our results do not show any, and neither we found any evidence in literature.

Multivariate statistical models are rarely used in the study of reptile habitats. This method is widespread, especially in botany and invertebrate zoology; specifically, it is often used to predict the occurrence of selected species. However, in studies focused on aspects of species protection, these are very valuable methods, the outputs of which can have direct application. For example, the discrimination of a certain type of habitat in reptiles (Hacking et al. 2014) using the MANOVA method. Their results demonstrated that Schmeltz's skink (*Carlia schmeltzii*) avoids microhabitats with a high proportion of invasive grasses. A similar method was used in a study of the local population of *Lacerta bilineata* in northern Italy (Sacchi et al. 2011). According to the authors, individuals in the monitored population purposefully seek out ecotones for their microhabitat; however, they do not discriminate based on the specific composition of these ecotones. Other authors also mention the importance of ecotones in *L. viridis* microhabitat usage (Harta et al. 2017).

The research site has been subject to active management since 2000. By 2013, both the population density and area usage significantly increased in comparison to 1995–1997 (Fischer and Rehák 2010), with the density and abundance corresponding to populations in similar habitats (Prieto-Ramírez 2023). Still, the isolation of the population means a high risk to its long-term survivability (Böhme et al. 2007b), and small isolated populations are presumed to be most threatened by habitat erosion due to climate change (Sinervo et al. 2010). The management measures were focused on keeping the landscape mosaic by retaining key microhabitat elements while avoiding excessive growth of vegetation coverage, ideally by combining grazing and cutting (Fischer and Rehák 2010; Rehák 2015; Fischer et al. 2016, 2023; Mizsei et al. 2023). Our study has identified some of these microhabitat elements and their combinations, which should be taken into account when planning management measures in similar areas. Most notably, we recommend maintaining the maximum distance between potential shelters (provided by vegetation or terrain) at 5 m and to keep the landscape mosaic heterogeneous. Cover against predation from above, mostly provided by shrubs, is also important, though impossible to test from our dataset. Significant avoidance of places covered by high vegetation also suggests that overgrowth of habitat needs to be prevented.

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Supplementary material 1

DFA dataset

Authors: Jan Chmelař, Daniel Frynta, Veronika Rudolfová, David Fischer, Ivan Rehák

Data type: xlsx

Explanation note: Primary dataset for DFA containing age categories and variable values for points of recorded presence and random points.

Link: https://doi.org/10.3897/herpetozoa.37.e120806.suppl1

ÖGH Herpetozoa
Austrian Herpetological Society

Herpetozoa 37: 295–298 (2024)
DOI 10.3897/herpetozoa.37.e132815

Lethal grip for an endangered endemic frog: The freshwater crab *Potamon karpathos* (Decapoda, Potamidae) preys on Karpathos water frog *Pelophylax cerigensis* (Anura, Ranidae)

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https://zoobank.org/EB2EC01C-19FA-4E4D-89B2-580B01C03350

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Academic editor: Lukas Landler ♦ **Received** 22 July 2024 ♦ **Accepted** 9 September 2024 ♦ **Published** 8 October 2024

Abstract

Invertebrate predation on amphibians is widespread, although unreported in Europe. Here, we present documented predation of the Karpathos water frog (*Pelophylax cerigensis*) by the crab *Potamon karpathos*. *Pelophylax cerigensis* is an endangered, endemic species exclusively found on the Rhodes and Karpathos Islands in the South Aegean Sea, Greece. The Karpathos population is at risk due to limited suitable habitats and a scarcity of freshwater bodies on the island. From April to October, when there is no rain on the island, frogs and crabs cohabitate, sharing the same shallow ditches. Incidents of frog predation by crabs are common, indicated by numerous individuals with amputated limbs and scars, representing an additional threat to the endangered frog. Further research on the interactions between the two species is urgently required, as it has direct conservation implications.

Key Words

Aegean Sea, amphibians, crustaceans, interspecific interactions, predation

Invertebrate predation on amphibians has been recorded numerous times worldwide, with insects and arachnids being the most common predators (e.g., Toledo 2005; Baracho et al. 2014; Maffei et al. 2014; Jablonski 2015; Christopoulos et al. 2022). Brachyura, both marine (Pyke et al. 2013; Pérez-Sorribes and Gil-Climent 2021) and freshwater species (Sichieri et al. 2021; Abraham and Hutter 2022), are also known to prey on anurans.

In this study, we present the first documented case of predation of the Karpathos frog by a freshwater crab. Reports of crab predation on frogs have been published in the past (Affonso and Signorelli 2011; Warrington and Cossel 2012; Hedrick and Cossel 2014). To the best of our knowledge, this is the first documented case of amphibian predation by a crustacean in Europe.

The freshwater crab genus *Potamon* Savigny, 1816 (Decapoda: Potamidae) includes 22 species distributed in Asia, Southern Europe, and Northern Africa (Cumberlidge and Ng 2009; Ghanavi et al. 2023). The Aegean Islands host seven species of the genus, and *Potamon karpathos* Giavarini, 1934, is the species living on Karpathos Island in the South Aegean Sea, Greece (Jesse et al. 2011). The feeding ecology of the species is largely understudied and remains elusive. Nonetheless, the literature on the diet of other *Potamon* crabs supports an opportunistic pattern based on omnivory, with a higher preference for plants (Bahuguna et al. 2016; Fadlaoui and Melhaoui 2022), which may also include extreme cases of animal consumption such as ophiophagy (Groen et al. 2023).

Until recently, the Karpathos frog (*Pelophylax cerigensis* Beerli et al., 1994) (Anura: Ranidae) was considered endemic to Karpathos and was classified as critically endangered (CR) (IUCN 2022). A recent phylogenetic study revealed that the populations of Rhodes Island also belong to the same species (Toli et al. 2018, 2023). Consequently, *P. cerigensis* is now categorized as endangered (EN) according to the newest assessment (NECCA 2024). However, the risk category of the species may change again in the future: the Karpathos populations are sparse, water bodies on the island are few and in bad condition, and wildfires that occurred on Rhodes in July 2023 may have had a negative impact on the frog populations. The ongoing implementation of the national action plan (NAP) for the conservation of the Karpathos frog (Pafilis et al. 2020) will shed light on these issues after its completion in December 2025.

The snout-vent length (SVL) of adult Karpathos frogs usually ranges from 5–7 cm (Valakos et al. 2008). The species is diurnal throughout the year, and its diet follows an opportunistic pattern, with coleoptera, Araneae, Isopoda, and Hymenoptera being the main prey groups (Pafilis et al. 2019).

The first unconfirmed reports of frog predation by crabs come from the guards of the local management unit of the Dodecanese. During the annual fieldwork of the Action Plan implementation, we recorded a number of incidents that confirmed the initial reports. First, we noticed many individuals with amputated limbs or digits (Fig. 1A) or body scars (Fig. 1B), indicating that a predator with a sharp instrument attempted to capture them. Following this, we observed two clear cases of predation in the field.

On 30 June 2021 (3:20 pm), along the Argoni Stream (35.7004°N, 27.1501°E; 142 m a.s.l.), we noticed a frog (SVL = 51 mm) that was immobilized by the chelae of an adult crab (carapace width (CW) = 75 mm) grasping the frog's hind limbs. Subsequently, although the crab was captured to release the frog, it continued to hold the frog very tightly (Fig. 2).

On 3 December 2023 (11:47 am), again in Argoni Stream (35.6917°N, 27.1554°E; 185 m a.s.l.), we observed an adult frog (SVL ≈ 52 mm) jump into the water. A crab (CW ≈ 70 mm) immediately emerged from under a stone and grabbed the frog with its chelae (Fig. 3A). The frog was trying to escape, but the crab was persistently holding it underwater using a firm grip on the frog's hind part of the trunk (Fig. 3B). The crab immobilized the frog, grasping its hind limb with one chela, while trying to grab the frog by the body with the other chela (Fig. 3C). When the crab squeezed the frog's neck for some seconds, the frog stopped reacting. After a few minutes, during which the frog passed out, the crab began to consume its prey from the outer side of the thigh base (Fig. 3D).

Our numerous observations suggest that crabs regularly prey on Karpathos frogs. *Pelophylax cerigensis* and *P. karpathos* have been sharing the same biotope for a long time. However, their main habitat, small river puddles, is becoming increasingly scarce due to prolonged drought, particularly during the long Aegean summer. Thus, the two species have to coexist in quite restricted ponds that do not exceed 30 cm^2 in width and 3 cm in depth. In such limited microhabitats, encounters are inevitable and, app arently, end up in predation attempts by the crab. These new observations should be taken into account to design and adapt appropriate mitigation measures that will ensure the viability of the frog population. At this stage, we cannot propose such measures due to the complexity of the problem: water scarcity forcing the two species to live in the same microhabitats, while it is not possible to eradicate the crab's because it is a native species and part of the ecosystem. Further future investigations should look at this in more detail.

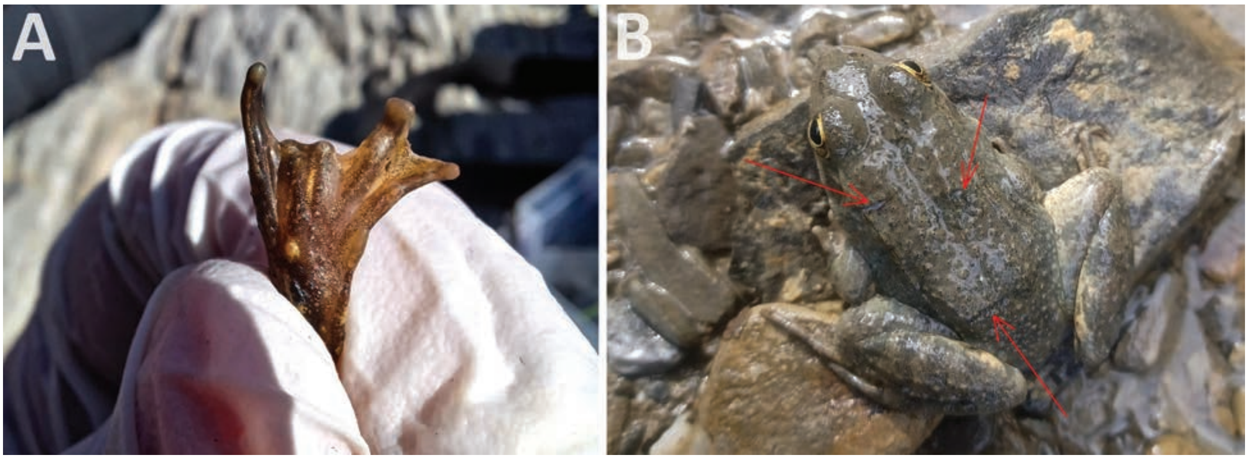

Figure 1. A. Amputated digits of the right hind limb of an adult *Pelophylax cerigensis*; **B.** Scars on the trunk of an adult frog marked with red arrows.

Figure 2. The first documented incidence of predation on *Pelophylax cerigensis* by *Potamon karpathos* on Argoni Stream, Karpathos Island, Dodecanese, Greece. Photos by Apostolos Christopoulos.

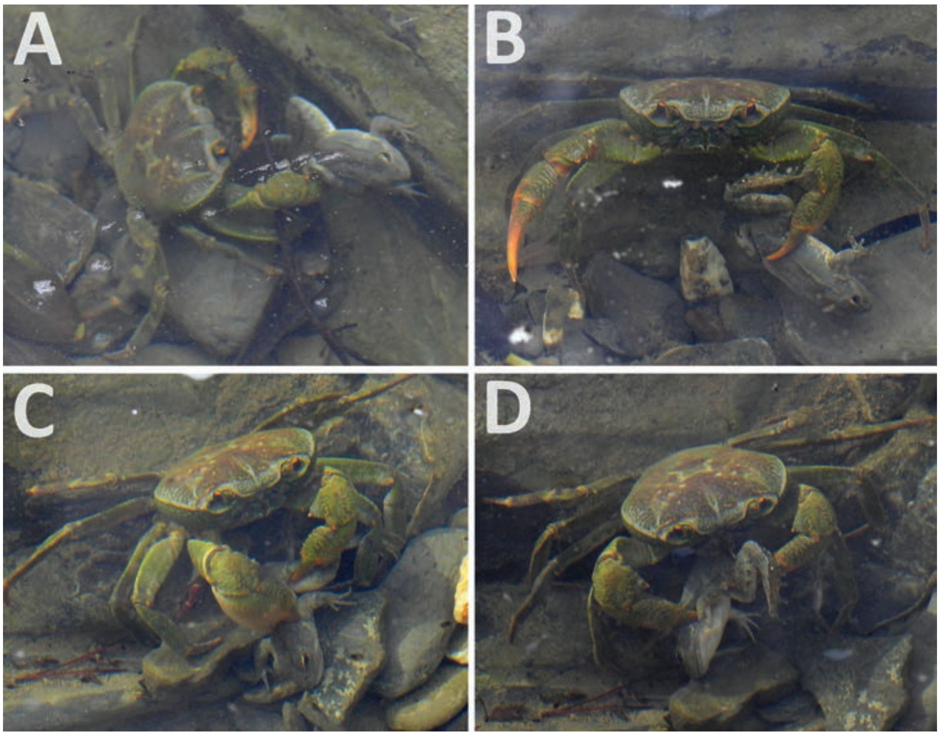

Figure 3. The second documented incidence of predation on *Pelophylax cerigensis* by *Potamon karpathos* in Argoni Stream, Karpathos Island, Dodecanese, Greece. **A.** The crab tries to grab the frog by its hind limbs; **B.** The crab holds the frog underwater with a firm grip; **C.** The crab keeps the frog immobilized while trying to strangle it between the trunk and head; **D.** The frog stops reacting and the crab starts to consume it. Photos by Apostolos Christopoulos.

Acknowledgements

The funding was provided by the GREEN FUND within the framework of the program LIFE-IP 4 NATURA (LIFE16 IPE/GR/000002) Integrated actions for the conservation and management of Natura 2000 network areas, species, habitats, and ecosystems in Greece, title “Implementation of the National Conservation Plan for the Karpathos frog (*Pelophylax cerigensis*, Beerli et al. 1994),” Priority Axis: AP 1: “Biodiversity conservation actions.” We are thankful to Giorgos Prearis, guard in the Management Unit of the Southeastern Aegean Protected Areas, for his assistance in the field. Fieldwork was conducted under permits 6ΔΘΘ4653Π8-1Β3 and ΨΟΥΩ4653Π8-ΘΨΠ issued by the Ministry of the Environment and Energy.

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ÖGH Herpetozoa
Austrian Herpetological Society

Herpetozoa 37: 299–303 (2024)
DOI 10.3897/herpetozoa.37.e128019

Crabs as snake predators? An observation from southern Italy leading to a comprehensive review

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Academic editor: Yurii Kornilev ◆ **Received** 21 May 2024 ◆ **Accepted** 4 September 2024 ◆ **Published** 18 October 2024

Abstract

The recent elevation of the Western Grass Snake, *Natrix helvetica*, from subspecies status prompted an assessment of its natural history and ecological traits compared to closely related species. We report an unusual predation attempt by the freshwater crab *Potamon fluviatile* on *N. helvetica sicula* from Sicily, indicating an ecological interaction previously overlooked in this species. The observation suggests that this crab may utilise snakes' muscle tissue as an additional nutrient source. This predator-prey interaction, although probably rare, adds to the understanding of the dynamics between crabs and snakes, shedding light on their interaction in freshwater habitats. To provide context for our observation and highlight its rarity, we searched through scientific literature and online sources to generate a comprehensive review of the phenomenon that examines the predatory behaviour of crabs on snakes.

Key Words

Decapoda, ecological interaction, grass snake, *Natrix helvetica*, natural history, *Potamon fluviatile*, predator-prey dynamics

The Western Grass Snake, *Natrix helvetica* (Lacépède, 1789), was recently elevated taxonomically from a subspecies of the *N. natrix* (Linnaeus, 1758) complex, based on a comprehensive genetic assessment (Kindler et al. 2017). Further molecular taxonomic evaluation examined the phylogeographic structure among populations of *N. helvetica* and its subspecific taxonomy (Kindler and Fritz 2018). According to these data, the species (comprising five subspecies) is distributed from Great Britain through central western Europe to Corsica, Sardinia, most of the Italian mainland, and Sicily. Furthermore, its elevation to species level provides renewed opportunities to compare natural history and ecological traits with the two other grass snake species, *N. natrix* and *N. astreptophora* (López-Seoane, 1884). Previous summaries treated the three grass snake species as one, even tough much information on predation referred to populations from western Europe, i.e., *N. helvetica* (Kabisch 1999; Mebert and Jablonski 2024).

Here we report an unusual predation attempt of a freshwater crab, *Potamon fluviatile* (Herbst, 1785), on a Sicilian Grass Snake, *N. helvetica sicula* (Cuvier, 1829), in the "Valle dell'Anapo" (37.142°N, 15.046°E), southeastern Sicily, Italy, observed on 21 March 2024. We encountered the adult freshwater crab while its claws tightly squeezed the tail of the adult snake (close to one meter in length), which was visibly alive (Fig. 1A). This predation attempt was observed for approximately 10 minutes, during which the grip of the crab's claws was strong enough to prevent the snake from freeing itself. Further search that day revealed three more grass snakes missing large portions of their tails (Fig. 1B–D).

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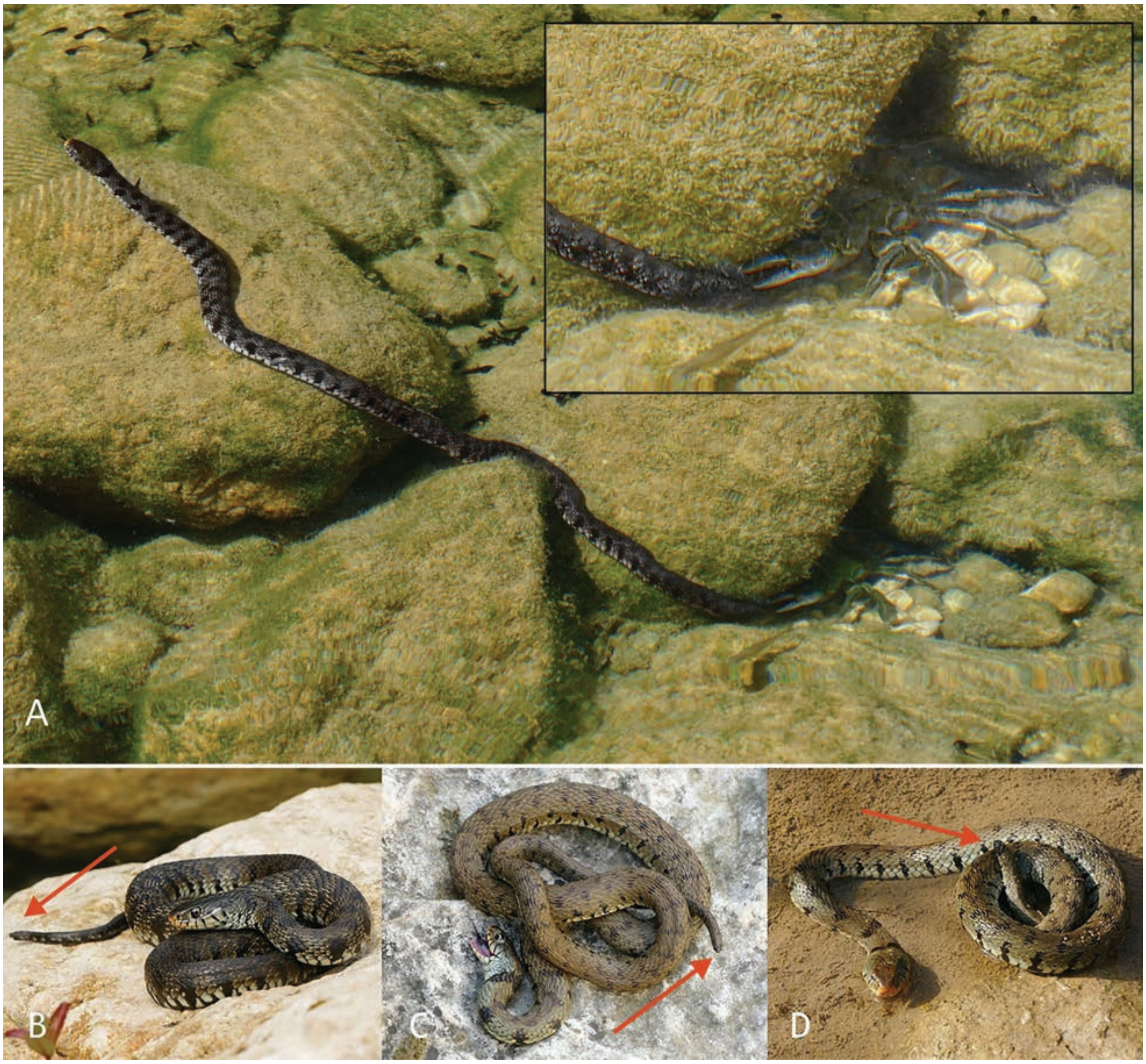

Figure 1. Predation attempt on *Natrix helvetica* by *Potamon fluviatile* from Sicily, Italy: A. adult *N. helvetica* with its tail held by the claw (inset) of a freshwater crab, *P. fluviatile*. B–D. different individuals of *N. helvetica* without the tip of the tail (marked by red arrows), suggesting previous crab-snake interactions. Photographs by David Hegner.

Based on additional 33 *N. helvetica* examined in Sicilian streams, in which *P. fluviatile* and *N. helvetica* coexist, 24.4% (n = 8) showed injured tails (F. P. Faraone, unpubl. data). Presumably, this freshwater crab, which has recently colonised Sicily and has expanded across the island (Vecchioni et al. 2017, 2022), may utilise snake (tail) muscle tissue as an opportunistic nutrition, offering an intriguing perspective on the flexible feeding networks of these freshwater animals. Eventually, tail loss in grass snakes may also result from predation by birds or mammals during the active season, from consumption by rodents, from freezing or dying off during hibernation, or even from pseudoautotomy as a defense against snake predation (Kabisch 1999; Gregory and Isaac 2005; Crnobrnja-Isailović et al. 2016 and references therein).

To compare our data in a global context, we conducted a thorough search of available scientific literature since Voris and Jeffries (1995) published their first topic-related review. We also searched online sources and the most common social network media—Facebook.com, Flickr.com, Instagram.com, Youtube.com—and the citizen-science platform inaturalist.org to compile a summary of this phenomenon that remained rarely observed.

We obtained 21 observations overall in which crabs engaged in a feeding interaction with snakes as prey. These observations encompassed 12 genera of crabs in eight families and 17 genera of snakes (with two cases lacking detailed identification) in eight families (Table 1). Although interactions between snakes and crabs are very rarely observed and reported (see references in Table 1), this is not the first observation of *Potamon* predating on

Table 1. A summary of crabs predating on snakes based on published literature and citizen-science data. cw = carapace width; SVL = snout-vent length.

Predator		Prey			Observed or inferred feeding behaviour	Study area			Date	Source
Species (cw in mm)	**Family**	**Species (SVL in mm)**	**Family**	**Condition**		**Locality/country**	**Coordinates (Lat, Long)**	**Habitat**		
Scylla serrata	Portunidae	Not determined	–	–	Unknown, snake remains in 5% of crab stomach (n = 86)	Phuket Island/ Thailand	–	Sea	–	Voris and Jeffries (1995)
Scylla serrata	Portunidae	*Cerberus rynchops*	Homalopsidae	Alive	Predation of 24% of 106 experimental trials	Phuket Island/ Thailand	–	Experimental enclosures	–	Voris and Jeffries (1995)
Eudaniela garmani (95)	Pseudothelphusidae	*Pseudoboa neuwiedii* (540)	Dipsadidae	Dead	Unknown, consumed tail first	Little Englishman's Bay/Tobago	–	2 m above the riverbank	27 August 1997	Maitland (2003)
Eudaniela garmani (42)	Pseudothelphusidae	*Atractus trilineatus*	Dipsadidae	Alive	Active predation, consumed tail first	Englishman's Bay/ Tobago	–	Above the water surface of the crab's burrow	23 August 1998	Maitland (2003)
Eudaniela garmani (92)	Pseudothelphusidae	*Erythrolamprus melanotus* (380)	Dipsadidae	Alive	Active predation, the prey's back was damaged	Gilpin Trace/ Tobago	–	1 m above the water level, river	5 June 2000	Maitland (2003)
Eudaniela garmani (~85)	Pseudothelphusidae	*Chironius carinatus*	Colubridae	Dead	Unknown, prey partially consumed	Little Englishman's Bay/Tobago	–	River	3 September 1997	Maitland (2003)
Eudaniela garmani (96)	Pseudothelphusidae	*Oxybelis aeneus* (620)	Colubridae	Dead	Active predation, the prey's back was damaged	Little Englishman's Bay/Tobago	–	Riverbank	6 June 2000	Maitland (2003)
Eudaniela garmani (70)	Pseudothelphusidae	*Sibon nebulatus* (203)	Dipsadidae	Alive	Active predation, consumed tail first	Arnos Vale/Tobago	–	Shallow water, river	22 August 1997	Maitland (2003)
Callinectes sapidus	Portunidae	*Nerodia clarkii* (404)	Natricidae	Alive	Attempted predation	West Ship Island/ USA	30.2091, -88.9783	Sea, shallow water	30 March 2006	Mohrman et al. (2008)
Potamon pelops	Potamidae	*Natrix natrix*	Natricidae	Alive	Active predation, consumed tail first	Charavgi/Greece	36.9847, 21.8550	Shallow water, stream	10 July 2020	Groen et al. (2023)
Exanthelphusa sp.	Gecarcinucidae	*Fowlea* cf. *flavipunctatus*	Natricidae	Dead	Unknown circumstances	Unknown, probably Asia	–	Muddy water (rice field?)	–	https://www.youtube.com/shorts/6VCVk1pI1g0
Parathelphusa convexa	Gecarcinucidae	Blindsnake	Gerrhopilidae or Typhlopidae?	–	Unknown circumstances	Java	–	Probably stream bank	–	https://create.vista.com/it/unlimited/stock-photos/583199136/stock-photo-field-crab-shows-expression-ready-attack-animal-has-scientific-name/
Parathelphusa convexa	Gecarcinucidae	*Xenodermus javanicus*	Xenodermidae	–	Unknown circumstances	Java	–	Probably stream bank	–	https://create.vista.com/it/unlimited/stock-photos/599183670/stock-photo-field-crab-eating-dragon-snake-animal-has-scientific-name-parathelphusa/
Ocypode sp.	Ocypodidae	*Hydrophis platurus*	Elapidae	Dead	Unknown, possible scavenging	Chandrabhaga beach/India	–	Sandy beach	9 February 2023	https://www.newsflare.com/video/543058/
Thalamita cf. *crenata*	Portunidae	*Hydrelaps darwiniensis*	Elapidae	–	Unknown circumstances	Broome/Australia	–	Sea shore	8 March 2017	https://www.flickr.com/photos/reptileshots/33332780516/in/photostream/
Candidiopotamon okinawense	Potamidae	*Hebius pryeri*	Natricidae	Dead	Unknown circumstances	Yanbaru forest, Okinawa/Japan	–	Forest	23 June 2016	https://www.flickr.com/photos/okinawaphotos/27655519790/in/photostream/
Potamonautes sp.	Potamonautidae	*Causus* cf. *rhombeatus*	Viperidae	Dead	Unknown circumstances	Unknown, probably Africa	–	Probably, backyard pavement	–	https://www.reddit.com/r/natureismetal/comments/17j0y18/crab_eating_a_snake/
Potamon fluviatile	Potamidae	*Natrix* cf. *helvetica*	Natricidae	Dead	Unknown circumstances	Italy (?)	–	Shallow water, stream	–	https://www.youtube.com/watch?v=jhiiofyj1lg
Potamon fluviatile	Potamidae	*Natrix helvetica*	Natricidae	Alive	Active predation	Valle dell'Anapo/ Italy	37.142, 15.046	Shallow water, stream	21 March 2024	This study
Birgus latro	Coenobitidae	*Boiga irregularis*	Colubridae	Alive	Attempted predation	Northern Guam	13.603, 144.871	Tree 0.7 m above ground	8 March 2010	Lardner et al. (2011)
Dilocarcinus pagei	Trichodactylidae	*Helicops leopardinus*	Colubridae	Dead	Scavenging	Macapá, Amapá state, Brazil	0.045, -51.067	Municipal pond	13 April 2018	Tavares-Pinheiro et al. (2018)
?	?	?	?	Dead	Feeding or scavenging	Indonesia	–	Shallow water, stream	–	https://www.facebook.com/reel/975446370681443

Natrix. Groen et al. (2023) described a similar predation attempt on a juvenile *N. natrix* by a Peloponnesian Freshwater Crab, *Potamon pelops* Jesse, Schubart, Klaus, 2010, in Greece. The authors did record the consumption of the snake's tail, the cloaca, and part of the lower abdomen (likely the most easily captured part of a snake's body by a crab) but did not complete their observation to record whether the crab fully consumed the snake. One of the cases reported in Table 1 is a video probably taken in Italy (https://www.youtube.com/watch?v=jhiiofyj1lg), which shows a *P. fluviatile* eating a dead adult *N. helvetica*, similarly to what we describe herein. However, no information was obtained to confirm the circumstances of this record, e.g., whether it shows a predation or scavenging event.

Further observations were gathered from all continents where snakes and crabs coexist and included interactions from various habitats and situations (i.e., active or attempted predation): for example, a video and text by Shakit Nanda (2023; https://www.newsflare.com/video/543058/) shows a Ghost Crab, *Ocypode* sp., pulling a deceased Yellow-bellied Sea Snake, *Hydrophis platurus* Linnaeus, 1766, into its sandy burrow on Chandrabhaga Beach, Eastern India. A photograph showing another beach crab holding on to a Black-ringed Mangrove Sea Snake, *Hydrelaps darwiniensis* Boulenger, 1896, in Broome, Western Australia (Brendan Schembri 2017 on flickr.com). In Sri Lanka, a Common Freshwater Crab, *Ceylonthelphusa sentosa* Bahir, 1999, grabbed the tail of a Schokar's Bronzeback Snake, *Dendrelaphis schokari* (Kuhl, 1820), but it became distracted by the observers a few minutes later and subsequently released/lost the snake (De Zoysa et al. 2015). Lardner et al. (2011) reported that a Coconut Crab, *Birgus latro* (Linnaeus, 1767) in northern Guam held onto, and later released, the tail of a large Cat-eyed Snake, *Boiga irregularis* (Bechstein, 1802) (SVL 1124 mm, total length 1423 mm). Additionally, the same authors mention that 40% of *B. irregularis* on the Orote Peninsula in Guam showed tail damage, indicating that crabs often attempt to prey on snakes in that area. The authors thus suggest a possible correlation between crab densities and the incidence of tail damage (Lardner et al. 2011). The aforementioned records thus support our assumption from Sicily that (mostly) snake tails are used as a food source by decapods, particularly crabs, likely due to the fact that this body part is small enough to be easily caught by their claws, aside from juveniles that also exhibit a small body circumference. We can alternatively hypothesise that predation pressure (where crabs represent a good example) on the tail may have contributed to the development of the little-studied pseudoautotomy (see review in Crnobrnja-Isailović et al. 2016), which has also been observed in semiaquatic snakes, including the genus *Natrix*.

Furthermore, Tavares-Pinheiro et al. (2018) observed an adult female freshwater crab, *Dilocarcinus pagei* Stimpson, 1861, feeding on a partly decomposed juvenile water snake, *Helicops leopardinus* (Schlegel, 1837), in a temporary pond in Macapá, Amapá State, Brazil. A predation attempt by a Blue Crab, *Callinectes sapidus* Rathbun, 1896 in Mississippi, USA, was described by Mohrman et al. (2008). Maitland (2003) documented six incidences of the freshwater crab *Rodriguezus* (formerly *Eudaniela*) *garmani* (Rathbun, 1898) from Tobago Island preying on three species of snakes: *Sibon nebulatus* (Linnaeus, 1758), *Atractus trilineatus* Wagler, 1828, and *Oxybelis rutherfordi* Jadin et al., 2020 (see details in Table 1).

Nonetheless, our rare observation contributes to the ongoing discussion on tail injuries in snakes resulting from predation pressure, confirming the occurrence of such ecological interactions between crabs and Grass Snakes. Furthermore, it represents the first well-documented instance of this interaction for both the Western Grass Snake *N. helvetica* and the geographic region.

Acknowledgements

We thank Elias Tzoras, Yurii Kornilev, and one anonymous reviewer for their valuable comments and corrections on an earlier version of the manuscript. This work was supported by the Scientific Grant Agency of the Slovak Republic VEGA 1/0242/21.

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ÖGH Herpetozoa
Austrian Herpetological Society

Herpetozoa 37: 305–318 (2024)
DOI 10.3897/herpetozoa.37.e131965

A revision of recent taxonomic changes to the eyelash palm pitviper, *Bothriechis schlegelii* (Serpentes, Viperidae)

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Academic editor: Peter Mikulíček ♦ **Received** 12 July 2024 ♦ **Accepted** 9 October 2024 ♦ **Published** 23 October 2024

Abstract

The taxonomy of the *Bothriechis schlegelii* species group has recently been expanded, nearly doubling the number of recognized species within this genus. However, the validity of these new species, defined primarily by mitochondrial DNA and without a robust evaluation of nuclear DNA and morphological data, warrants critical reassessment. This study re-examines the integrative data used in the initial analysis and employs additional visualizations of the genetic and morphological datasets. The results suggest that the genetic differences previously interpreted as species boundaries may instead reflect clinal variation, not independent lineages. Furthermore, DELINEATE species delimitation analysis and morphological assessments challenge the distinction of the proposed taxa. The findings indicate that only three species in the *B. schlegelii* group should be maintained as valid (*B. nigroadspersus*, *B. schlegelii*, and *B. supraciliaris*), while most taxa proposed by Arteaga et al. (2024) should be considered junior synonyms, or at most, as subspecies. This has significant implications for conservation priorities and resource allocation. In highlighting the need for a cautious and thorough approach to species delimitation, this study contributes to the broader discussion of taxonomic practices, the issue of taxonomic inflation, and their impact on biodiversity conservation.

Resumen

La taxonomía del grupo de especies *Bothriechis schlegelii* se ha expandido recientemente, casi duplicando el número de especies reconocidas dentro de este género. Sin embargo, la validez de estas nuevas especies, definidas principalmente por ADN mitocondrial y sin una evaluación robusta de datos nucleares, requiere una reconsideración crítica. Este estudio reexamina los datos integrativos utilizados en el análisis inicial y empleando visualizaciones adicionales de los conjuntos de datos genéticos y morfológicos. Los resultados sugieren que las diferencias genéticas previamente interpretadas como límites de especies podrían en cambio reflejar variación clinal, no linajes independientes. Además, el análisis de delimitación de especies DELINEATE y las evaluaciones morfológicas desafían la distinción de los taxones propuestos. Los hallazgos indican que solo tres especies del grupo *B. schlegelii* deben mantenerse como válidas (*B. nigroadspersus*, *B. schlegelii* y *B. supraciliaris*), mientras que la mayoría de los taxones propuestos por Arteaga et al. (2024) se consideran como sinónimos, o máximo, como subespecies. Esto tiene implicaciones significativas para las prioridades de conservación y la asignación de recursos. Al resaltar la necesidad de un enfoque cauteloso y exhaustivo para la delimitación de especies, este estudio contribuye a la discusión más amplia sobre las prácticas taxonómicas, el problema de la inflación taxonómica y su impacto en la conservación de la biodiversidad.

Kurzfassung

Die Taxonomie der *Bothriechis schlegelii*-Artengruppe wurde kürzlich erweitert, wodurch sich die Anzahl der anerkannten Arten innerhalb dieser Gattung nahezu verdoppelt hat. Die Gültigkeit dieser neuen Arten, die hauptsächlich durch mitochondriale DNA definiert wurden, und aber ohne eine robuste Bewertung nuklearer Daten auskommen, bedarf jedoch einer kritischen Neubewertung. Diese Studie untersucht die integrativen Daten, die in der ursprünglichen Analyse verwendet wurden, und nutzt zusätzliche Visualisierungen der genetischen und morphologischen Datensätze. Die Ergebnisse deuten darauf hin, dass die genetischen Unterschiede, die zuvor als Artgrenzen interpretiert wurden, stattdessen klinale Variationen und keine unabhängigen Linien darstellen könnten. Darüber hinaus stellen DELINEATE-Artabgrenzungsanalysen und morphologische Bewertungen die Unterscheidung der vorgeschlagenen Taxa in Frage. Die Ergebnisse zeigen, dass nur drei Arten in der *B. schlegelii*-Gruppe als gültig anerkannt werden sollten (B. nigroadspersus, *B. schlegelii* und *B. supraciliaris*),

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während die meisten von Arteaga et al. (2024) vorgeschlagenen Taxa als jüngere Synonyme oder höchstens als Unterarten betrachtet werden sollten. Dies hat erhebliche Auswirkungen auf die Prioritäten im Naturschutz und die Ressourcenzuweisung. Indem die Notwendigkeit eines vorsichtigen und gründlichen Ansatzes zur Artabgrenzung hervorgehoben wird, trägt diese Studie zur breiteren Diskussion über taxonomische Praktiken, das Problem der taxonomischen Inflation und deren Auswirkungen auf den Biodiversitätsschutz bei.

Key Words

conservation priorities, taxonomic inflation, species delimitation

Palabras clave

Inflación taxonómica, delimitación de especies, prioridades de conservación

Schlüsselwörter

Artenabgrenzung, Naturschutzprioritäten, taxonomische Inflation

Introduction

The delimitation of species has evolved significantly over time, transitioning from morphology-based approaches to molecular-based methods. With the advent of DNA sequencing, particularly the rapid amplification of mitochondrial DNA (mtDNA), there has been a surge in species delimitation based on genetic data (Hebert et al. 2003; DeSalle et al. 2005; Ahrens 2024). However, this shift has also brought challenges and potential pitfalls (Hillis 2019; Ahrens 2024).

Species delimitation is a complex process that should not rely solely on a single type of data, such as mtDNA. While mtDNA can provide valuable insights, it often leads to the over-splitting of species due to deep intraspecific genetic divergences, reduced gene flow, lack of recombination, incomplete lineage sorting, sex-biased dispersal, and asymmetrical introgression (Funk et al. 2003; Hebert et al. 2003; DeSalle et al. 2005). These issues can obscure the true nature of species boundaries. Therefore, an integrative approach that includes nuclear DNA, morphological data, and ecological information is necessary for accurate species delimitation (Padial et al. 2010). Integrating these methods can lead to a more accurate reflection of evolutionary history and avoid the pitfalls of premature taxonomic changes based solely on mitochondrial genetic data.

Recent studies on North American snakes that claim to use an integrative taxonomic approach have, in fact, made taxonomic changes based on mtDNA clades alone, with nuclear or morphological data playing a minimal role in diagnosing supposedly new species (e.g., Krysko et al. 2016; Carbajal-Márquez et al. 2020; Jadin et al. 2020; Tepos-Ramírez et al. 2021—but see Folt et al. 2019 for an example of articles fixing some of the issues).

To illustrate these challenges, the case of the palm pit vipers (genus *Bothriechis*) is particularly instructive. *Bothriechis* species have long captivated the fascination of both professional herpetologists and enthusiasts, taking on an almost mythic status due to their enigmatic behaviors, remarkable chromatic diversity, and potent venom. *Bothriechis* was historically composed of 11 species that mostly occur at relatively high elevations in mesic environments from Oaxaca to Ecuador and Venezuela (Campbell and Lamar 2004; Wylie and Grünwald 2016). The eyelash pit viper, *Bothriechis schlegelii* (Berthold, 1846) sensu lato, celebrated for its kaleidoscopic color variations, is particularly renowned. This species is distributed across a vast expanse of over 3,000 km, encompassing a myriad of eco-geographic zones, leading to the hypothesis that it might actually comprise multiple distinct species (Hurtado-Gómez 2009).

In a recent, exquisitely illustrated study, Arteaga et al. (2024) undertook a comprehensive taxonomic review of *Bothriechis schlegelii*. Employing an integrative approach, they analyzed morphological characteristics and molecular markers, including both nuclear and mitochondrial DNA sequences. Their work defined five novel species and reinstated three others previously subsumed under *B. schlegelii*. These new species came as a surprise, as these taxonomic findings nearly doubled the number of species in the genus *Bothriechis*. According to their findings, the nominal species is now geographically confined to Colombia's Cordillera Central (Fig. 1). Of the newly described and resurrected species, four are endemic to Colombia (*B. khwargi*, *B. klebbai*, *B. rasikusumorum*, and *B. schlegelii*), two occupy exclusive ranges within Ecuador (and possibly Peru, *B. hussaini and B. nitidus*), one straddles Ecuador and Colombia (*B. rahimi*), another between Colombia and Panama (*B. torvus*), and one spans from the Panama-Colombia border to southern Mexico (*B. nigroadspersus*). From now on, I refer to the species described or resurrected by Arteaga et al. (2024) as the *B. schlegelii* species group.

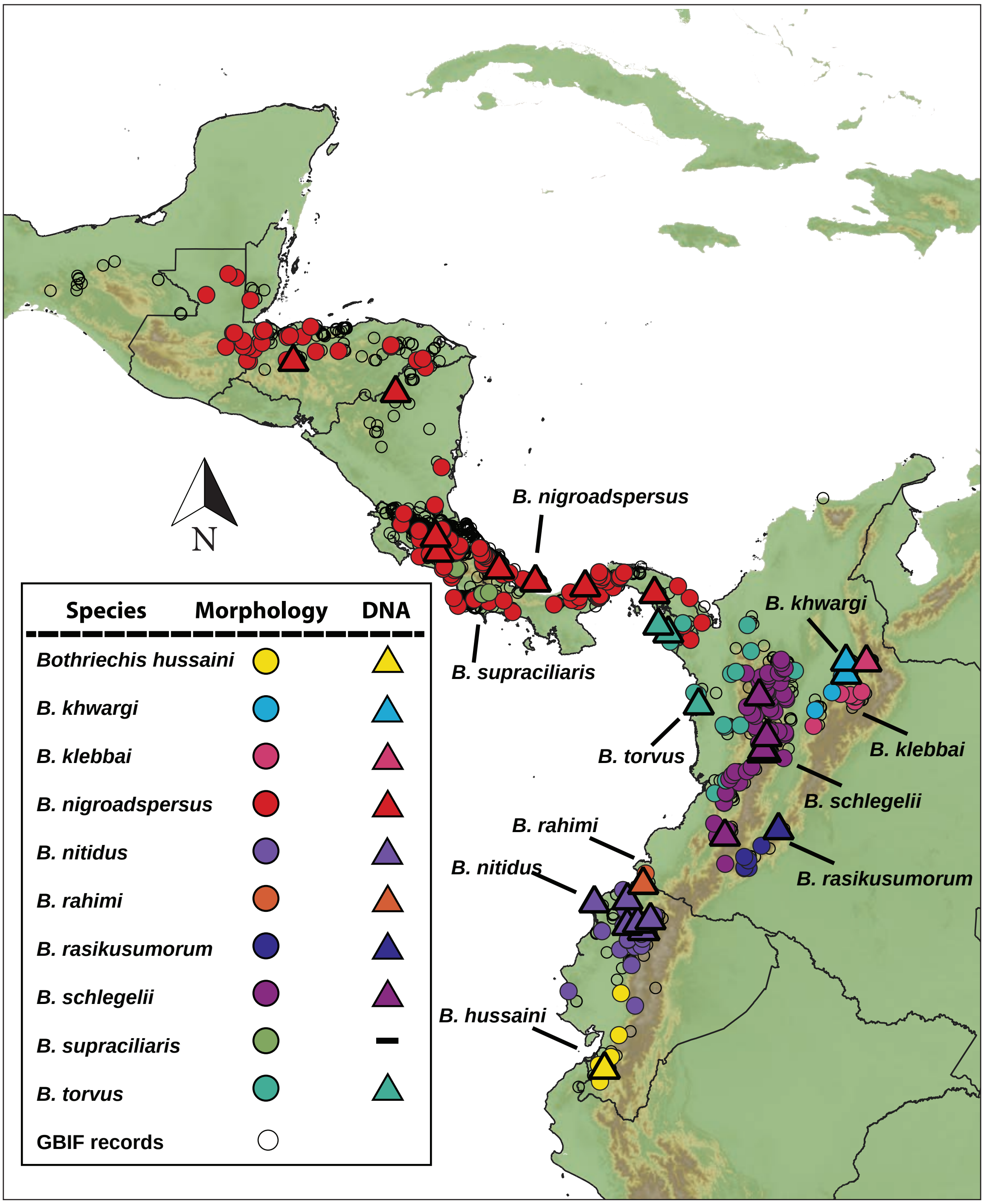

Figure 1. Geographic distribution map of the *Bothriechis schlegelii* species group. Colored circles indicate individuals assessed in Arteaga et al.'s morphological analysis. Colored triangles mark individuals for which molecular sequence data and locality information are available. Open circles correspond to occurrences from the Global Biodiversity Information Facility (GBIF) database.

Considering their molecular findings and methodology, questions arise regarding whether the newly described or resurrected taxa in fact represent separate species or if they only represent mitochondrial intraspecific diversity and not necessarily separate species within this complex. Herein, I reassess their datasets, employ other visualization methods on their genetic and morphological datasets, and re-evaluate the species designations based on their own rationale for the definition of species-level candidate taxa to rigorously test the species boundaries within the *Bothriechis schlegelii* complex.

Materials and methods

Molecular analyses

To re-evaluate the taxonomic conclusions of Arteaga et al. (2024), I first retrieved the same sequences that they analyzed in their study (Arteaga et al. 2024—supplementary table S1). They used seven loci: two nuclear (RAG1 and NT3), as well as five mitochondrial, including two non-coding (12S and 16S) and three coding (COI, Cyb7, and ND4) loci. Despite their extensive sampling of loci, only three mitochondrial loci had less than 50% of missing data (16S, Cytb, and ND4), while their two nuclear loci had ~90% missing data. Each locus was aligned separately in MAFFT version 7 (Katoh et al. 2019) using the Q-INS-I option. The alignments were then concatenated using FASconCAT v.1.04 (Kück and Longo 2014). The final alignment comprised 4,959 base pairs, which included sequences from 88 representatives of the genus *Bothriechis*, as well as *Tropidolaemus wagleri* used as an outgroup.

For Bayesian phylogenetic analysis (BI), I used the same model for nucleotide substitution for each gene segment and codon position as presented by Arteaga et al. (2024, table 3). Subsequently, BI was executed through Mr. Bayes v3.2.2 (Ronquist et al. 2012) using the CIPRES science gateway (Miller et al. 2011). This involved four separate runs with a total of 10 million generations each, sampled every 1,000 generations. This included three hot chains and a single cold chain. I assessed the convergence of the runs by examining the overlap in likelihoods and parameter estimates, as well as the effective sample sizes and the potential scale reduction factor (PSRF), using Tracer v1.7.1 (Rambaut et al. 2022). The runs converged after 200,000 generations, which allowed for the initial 25% of the data to be excluded as burn-in. The remaining trees were combined using TreeAnnotator (Drummond and Rambaut 2007) and viewed using Figtree v1.4.2 (Rambaut 2014). Additional analyses, which included only the nuclear loci and only mtDNA data, were carried out the same way as previously described.

Maximum likelihood analyses (ML) were carried out on the combined data sequence in IQ-TREE (Nguyen et al. 2015) through its online platform (Trifinopoulos et al. 2016). An automatic model selection was used, and the analysis included 1,000 bootstrap iterations to determine support levels.

Due to the resemblance of the trees derived from ML and BI methods, I present only the phylogenetic tree resulting from BI of concatenated data, while the ML trees and mtDNA analyses are included as Suppl. material 3: figs S1–S3.

SplitsTree

I employed SplitsTree (Huson and Bryant 2006) to generate phylogenetic networks for the combined Cytb and ND4 mitochondrial DNA sequences of Arteaga et al. (2024). SplitsTree is a tool that generates phylogenetic networks from molecular data, which can reveal complex evolutionary relationships or homoplasy in the data not captured by traditional trees, thereby offering deeper insights into species divergence.

Genetic distances

Arteaga et al. (2024) highlighted sequence divergence as a critical factor for delineating new species, specifically using a 4% divergence threshold in the mitochondrial Cytb gene. Reexamining this threshold, I calculated pairwise genetic distances in the mitochondrial Cytb and ND4 genes using MEGA X software (Kumar et al. 2018). These analyses considered all codon positions and incorporated both nucleotide substitutions and variations in substitution rates, the latter modeled with a Gamma distribution.

Species delimitation analysis

Recent studies have shown that species delimitation analyses delimit populations and not necessarily species (Sukumaran and Knowles 2017; Chambers and Hillis 2020; Sukumaran et al. 2021), therefore, they can be misleading when defining species and tend to over-split species in the case of geographically widespread taxa (Chambers and Hillis 2020). In order to test for "species" (or populations) limits in *Bothriechis*, I employed the program DELINEATE (Sukumaran et al. 2021), which uses a protracted speciation model (PSM). DELINEATE allows the user to specify what lineages are considered as species validated with other types of data (morphology, ecology, etc.) and to test if certain molecular lineages could be considered species based on previous taxonomic cutoffs.

I set up a StarBeast3 configuration file using the program BEAUTI, part of the BEAST software package (Drummond and Rambaut 2007). Due to computational constraints, I used one individual per species, with the exception of *Bothriechis nigroadspersus*, in which I used one individual of each of the three major mitochondrial clades of Arteaga et al. (2024). I included the mitochondrial loci 12S, 16S, Cytb, and ND4, with a single strict clock model of substitution across genes and an HKY+G substitution model. The BEAST analysis ran for 200 million generations, sampling every 10,000 generations, for a total of 20,000 trees.

In order to check if the BEAST2 analysis had run for long enough, I examined the overlap in likelihoods and parameter estimates, as well as the effective sample sizes and the potential scale reduction factor (PSRF), using Tracer v1.7.1 (Rambaut et al. 2022). I then used Tracer v. 1.7.1 (Rambaut et al. 2022) to discard the first 10% of trees as burn-in. The remaining trees were combined using the Maximum Clade Credibility tree option in TreeAnnotator (Drummond and Rambaut 2007). I then prepared the population assignment input file for DELINEATE and constrained all species of *Bothriechis*, with the exception of the eight taxa described or resurrected by Arteaga et al. (2024).

Revision of morphological characters

Re-valuation of morphological characters and principal component analyses

In order to better visualize variation in the morphological characters of the new species of *Bothriechis* presented by Arteaga et al. (2024), I performed a principal component analysis (PCA) on the data they provided in their Suppl.

material 2: table S1. First, I excluded individuals that had more than 20% missing morphological character data. Separate analyses were conducted for scale count data and measurement data for both males and females.

For scale count data, I used 78 males and 95 females. For measurement data, I used two different standardization methods: standardization by snout-vent length (SVL) and regression residuals (Thorpe 1975, 1983). The number of individuals used in these analyses varied due to the specific requirements of each method. For males, I used 65 individuals for the SVL-standardized data and 67 for the regression residuals, for females, I used 84 individuals for the SVL-standardized data and 85 for the regression residuals.

I performed PCA in Python using multiple computing libraries: Pandas for data manipulation, NumPy for numerical computations, Scikit-learn for applying PCA, and Matplotlib for data visualization. The analysis began with preprocessing the dataset to exclude individuals with more than 20% missing data and standardizing the features according to the three methods mentioned above. PCA was then conducted to reduce the dimensionality of the data, extracting principal components that capture the most significant variance and patterns within the morphological traits.

Results

Molecular phylogenetic analysis

Concatenated analysis

The resulting phylogenetic relationships within *Bothriechis* inferred using BI were almost identical to those of Arteaga et al. (2024), which is not surprising, as the same samples and loci were used (Fig. 2). However, a number of differences should be pointed out. In Fig. 2 of Arteaga et al. (2024), it was claimed that the support values on intra-specific branches were not shown for clarity, however, they did show the support for some of the branches but not others. In the present study, the sister relationship between *B. rasikusumorum* and all other members of *B. schlegelii* was recovered with low posterior support (pp = 0.58), as well as *B. rahimi*, sister to *B. hussaini* + *B. nitidus*. All other species and relationships, despite being recovered as monophyletic (with one exception), were recovered with low support, which coincides with the branches not reported by Arteaga et al. (2024). The relationships between *B. klebbai*, *B. khwargi*, *B. torvus*, and *B. schlegelii* formed a polytomy. An individual of *B. khwargi* (UIS-R2529) was recovered as nested within *B. klebbai*. This was not an error when making the alignment, as when the sequence was blasted in GenBank, the closest matches were individuals of *B. klebbai*, however, it could be an issue that occurred when the authors submitted their sequences to GenBank.

In the ML analysis, the monophyly of South American *Bothriechis* was recovered with high support (bootstrap support (bs) = 100%), while *B. khwargi* was recovered as sister to all other species described or resurrected by Arteaga et al. (2024), but with low support (bs = 60%). *Bothriechis torvus* was recovered as sister to all remaining species, however, most nodes had very low bootstrap values, with the exception of *B. rahimi*, sister to *B. hussaini* + *B. nitidus,* which had relatively high support (bs = 98%). Once again, an individual of *B. khwargi* (UIS-R2529) was recovered as nested within *B. klebbai* (Suppl. material 3: fig. S1).

mtDNA analysis

Both ML and BI analysis of the concatenated mtDNA data resulted in a very similar topology to the concatenated phylogenies, which included nuclear loci (Suppl. material 3: figs S2, S3). This is no surprise, as the vast majority of the data in the mtDNA-nuclear concatenated analysis corresponds to mtDNA sequences.

Nuclear analysis

Of the individuals of *Bothriechis* sequenced by Arteaga et al. (2024), only 12 individuals from 5 species had nuclear DNA data, which limits its utility for estimating relationships within the group. The Bayesian inference analysis of the concatenated nuclear loci produced a poorly supported phylogeny in which none of the species formed a monophyletic group (Fig. 3). Most nodes in the phylogeny were poorly supported (posterior probability (pp) < 0.5), with only a handful of nodes with values higher than 0.5 pp (Fig. 3).

SplitsTree phylogenetic networks

The resulting phylogenetic networks from SplitsTree showed that many of the members of the *B. schlegelii* group had very low levels of genetic differentiation, with the genetic differentiation between species in that group being even lower than between populations of *B. nigroadspersus* (Fig. 4).

Genetic distances

Upon reevaluating the Cytb mitochondrial data presented by Arteaga et al. (2024), it was observed that within the *B. schlegelii* group, only four species exhibited an average genetic distance exceeding 4% in relation to all other members (as shown in Table 1). Arteaga et al. (2024) classified individual clusters as distinct species when their average pairwise (p) genetic distance was greater than 4%. They justified this cutoff by noting its applicability to species delineation in other *Bothriechis* taxa. Yet, such a level of interspecific divergence seems exclusive to *B. lateralis* vs. *B. guifarroi,* where other pairwise comparisons within the genus show divergences ranging from 6.3% to 20.3% in the Cytb gene, and only four comparisons yielded a divergence of less than 7% (Table 1).

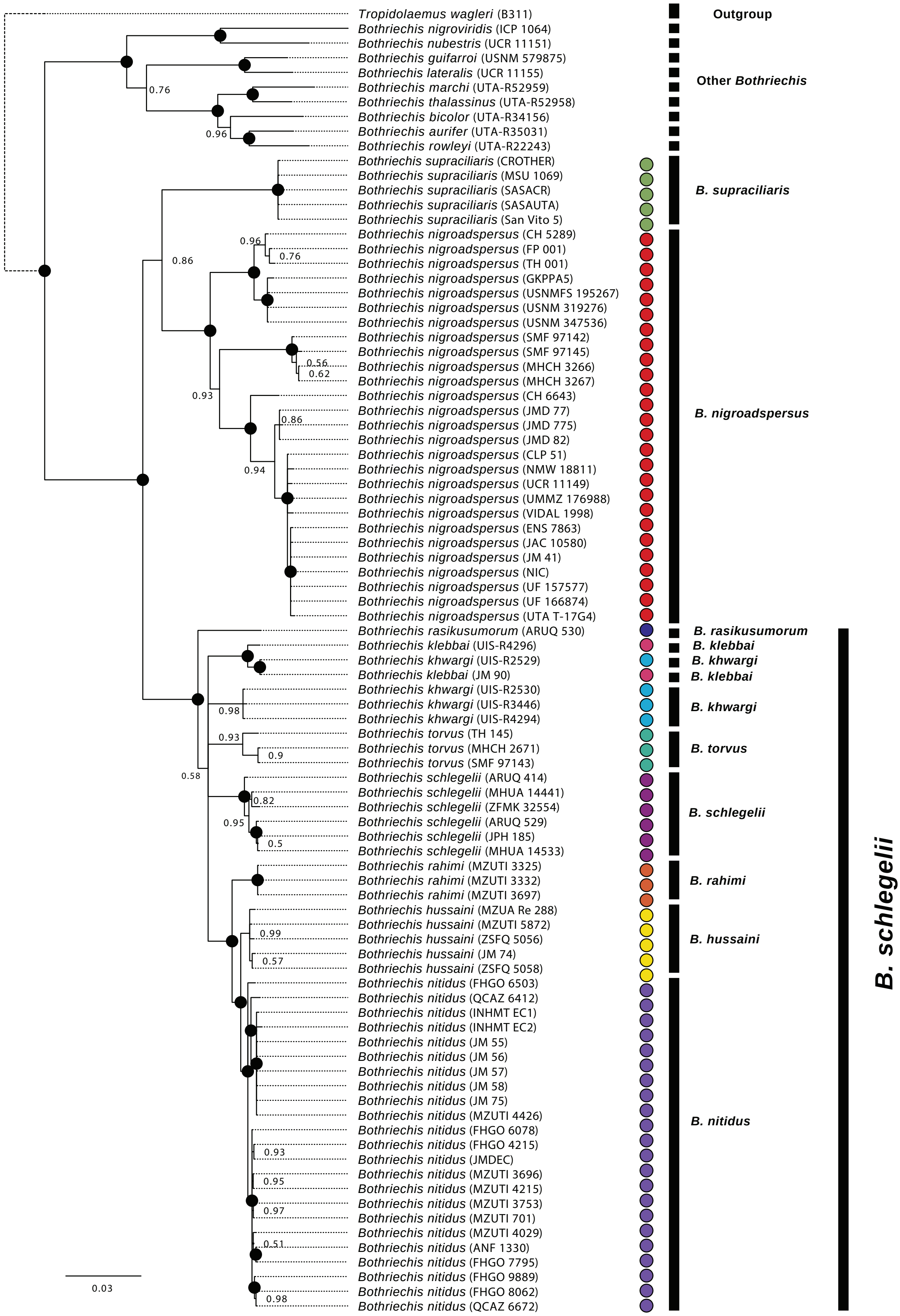

Figure 2. Phylogenetic relationships of *Bothriechis* species based on Bayesian inference from concatenated nuclear and mitochondrial loci. Node numbers indicate posterior support values, with black circles denoting posterior support of 1.

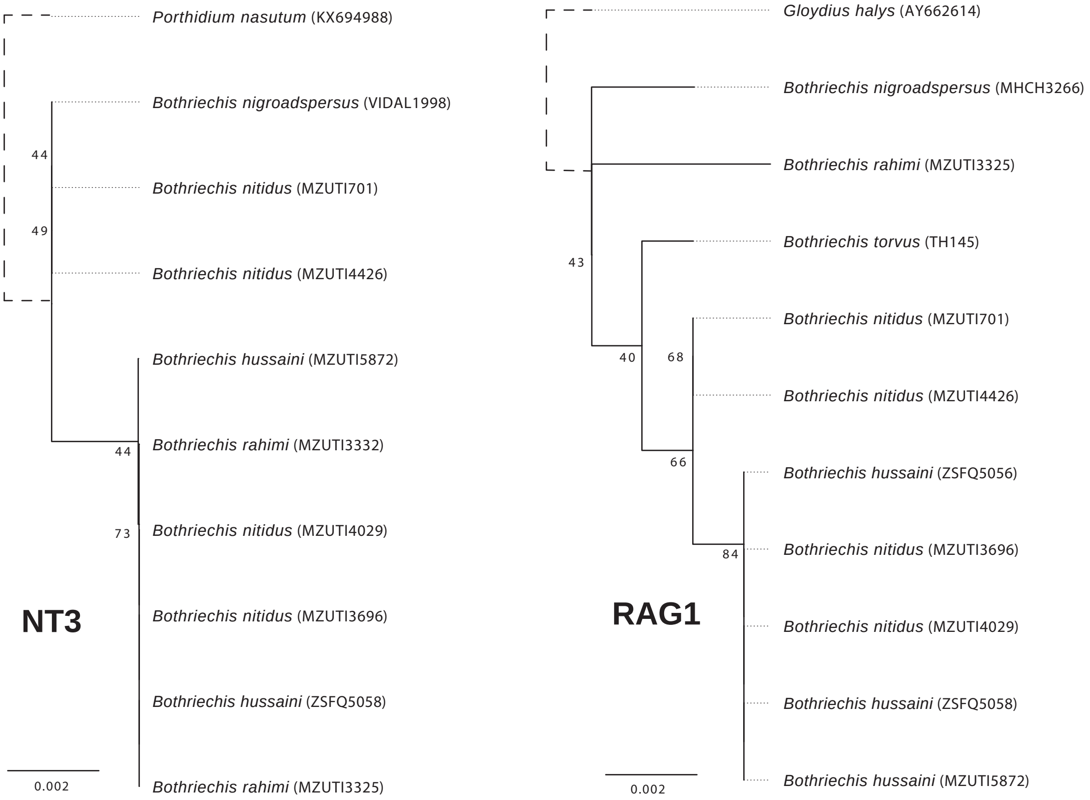

Figure 3. Phylogenetic trees derived from Bayesian inference of the nuclear genes NT3 (left) and RAG1 (right) for species of *Bothriechis*. Node labels indicate posterior probabilities reflecting support for each clade.

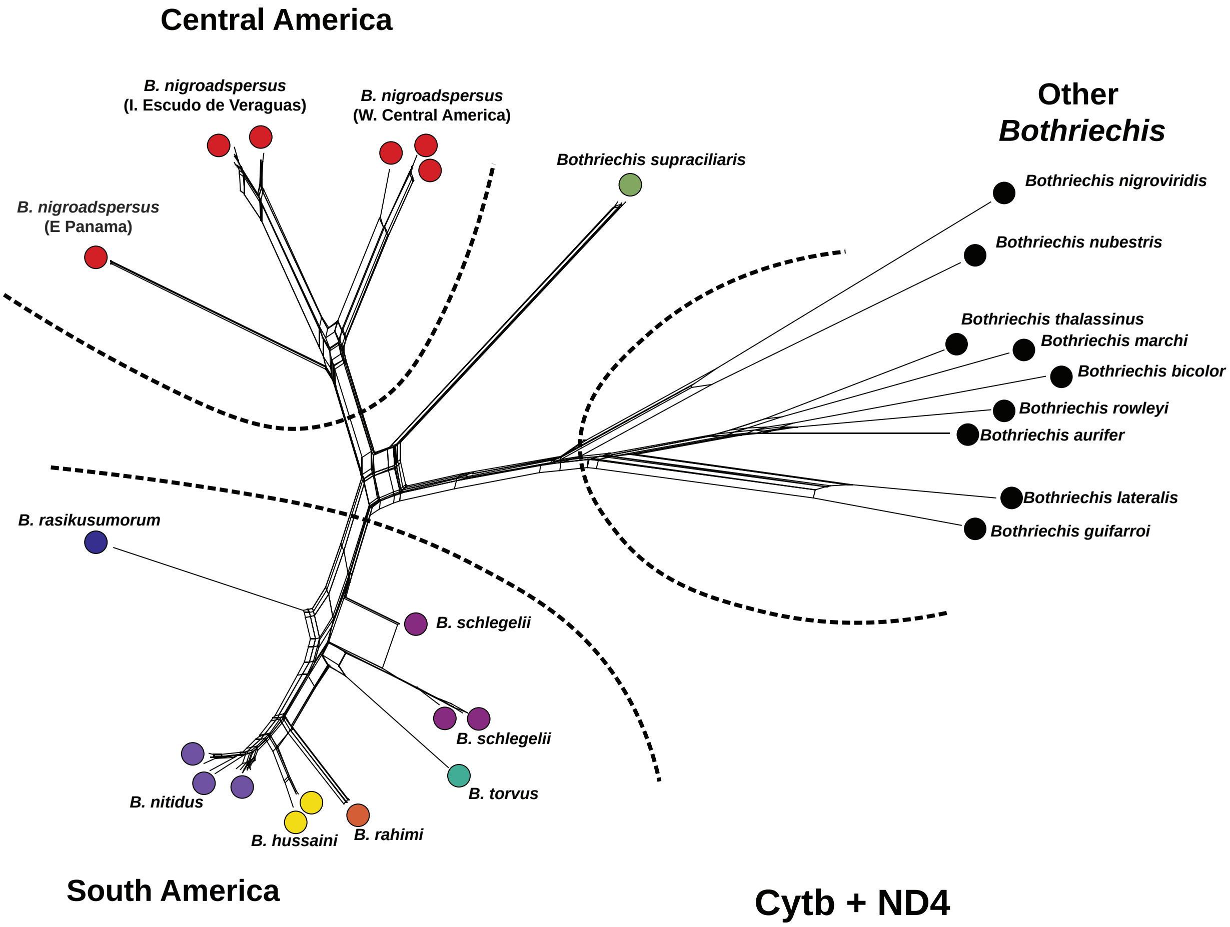

Figure 4. SplitsTree phylogenetic network of the concatenated mitochondrial DNA sequences Cytb and ND4 for members of *Bothriechis*.

Specifically, for the *B. schlegelii* species group, B. *rasikusumorum* was the sole species demonstrating consistent genetic distances above 5% when compared to the rest of the members of the group. In the case of ND4, genetic distances were generally lower than in Cytb (Table 2). Once again, only *B. rasikusumorum* had genetic distances consistently larger than 5% compared to the rest of the South American *B. schlegelii* group. Genetic distances were also generally lower in other members of *Bothriechis*. The differences in genetic distances between South American members of the *B. schlegelii* group and the Central American *B. nigroadspersus* and *B. supraciliaris* ranged between 8.0–9.8% (ND4) and 9.9–13.4% (Cytb), while the genetic differences between *B. supraciliaris* and *B. nigroadspersus* were 8.9% (ND4) and 11.5% (Cytb). These genetic differences are as high or higher than genetic distances between other members of *Bothriechis*, which could indicate species-level divergence between South and Central American populations.

The use of a 4% threshold in mtDNA for possible species-level differentiation should be treated as a way to pinpoint clades that could potentially represent distinct species and then use other sources of data to test these species' hypotheses, not as the sole evidence for species differentiation, as in the case of the study by Arteaga et al. (2024).

Table 1. Mean between-group genetic distance for *Bothriechis* species using the mitochondrial locus Cytb, expressed as a percentage.

	B. nitidus	***B. hussaini***	***B. rahimi***	***B. schlegelii***	***B. torvus***	***B. khwargi***	***B. klebbai***	***B. rasikusumorum***	**Average in *B. schlegelii* group**	***B. nigroadspersus***	***B. supraciliaris***	***B. rowleyi***	***B. aurifer***	***B. bicolor***	***B. marchi***	***B. thalassinus***	***B. guifarroi***	***B. lateralis***	***B. nubestris***	***B. nigroviridis***
B. nitidus	-	1.5	2.8	4.2	2.3	4.4	5.4	5.8	**3.8**	11.7	13.1	17.4	17.8	18.1	18.2	15.1	15.5	17.1	18.2	20.7
B. hussaini	1.5	-	2.7	4.5	1.6	5.1	5.8	6.6	**4.0**	12.1	12.7	17.9	17.4	18.0	18.2	15.4	16.2	17.2	19.3	20.9
B. rahimi	2.8	2.7	-	4.7	3.8	5.3	5.1	6.9	**4.5**	13.4	12.8	18.3	18.3	18.3	18.2	18.0	15.5	15.1	14.5	12.2
B. schlegelii	4.2	4.5	4.7	-	2.9	4.5	6.2	6.2	**4.7**	9.9	11.8	16.2	16.4	16.5	16.4	16.4	15.5	15.5	15.5	14.6
B. torvus	2.3	1.6	3.8	2.9	-	4.2	5.2	5.6	**3.7**	10.5	12.8	16.5	16.6	16.9	17.2	17.0	17.8	18.1	19.4	20.4
B. khwargi	4.4	5.1	5.3	4.5	4.2	-	4.4	6.9	**5.0**	10.6	12.5	17.0	17.1	17.2	17.2	17.1	17.9	18.7	19.8	20.5
B. klebbai	5.4	5.8	5.1	6.2	5.2	4.4	-	7.1	**5.6**	12.2	12.0	17.7	17.8	17.9	18.0	17.9	18.7	19.3	20.1	20.1
B. rasikusumorum	5.8	6.6	6.9	6.2	5.6	6.9	7.1	-	**6.4**	11.3	13.2	18.7	18.8	18.9	18.9	18.8	19.2	19.8	21.1	22.2
Average in *B. schlegelii* group	**3.8**	**4.0**	**4.5**	**4.7**	**3.7**	**5.0**	**5.6**	**6.4**												
B. nigroadspersus	11.7	12.1	13.4	9.9	10.5	10.6	12.2	11.3		-	11.5	16.9	17.0	17.2	17.3	17.1	17.7	18.6	19.8	20.3
B. supraciliaris	13.1	12.7	12.8	11.8	12.8	12.5	12.0	13.2		11.5	-	18.0	17.9	18.0	18.0	17.8	18.2	19.1	20.3	20.2
B. rowleyi	17.4	17.9	18.3	16.2	16.5	17.0	17.7	18.7		16.9	18.0	-	6.8	7.0	10.3	9.1	11.7	10.7	15.1	16.9
B. aurifer	17.8	17.4	18.3	16.4	16.6	17.1	17.8	18.8		17.0	17.9	6.8	-	7.2	9.4	7.5	11.6	11.6	16.9	16.1
B. bicolor	18.1	18.0	18.3	16.5	16.9	17.2	17.9	18.9		17.2	18.0	7.0	7.2	-	9.7	6.7	11.4	10.7	15.3	16.8
B. marchi	18.2	18.2	18.2	16.4	17.2	17.2	18.0	18.9		17.3	18.0	10.3	9.4	9.7	-	6.3	12.8	12.3	14.7	15.9
B. thalassinus	15.1	15.4	18.0	16.4	17.0	17.1	17.9	18.8		17.1	17.8	9.1	7.5	6.7	6.3	-	11.9	11.8	14.9	16.9
B. guifarroi	15.5	16.2	15.5	15.5	17.8	17.9	18.7	19.2		17.7	18.2	11.7	11.6	11.4	12.8	11.9	-	4.4	14.4	16.1
B. lateralis	17.1	17.2	15.1	15.5	18.1	18.7	19.3	19.8		18.6	19.1	10.7	11.6	10.7	12.3	11.8	4.4	-	13.7	16.6
B. nubestris	18.2	19.3	14.5	15.5	19.4	19.8	20.1	21.1		19.8	20.3	15.1	16.9	15.3	14.7	14.9	14.4	13.7	-	11.3
B. nigroviridis	20.7	20.9	12.2	14.6	20.4	20.5	20.1	22.2		20.3	20.2	16.9	16.1	16.8	15.9	16.9	16.1	16.6	11.3	-

Table 2. Mean between-group genetic distance for *Bothriechis* species using the mitochondrial locus ND4, expressed as a percentage.

	B. nitidus	***B. hussaini***	***B. rahimi***	***B. schlegelii***	***B. torvus***	***B. rasikusumorum***	**Average in *B. schlegelii* group**	***B. nigroadspersus***	***B. supraciliaris***	***B. rowleyi***	***B. aurifer***	***B. bicolor***	***B. marchi***	***B. thalassinus***	***B. guifarroi***	***B. lateralis***	***B. nubestris***	***B. nigroviridis***
B. nitidus	-	1.6	2.1	3.9	5.5	5.2	**3.7**	8.2	8.4	11.6	10.6	13.4	11.9	12.2	11.6	12.3	11.0	11.2
B. hussaini	1.6	-	2.1	4.3	5.3	5.1	**3.7**	8.5	8.6	9.8	10.5	13.3	11.5	12.1	11.5	12.6	10.6	11.0
B. rahimi	2.1	2.1	-	4.0	4.7	5.3	**3.6**	8.0	8.8	9.9	10.6	12.5	11.0	11.6	11.6	12.3	10.2	10.9
B. schlegelii	3.9	4.3	4.0	-	5.2	5.8	**4.7**	8.0	8.4	9.7	10.7	13.8	11.7	12.2	13.3	12.6	11.5	11.6
B. torvus	5.5	5.3	4.7	5.2	-	6.8	**5.5**	9.5	9.8	10.5	11.5	14.6	11.8	11.8	13.9	13.5	11.9	12.2
B. rasikusumorum	5.2	5.1	5.3	5.8	6.8	-	**5.6**	9.1	9.3	10.4	10.9	14.0	12.7	12.1	13.0	13.7	12.1	12.8
Average in *B. schlegelii* group	**3.7**	**3.7**	**3.6**	**4.7**	**5.5**	**5.6**												
B. nigroadspersus	8.2	8.5	8.0	8.0	9.5	9.1		-	8.9	10.6	13.1	16.0	14.3	14.5	15.2	14.7	13.2	13.3
B. supraciliaris	8.4	8.6	8.8	8.4	9.8	9.3		8.9	-	6.2	11.3	14.9	13.4	13.6	14.0	13.4	13.0	13.0
B. rowleyi	11.6	9.8	9.9	9.7	10.5	10.4		10.6	6.2	-	3.8	6.6	6.4	6.4	10.2	8.9	10.3	11.6
B. aurifer	10.6	10.5	10.6	10.7	11.5	10.9		13.1	11.3	3.8	-	6.7	5.9	6.2	10.5	9.6	10.2	11.9
B. bicolor	13.4	13.3	12.5	13.8	14.6	14.0		16.0	14.9	6.6	6.7	-	7.5	8.7	12.5	11.6	11.8	13.3
B. marchi	11.9	11.5	11.0	11.7	11.8	12.7		14.3	13.4	6.4	5.9	7.5	-	5.2	11.8	10.8	11.0	12.7
B. thalassinus	12.2	12.1	11.6	12.2	11.8	12.1		14.5	13.6	6.4	6.2	8.7	5.2	-	11.5	10.2	11.4	12.3
B. guifarroi	11.6	11.5	11.6	13.3	13.9	13.0		15.2	14.0	10.2	10.5	12.5	11.8	11.5	-	4.5	11.3	12.8
B. lateralis	12.3	12.6	12.3	12.6	13.5	13.7		14.7	13.4	8.9	9.6	11.6	10.8	10.2	4.5	-	11.1	11.6
B. nubestris	11.0	10.6	10.2	11.5	11.9	12.1		13.2	13.0	10.3	10.2	11.8	11.0	11.4	11.3	11.1	-	8.5
B. nigroviridis	11.2	11.0	10.9	11.6	12.2	12.8		13.3	13.0	11.6	11.9	13.3	12.7	12.3	12.8	11.6	8.5	-

Species delimitation

The species delimitation analysis conducted using DELINEATE did not support the species distinctions proposed by Arteaga et al. (2024). Instead, the maximum likelihood approach recognized only four species within the group as distinct (Fig. 5). Specifically, *B. rasikusumorum* and *B. schlegelii* were validated as separate species. Conversely, *B. khwargi* and *B. klebbai* were not differentiated and were grouped as a single taxon. Similarly, *B. torvus*, *B. rahimi*, *B. hussaini*, and *B. nitidus* were collectively treated as one taxon. Notably, the analysis's uncertainty is reflected in the variation of the 10 most probable phylogenetic trees, which identified between two and seven potential species within the *B. schlegelii* group (see Suppl. material 1 for details).

Morphological analyses

In the PCA examining morphological characters across the *B. schlegelii* group, the data did not resolve distinct clusters for the various species (Figs 6, 7, Suppl. material 3: figs S4–S9). Some clustering was observed for *B. nigroadspersus* and *B. nitidus*, but because these species represent the bulk of the individuals studied, the clusters may not signify distinct groupings but rather reflect sample size. Separate PCA analyses were conducted for scale count data and measurement data, standardized by snout-vent length (SVL) and regression residuals, for both females (Fig. 6) and males (Fig. 7). Overall, the PCA results indicated that while there is some morphological variation within the *B. schlegelii* group, particularly in *B. nigroadspersus* and *B. nitidus*, the characters examined do not provide clear distinctions between the species. The overlap in morphological traits suggests that morphological data do not differentiate between the species described by Arteaga et al. (2024).

Discussion

A recent trend in taxonomy involves grouping individuals into lineages and describing them as species without testing if the groups are evolving independently from one another (Hillis 2019). In many cases, these groupings do not consider clinal variation and are based on mtDNA data alone. The recent study of *B. schlegelii* by Arteaga et al. (2024) is one such example. Despite the authors claiming that their study follows an integration by congruence approach (Padial et al. 2010), the definition of the new or resurrected taxa in their study is mostly based on mtDNA data, as their nuclear dataset is extremely limited and does not result in monophyletic lineages (see Fig. 3).

The primary function of nuclear loci in species delimitation is to test for congruence with mtDNA and to assess

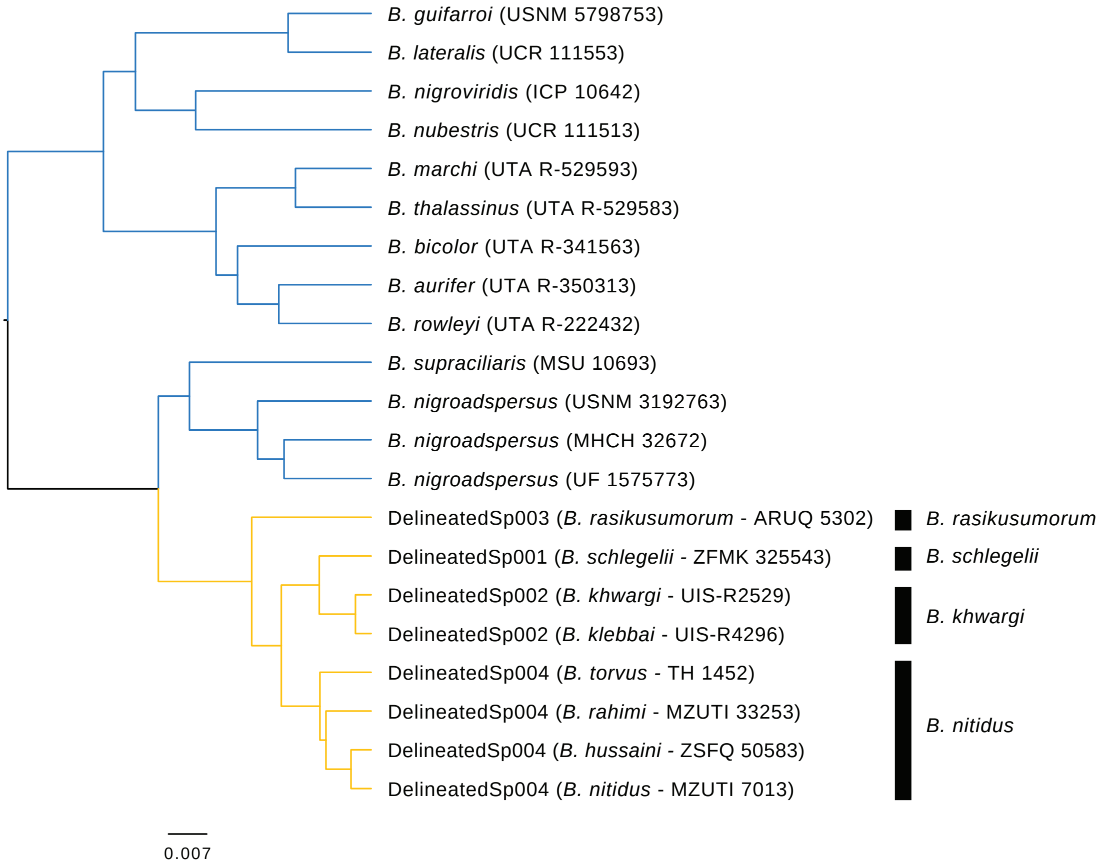

Figure 5. Species delimitation analysis using DELINEATE. Blue clades indicate constrained species, while yellow clades denote lineages undergoing species designation testing. Terminal nodes labeled 'DelineatedSp' identify lineages that DELINEATE distinguishes as candidate separate species (sp1–4), with their former species assignations provided in parentheses. Names at right represent names that have priority.

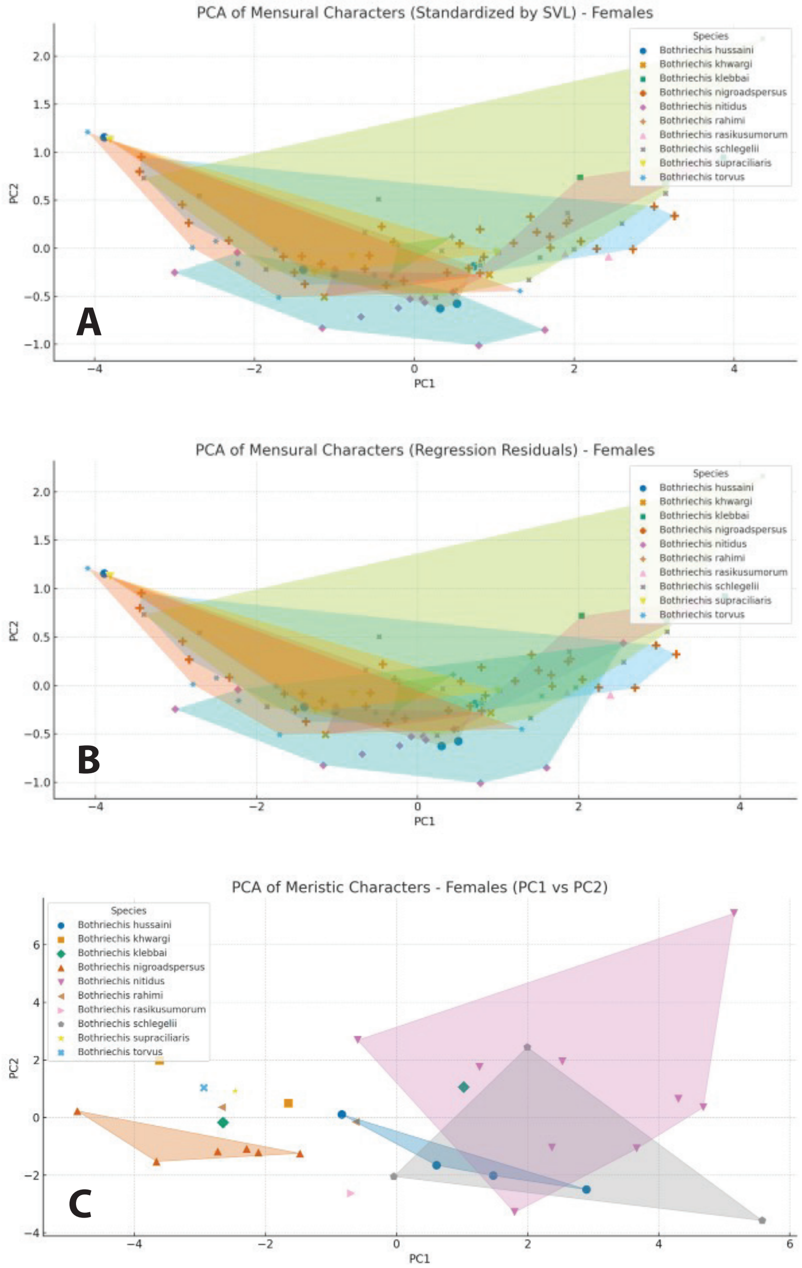

Figure 6. PCA plots for females of *Bothriechis* species. **A.** PCA of measurement characters standardized by SVL (PC1 vs. PC2), **B.** PCA of measurement characters standardized by regression residuals (PC1 vs. PC2), **C.** PCA of meristic data (PC1 vs. PC2).

genetic exchange between mtDNA-based candidate species. In species delimitation, nuclear loci should not merely serve as additional genes in a concatenated analysis. A mixed nuclear-mtDNA concatenated analysis effectively becomes a mtDNA analysis with additional noise (such as in the study of Arteaga et al. 2024) rather than a true multilocus analysis. Thus, the sequencing strategy employed by Arteaga et al. (2024), which focused heavily on mitochondrial genes and included only two nuclear genes for a small number of specimens, limits the ability to test for genetic exchange and congruence with mtDNA-based candidate species.

Although the majority of the new or resurrected species proposed by Arteaga et al. (2024) formed monophyletic groups (but not all), the relationships between the different lineages were poorly supported, likely due to incomplete lineage sorting in the loci used or rapid diversification. Furthermore, genetic distances between these putative species are very small, and genetic network visualization shows less differentiation within them than within the various populations of *B. nigroadspersus*. Only *B. rasikusumorum* displays more divergence in two mitochondrial loci than the rest of the other species in the *B. schlegelii* group, yet it was nested within other members of the group in the ML analysis (Suppl. material 3: figs S1, S2). Genetic distances of the mitochondrial genes between the Central American *B. supraciliaris* and *B. nigroadspersus* and the South American *B. schlegelii* group were quite high (8.0–13.4%), which is as high or even higher than the genetic distances between other species of *Bothriechis* (Tables 1, 2), suggesting species-level differentiation between the South and Central American populations.

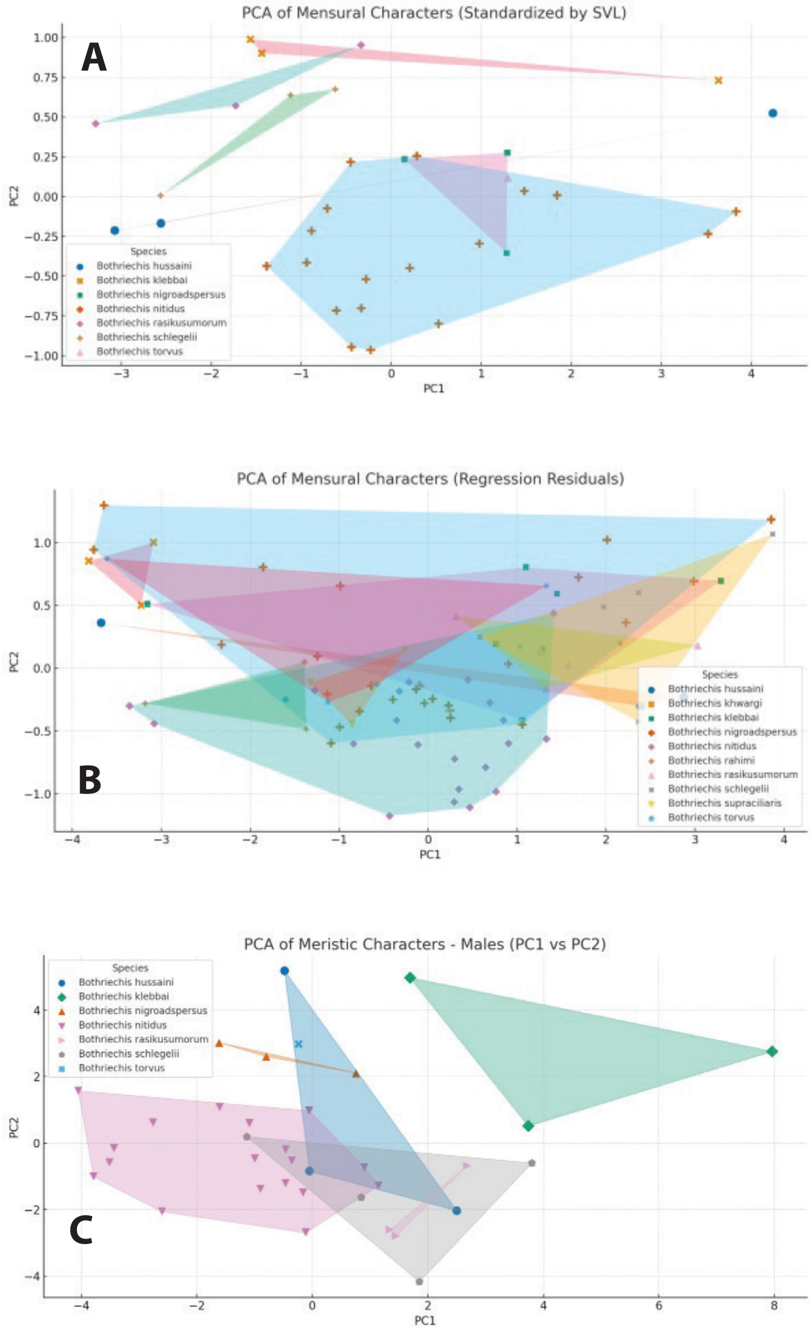

Figure 7. PCA plots for males of *Bothriechis* species. **A.** PCA of measurement characters standardized by SVL (PC1 vs. PC2), **B.** PCA of measurement characters standardized by regression residuals (PC1 vs. PC2), **C.** PCA of meristic data (PC1 vs. PC2).

A re-analysis of the morphological characters used by Arteaga et al. (2024) failed to distinguish between the multiple putative species. The study lists 22 morphological characters purported to distinguish species within the *B. schlegelii* group (table 2 in their study). Eight of these traits concern coloration, which is unreliable due to the well-documented color variation within this group. None of these color traits is exclusive to a single taxon, diminishing their discriminative power. This problem extends to scale characteristics, none of the 10 scale-related traits were found to be diagnostic, with overlaps observed among multiple species. This is particularly noticeable with ventral scales in both sexes and midbody scale rows. Furthermore, traits such as maximum total length, which are not consistent or unique to specific lineages, are not suitable for species identification.

Venom potency, measured by LD50 values, was another character considered, however, it is only available for some species. Moreover, studies by Fernandez et al. (2010) and Lomonte et al. (2012) have demonstrated significant intraspecific variation in LD50 among *Bothriechis* species, and this character is known to show intraspecific variation in other species of pitvipers (Colis-Torres et al. 2022), suggesting that these values are not species-specific.

Additionally, Arteaga et al. (2024) described the basal hooks on the hemipenial body as a distinguishing feature, but with a small sample size for most species, this trait's reliability is compromised. Only *B. schlegelii* had a sample size greater than three, further undermining the diagnostic utility of this character. Additionally, Dowling (2002) refers to the existence of intraspecific variation in the size of these structures, indicating that they cannot reliably differentiate between species within this group. Basal hooks are known to change over the lifetime of snakes (Jadin and King 2012), with the spines (hooks) becoming more prominent with age (Lara Granados et al. 2022). Therefore, the size of the hooks is not necessarily a diagnostic character, especially when sample sizes are small. At first glance, the images of the hemipenes in Arteaga et al. (2024) might look very different from each other, especially in coloration and shape, however, the multiple colorations are the result of the preparation techniques and are not unique to each hemipenis. The same occurs with the shape of the hemipenes, as if the lobes are not completely everted, they might look more pointed (vs. rounded) at the tip. These differences are particularly noticeable in figs 15, 21, and 31 of Arteaga et al. (2024).

Lastly, the DELINEATE analysis failed to recover all species described or resurrected by Arteaga et al. (2024) (Fig. 5). Although DELINEATE has limitations when used with single-locus datasets, it was selected for its unique ability to incorporate previous taxonomic work when testing species limits (Sukumaran et al. 2021). This feature allows the integration of species concepts based on morphology, ecology, and other markers, providing a more comprehensive approach to species delimitation. However, it is important to acknowledge that this method also has its drawbacks, especially with the limited molecular dataset presented by Arteaga et al. (2024).

Previous research has shown that other single-locus species delimitation methods tend to over-split taxa (Ahrens 2024). Therefore, despite the challenges associated with using DELINEATE on a single-locus dataset, it was preferred over alternative methods to avoid artificially inflating the number of species. The results, while indicating that several of the species proposed by Arteaga et al. (2024) remain invalid, should be interpreted with caution. The data used (mtDNA) is not recommended for DELINEATE analysis, and multiple, unlinked loci should be used for this type of analysis, which are not available for this group at the present time.

Inaccurate taxonomy can dilute the already scarce conservation resources. Currently, species diversity is a major metric for biodiversity, hence, sound taxonomic practices are vital to positively impact conservation efforts and inform biological diversity studies, preventing taxonomic inflation and its associated issues. Recently, Dufresnes et al. (2023) suggested recognizing subspecies to show intra-specific diversity without resulting in taxonomic inflation. This approach could potentially be applied to some (but not all) of the clades of the *B. schlegelii* group, for example, *B. rasikusumorum*, however, if this is the case, the same should be done for the multiple populations of *B. nigroadspersus*, which I do not recommend doing with the data at hand.

Conclusion

A comprehensive review of the data challenges the validity of the taxa described or resurrected by Arteaga et al. (2024). The evidence suggests that within the *B. schlegelii* group, only *B. nigroadspersus*, *B. schlegelii*, and *B. supraciliaris* should be recognized as distinct species, however, morphological analyses failed to separate these three species. Therefore, while the current data are insufficient to definitively determine the exact number of species, it is proposed that *B. hussaini*, *B. khwargi*, *B. klebbai*, *B. nitidus*, *B. rahimi*, *B. rasikusumorum*, and *B. torvus* be considered junior synonyms of *B. schlegelii*. To fully understand the genetic relationships within the *Bothriechis schlegelii* group, future studies should incorporate comprehensive nuclear data alongside mitochondrial data to test for potential cytonuclear discordance, ensuring a more robust evaluation of species boundaries.

Acknowledgements

I thank Stephane Boissinot and Christoph Grünwald for comments that improved this manuscript. Two reviewers greatly helped to improve an earlier version of this manuscript. I thank ChatGPT for its help running code in Python and R and for translating the abstract into German.

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Supplementary material 1

Results of the DELINEATE analysis

Author: Jacobo Reyes-Velasco

Data type: trees

Explanation note: The file presents the ten most probable species assignations, ranked in descending order of likelihood.

Link: https://doi.org/10.3897/herpetozoa.37.e131965.suppl1

Supplementary material 2

Supplementary tables

Author: Jacobo Reyes-Velasco

Data type: xlsx

Explanation note: **table S1.** Principal component loadings for measurement characters standardized by SVL in females of *Bothriechis* species. **table S2.** Explained variance for principal components of measurement characters standardized by SVL in females of *Bothriechis* species. **table S3.** Principal component loadings for measurement characters standardized by SVL in males of *Bothriechis* species. **table S4.** Explained variance for principal components of measurement characters standardized by SVL in males of *Bothriechis* species. **table S5.** Principal component loadings for measurement characters standardized by regression residuals in females of *Bothriechis* species. **table S6.** Explained variance for principal components of measurement characters standardized by regression residuals in females of *Bothriechis* species. **table S7.** Principal component loadings for measurement characters standardized by regression residuals in males of *Bothriechis* species. **table S8.** Explained variance for principal components of measurement characters standardized by regression residuals in male *Bothriechis* species. **table S9.** Principal component loadings for meristic characters in females of *Bothriechis* species. **table S10.** Explained variance for principal components of meristic characters in females of *Bothriechis* species. **table S11.** Principal component loadings for meristic characters in males of *Bothriechis* species. **table S12.** Explained variance for principal components of meristic characters in males of *Bothriechis* species.

Link: https://doi.org/10.3897/herpetozoa.37.e131965.suppl2

Supplementary material 3

Supplementary figures

Author: Jacobo Reyes-Velasco

Data type: docx

Explanation note: **figure S1.** Maximum likelihood phylogenetic tree derived from all seven nuclear and mitochondrial concatenated loci using IQ-TREE. Bootstrap support values are displayed at the nodes. **figure S2.** Maximum likelihood phylogenetic tree derived from five mitochondrial concatenated loci using IQ-TREE. Bootstrap support values are displayed at the nodes. **figure S3.** Phylogenetic relationships of *Bothriechis* species based on Bayesian inference from concatenated mitochondrial loci. Node numbers indicate posterior support values. **figure S4.** PCA of measurement characters standardized by SVL in females of *Bothriechis* species (PC1 vs. PC3). **figure S5.** PCA of measurement characters standardized by SVL in males of *Bothriechis* species (PC1 vs. PC3). **figure S6.** PCA of measurement characters standardized by regression residuals in females of *Bothriechis* species (PC1 vs. PC3). **figure S7.** PCA of measurement characters standardized by regression residuals in males of *Bothriechis* species (PC1 vs. PC3). **figure S8.** PCA of meristic data in females of *Bothriechis* species (PC1 vs. PC3). **figure S9.** PCA of meristic data in males of *Bothriechis* species (PC1 vs. PC3).

Link: https://doi.org/10.3897/herpetozoa.37.e131965.suppl3

ÖGH Herpetozoa
Austrian Herpetological Society

Herpetozoa 37: 319–326 (2024)
DOI 10.3897/herpetozoa.37.e133456

Guilty or innocent? Could the exotic lizard *Hemidactylus mabouia* (Squamata, Gekkonidae) be responsible for the displacement of the native species *Notomabuya frenata* (Squamata, Mabuyidae) in anthropic environments?

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Academic editor: Peter Mikulíček ♦ **Received** 30 July 2024 ♦ **Accepted** 10 October 2024 ♦ **Published** 25 October 2024

Abstract

Hemidactylus mabouia is a gecko species that has successfully colonized southern North, Central, and South America. In Paraguay, there is a commonly held belief that this species has displaced the native skink *Notomabuya frenata* in urban areas. In this study, we compared several ecological features of *N. frenata* and *H. mabouia* to better understand the degree of their niche overlap. A comparative analysis was conducted based on a literature survey and the examination of museum specimens from Asunción (Paraguay). Furthermore, differences in microhabitat use between *H. mabouia* and *N. frenata* were investigated through a review of observational records available on iNaturalist. Our findings indicate that the two species share only a few biological traits, such as an insectivore diet, an ability to adapt to living close to human dwellings, and the use of mimesis as a primary defensive strategy. Both lizards are generalist feeders, although there are notable differences in their frequency of prey consumption. Regarding habitat use, the majority of *N. frenata* specimens were documented on grass or other natural substrates, as well as on trees and shrubs. In the case of *H. mabouia*, the majority of records originate from smooth and brick walls, including metal and glass surfaces. Consequently, there is no evidence to suggest that dietary overlap could induce competition between these species. Therefore, it can be posited that the displacement of the native lizard *N. frenata* is a consequence of human activities that have altered its preferred microhabitat while expanding potential habitat for *H. mabouia*.

Key Words

iNaturalist, invasive species, Paraguay, Reptilia, urban biodiversity

Introduction

Exotic species may constitute a major problem in conservation biology, especially when they become invasive (Lowe et al. 2000). Nevertheless, in some cases, exotic species do not develop invasive capacities and may reach a populational climax without harming local wildlife (Ricciardi and Cohen 2007). In several cases, exotic invaders have outcompeted native species from ecological niches (Spencer et al. 1991; Holway 1999; Madjidian et al. 2008; Sanders and Suarez 2011). *Hemidactylus mabouia* (Moreau de Jonnès, 1818) is one example of a species that has successfully colonized and invaded southern North, Central, and South America (Ávila-Pires 1995; Fuenmayor et al. 2005; Pinho et al. 2023), most likely due to its broad dietary

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range as an opportunistic and generalist species (Rocha and Anjos 2007; Iturriaga and Marrero 2013; Agarwal et al. 2021).

Hemidactylus mabouia probably entered first in South America in northeastern Brazil as early as ~1640 (Agarwal et al. 2021), and the further dispersion was facilitated by human-modified environments (Rocha et al. 2011). It is difficult to pinpoint the exact date of colonization in Paraguay, but the oldest verified record for *H. mabouia* in the country is from 21 January 1983 in the city of Concepción (Baldo et al. 2008; Cacciali and Motte 2009). Since then, the species has successfully colonized Asunción and Ciudad del Este. Nevertheless, the species is more widespread in the country (Cacciali et al. 2016). All records belong to anthropogenic environments, common in human dwellings.

However, Rocha and Anjos (2007) recorded a population of *H. mabouia* in a non-urban environment in thirteen Brazilian states, indicating that the species is encroaching into wild areas. This expansion is a major concern, as the exotic population could potentially spread without constraint. The actual impact of *H. mabouia* on native fauna in Paraguay remains unknown.

In Paraguay, it is widely believed that *H. mabouia* has displaced the native skink lizard *Notomabuya frenata* (Cope, 1862) (Motte et al. 2004; NLT 2007), but there is no rigorous evidence for this claim, only anecdotal. For instance, many residents of Asunción, the capital of Paraguay, informed us that ~30 years ago all houses were inhabited by *N. frenata* (or "Amberé," as the locals call them), but with the invasion of *H. mabouia*, the original "Amberé" disappeared.

In this study, we compared several ecological features of *N. frenata* and *H. mabouia* to better understand the degree of their niche overlap. We hypothesize that the two species do not compete for resources, mainly due to differences in diel activity and microhabitat use, and therefore *H. mabouia* does not outcompete *N. frenata.* To test this hypothesis, we extracted data from the literature, examined museum specimens, and analyzed records available on an online social network, iNaturalist.

Material and methods

According to Colwell and Futuyama (1971), niche overlap refers to the shared utilization of resources by two or more taxa. However, interspecific competition can be inferred when there is a conflict for the resources trending to the displacement or exclusion of any of such taxa. Thus, to assess the degree of niche overlap between *H. mabouia* and *N. frenata,* we performed a comparison of their biological aspects based on the following literature: Dixon and Soini (1986), Martínez Rivera et al. (2003), Regalado (2003), and Rocha and Anjos (2007) for *H. mabouia*; and Norman (1994), Vrcibradic and Rocha (1998a, b), and Cassel et al. (2012) for *N. frenata*. We followed the PRISMA statement for ecology and evolutionary biology (O'Dea et al. 2021) using a proposed flow diagram by Haddaway et al. (2022) summarized in Suppl. material 1.

One of the key dimensions of the ecological niche that can lead to interspecific competition is the overlap of dietary resources (Duncan et al. 2002; Fabre et al. 2004). We investigated this aspect through an analysis of literature and scientific collections. Information regarding the trophic diversity of *H. mabouia* is based on Rocha and Anjos (2007), while data for *N. frenata* was taken from Vrcibradic and Rocha (1998a). These references were chosen as they both focus on sympatric populations at Valinhos, São Paulo, Brazil, although separated in time by almost a decade. We used percentages because of the differences in sample size. Insect larvae records were not included because of the high rate of quick decomposition.

Extra data from a diet of these two lizard species were taken from specimens from the Museo Nacional de Historia Natural del Paraguay from the same location: Asunción, Paraguay (*Hemidactylus mabouia*, n = 8, voucher specimens MNHNP 3129, 3158, 3161-2, 3165, 3814, 3816, 8067; *Notomabuya frenata*, n = 5, voucher specimens MNHNP 2921, 2926-7, 3802, 4314). Stomach contents were extracted and analyzed under a stereoscopic microscope to identify prey items to the deepest taxonomic level possible. Literature for stomach content identification is listed in Suppl. material 2.

To assess differences in trophic composition between *H. mabouia* and *N. frenata*, we performed a chi-square test using results from bibliographic data. Rarefaction curves were estimated to compare differences in stomach content richness among dissected individuals. Analyses were performed with the software PAST v. 4.03 (Hammer et al. 2001).

We also examined differences in temporal patterns of *H. mabouia* and *N. frenata* occurrence in Asunción city. This analysis involved studying collection dates (based on MNHNP records) and observations from iNaturalist, an online citizen science-based website, to detect the contemporaneous occurrence of the two species. We chose this digital repository because of its visibility, accessibility, and visualization of data, which makes it a valuable tool for research nowadays (Forti and Szabo 2023; White et al. 2023). We assessed the microhabitat use of both lizards by reviewing records available at iNaturalist and categorizing the microhabitat type as follows: smooth wall, brick wall, wooden wall, tile floor, grass (or other natural ground), bark tree or shrubs, rocky substrate, metal or glass surfaces, and out of habitat (for animals that were deceased, manually handled, or in scientific collections). To eliminate potential latitudinal biases, we used the distribution of *N. frenata* and performed a search for *H. mabouia* within the same range (Lat -6.061683 to -29.280589, Long -65.986297 to -41.32117).

Results

Notomabuya frenata and *H. mabouia* share only a few biological traits, such as diet and defensive behavior (Table 1). Most aspects of those species' lives differ, including diel activity, habitat, reproduction, and communication. As shown in Suppl. material 3, there is a high degree of overlap in the items consumed by both species (at least at the order level) based on literature data. Both lizards are rather generalist feeders, consuming a wide variety of available invertebrates. Nevertheless, Fig. 1 shows a difference in the frequency of prey consumption. *Hemidactylus mabouia* shows a higher number of arachnids in its diet, whereas *N. frenata* ingests a substantial quantity of isopterans. The chi-square test shows a significant difference between items consumed by both predators ($\chi^2 = 92.89$, DF = 23, $p < 0.001$).

The analysis of stomach contents of MNHNP specimens from Asunción yielded limited information due to the small sample size and the number of items found. Notably, we discovered inorganic and vegetal substances in the stomachs of *H. mabouia*, including sand, fabric, and remnants of shed skin, presumably from the own lizard (Table 2). None of these items were found in *N. frenata*.

Regarding the items consumed by both species in Asunción, they coincide with the ingestion of spiders, coleopterans, and homopterans (Table 2). The remaining items were not shared by both species (Table 2). Furthermore, there appears to be a difference in the types of species consumed. Rarefaction curves show that dissected specimens of *N. frenata* and *H. mabouia* have a similar dietary structure (Fig. 2). Curves overlap, and the confidence interval for *N. frenata* is entirely encompassed by the confidence interval of *H. mabouia*, meaning that in the city, perhaps with limited resources, the diet coincides.

In terms of recorded dates, *N. frenata* was last recorded in Asunción in 1986 (MNHNP 3802), with two additional records in iNaturalist (3-Feb-1995 and 30-Mar-2017). On the other hand, *H. mabouia* has one voucher record dated in 2016 (MNHNP 12128) and 15 records on iNaturalist (from 21-Nov-2013 to 24-Feb-2022). Based on this fragmented information (these species were not exhaustively sampled), we present the number of records over time in Fig. 3. This figure illustrates that most of the records of *N. frenata* are dated up to 1995 and one in 2017, while the majority of *H. mabouia* records are more recent, and from 2017, the records of this species are notoriously frequent. Notably, there is a significant data gap from 1996 to 2012.

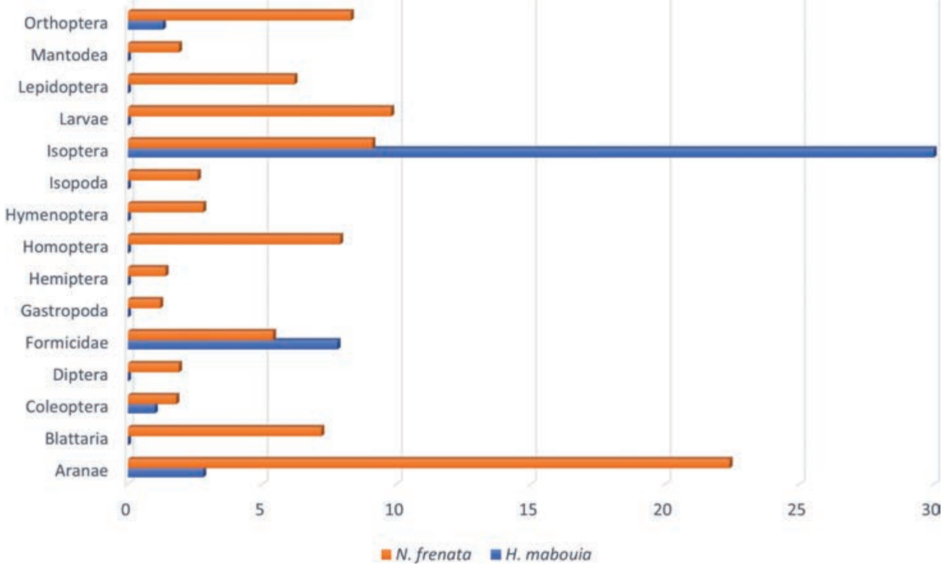

Figure 1. Frequency of prey items (expressed in percentage) consumed by *Notomabuya frenata* and *Hemidactylus mabouia* based on Vrcibradic and Rocha (1998a) and Rocha and Anjos (2007). Only frequencies ≥ 1% are shown. Full graphic available in Suppl. material 3.

Table 1. Biological traits of *Notomabuya frenata* and *Hemidactylus mabouia*. See Methods for bibliographic sources.

	Notomabuya frenata	***Hemidactylus mabouia***
Diet	Insectivore	Insectivore
Diel activity	Diurnal	Nocturnal, rarely diurnal
Habitat	Wide preferences in natural habitats, as well as in anthropogenic environments	Human dwellings
Microhabitat	Shrubs, logs and planks; climbs by claws; cannot climb smooth surfaces	Houses walls; climbs by lamellae; can climb smooth surfaces
Shelter	Fissures	Any available refugee, even inside electric installations
Reproduction	Ovoviviparous, reproductively active throughout the year	Oviparous, reproductively active throughout the year
Defense	Mimesis, caudal autotomy	Mimesis (including color change), caudal autotomy
Communication	Visual and chemosensory behavior	Acoustic, visual and chemosensory displays

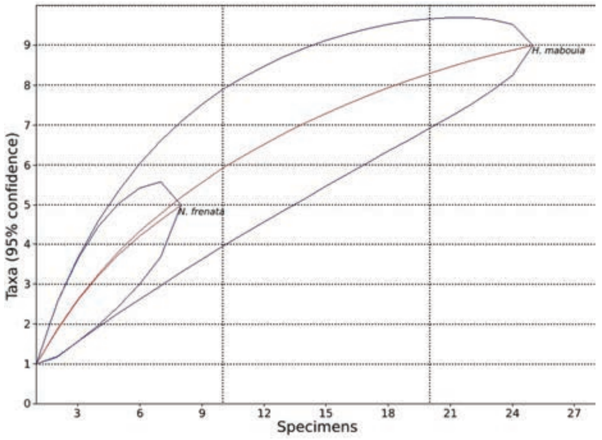

Figure 2. Rarefaction curves showing similar niche amplitude for the species *Notomabuya frenata* and *Hemidactylus mabouia*.

Regarding habitat use, we assessed 126 records with 130 observations of *N. frenata* on iNaturalist (a simple record may contain more than one observation or photograph). For *H. mabouia*, we had a total of 1,761 observations with the classification based on the first 1,000 records. For *N. frenata* and *H. mabouia*, 32 and 209 observations of individuals outside our habitat categories were deleted, leaving 98 and 791 observations, respectively, for comparison. Most specimens of *N. frenata* were documented on grass or natural substrate (30%) or on trees or shrubs (27%) (Fig. 4). Fewer records were observed in human environments such as brick or wooden walls (11% in each case) and tiled floors (9) (Fig. 4). In the case of *H. mabouia*, most of the records come from smooth walls and brick walls (71%), including metal and glass surfaces (3%) that were not used by *N. mabouia* (Fig. 4). A considerably low number of specimens were photographed on tile floors (1%). Comparing habitat use by both species, it is possible to see that wooden walls are the only habitat relatively used with the same frequency (approx. 10%) (Fig. 5).

Table 2. Stomach contents of specimens of *Notomabuya frenata* and *Hemidactylus mabouia* from Asunción (Paraguay). No id. refers to prey items that could not be identified below the major group classification. Lepidoptera marked with * indicate several fragments, probably more than one specimen. Number of specimens (including prey items) in parenthesis. X indicates presence (without quantification).

Major prey group	***N. frenata* (5)**	***H. mabouia* (8)**
Arachnida		
Acari		No id. (1)
Araneae	Salticidae (2)	*Kukulcania hibernalis* (Filistatidae) (2)
Insecta		
Blattaria		No id. (2)
Coleoptera	No id. (1)	Elateridae (1) No id. (2)
Ephemeroptera		No id. (2)
Dermaptera	*Euborellia annulipes* (Anisolabididae) (1)	
Diptera		Nematocera (~6) Cyclorrhapha (1)
Homoptera	No id. (1)	No id. (1)
Lepidoptera		No id. (*)
Orthoptera	*Gryllodes sigillatus* (Gryllidae) (1) No id. (2)	
Psocoptera		Liposcelidae (1)
Others		
Sand		X
Ecdysed lizard skin		X
Fabric		X
Vegetal matter		X

Discussion

Rocha and Anjos (2007) found that even in the wild, when *H. mabouia* shares food resources with native diurnal lizards such as *Tropidurus itambere* Rodrigues, 1987 (Tropiduridae) and *N. frenata*, interspecific competition remains low due to differences in their activity patterns. Additionally, these species have differential preferences in microhabitat use (Van Sluys 1992, 1993; Vrcibradic and Rocha 1998a).

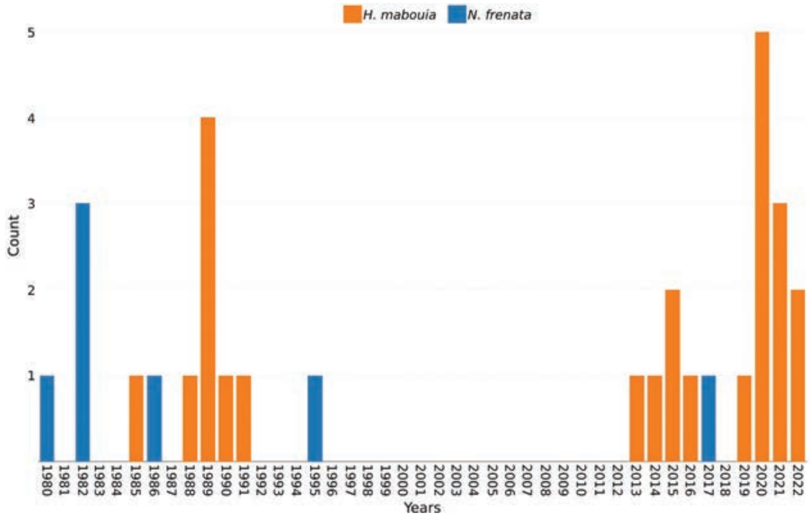

Figure 3. Records of *Hemidactylus mabouia* and *Notomabuya frenata* in Asunción shown by year, based on specimens from the MNHNP and iNaturalist data.

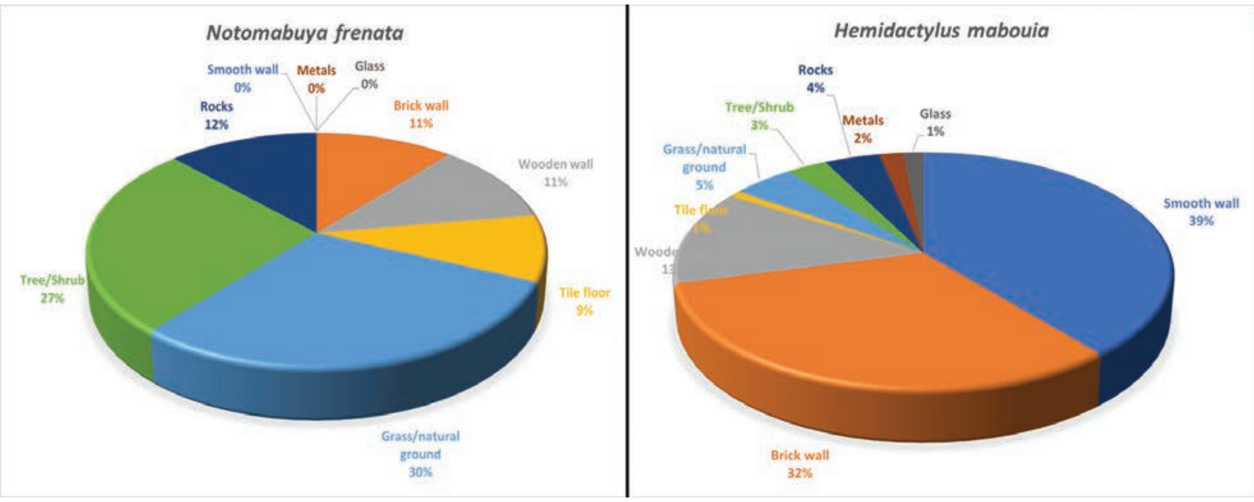

Figure 4. The proportion of microhabitat use by *Notomabuya frenata* (n = 126) and *Hemidactylus mabouia* (n = 1,000) according to records in iNaturalist.

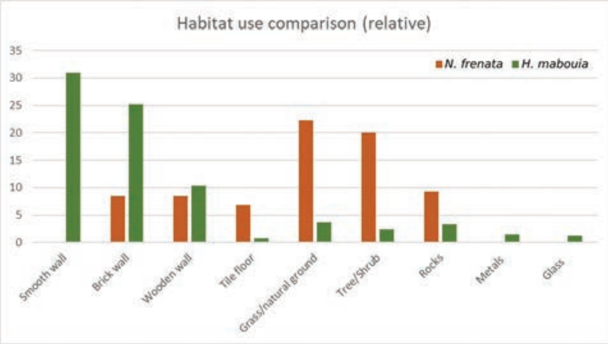

Figure 5. Comparison between the use of habitat in *Notomabuya frenata* and *Hemidactylus mabouia*. Relative values are expressed in percentages.

The use of the same resource by two or more species does not necessarily indicate competition. As Colwell and Futuyma (1971) explain, when a resource is abundant or oversupplied, competition between species may not occur, even if they share the same resource. In the case of the dietary resources for *H. mabouia* and *N. frenata*, the large diversity of insects consumed (Garcete and Molinas 2021) indicates that the species do not compete for this resource. Furthermore, we found significant differences between the dietary composition of *H. mabouia* and *N. frenata* based on bibliographic data. The examination of specimens from the same locality also revealed some differences, even with the limited sample size. As shown in Table 2, *N. frenata* primarily feeds on non-flying insects (e.g., *Euborellia annulipes* (Lucas, 1847)), along with terrestrial and diurnal arthropods (Salticidae and orthopterans). Conversely, the spiders found in the dietary content of *H. mabouia* are nocturnal, typically inhabiting walls. Moreover, the majority of prey items for this lizard consist of flying insects that are often attracted to artificial lights (Elateridae, Ephemeroptera, Diptera, Lepidoptera). We also found synthetic fibers of fabric, probably due to the urban environment. Although ants are a primary food source for many vertebrates, particularly lizards (Cavalcanti et al. 2023), our results show that they are not a significant component of the diet for *N. frenata* or *H. mabouia*. This suggests that these lizards may rely on other prey items or have adapted to different feeding strategies despite the general importance of ants in many lizards' diets.

In conclusion, there is no evidence that dietary overlap could induce competition between *H. mabouia* and *N. frenata*. In the specific case of Asunción, the diet strongly overlaps, and then prey availability could influence competition. Nevertheless, we argue that if local extinctions of *N. frenata* (mainly in large cities in Paraguay) are related to the invasion of *H. mabouia*, this fact cannot be explained by dietary overlap. Ecological invasions can arise from fitness advantages favoring the invader over the resident species or from niche differences that enable the establishment of the invader even if it may have lower overall fitness (MacDougall et al. 2009). However, many invasions are primarily the result of occupying available niches (Ricciardi and MacIsaac 2011). The latter scenario appears to be the case for *H. mabouia* given that the architectural features of urban development coincide with microhabitats used by this species, which are not suitable for skinks.

Regardless of the presence of *H. mabouia*, *N. frenata* is still commonly found in urban gardens where architectural design allows connections among populations. In areas of the city where *N. mabouia* has already disappeared, there are paved streets, tall and smooth walls, and fewer green areas, which hinder the dispersal of the species and lead to significant isolation of individuals. Therefore, the displacement of the native lizard *N. frenata* is a consequence of human activities, which is not surprising. These same anthropogenic changes that negatively influence *N. frenata* are likely to positively influence human commensal geckos such as *H. mabouia*. Fig. 6 provides an example of city development in an area between the botanical garden and Parque Guasu Metropolitano, two major green areas in Asunción. This figure highlights the expansion of concrete structures and buildings and the loss of gardens, green areas, and unpaved roads.

Figure 6. Development of an area of Asunción between a botanical garden and Parque Guasu Metropolitano, major green areas in Asunción, showing the expansion of concrete structures and buildings. Images obtained in Google Earth Pro 7.3.6. Years 2003 (above) and 2023 (below).

We must clarify that we do not assert that *H. mabouia* is incapable of competing and displacing other species. In fact, in certain areas where *H. mabouia* has been introduced, it has been proven to not only have conquered urban environments but also impact local fauna (Anjos and Rocha 2008). These geckos are highly adaptable and thrive in a variety of environments, from urban areas to natural habitats, and their presence often leads to the displacement of not only native gecko species through direct competition for resources and predation but also to other lizards (Oliveira et al. 2016; Lamb et al. 2021). Additionally, their aggressive behavior and high reproductive rates further exacerbate their impact, making them formidable invasive species that pose a threat to biodiversity and the stability of native ecosystems (Weterings and Vetter 2018; Agarwal et al. 2021).

However, it is worth noting that this exotic lizard is mostly confined to cities in Paraguay (Baldo et al. 2008; Cacciali and Motte 2009; Mackenzie and Vladimirova 2022), and, at least for now, no direct evidence of its possible negative effects on native lizards has been recorded (Cacciali 2024). Hiscock et al. (2023) reported trophic niche overlap, indicating the potential for shared ecological roles, but not necessarily competition, between *H. mabouia* and the common toad, *Rhinella diptycha* (Cope, 1862). An important fact highlighted by Cacciali et al. (2016) is that in the riverine town of Bahía Negra (Alto Paraguay department, northern Paraguay), the largest native Paraguayan gecko, *Phyllopezus przewalskii* Koslowsky, 1895 (12 cm of total length like *H. mabouia*), is found inhabiting human dwellings where *H. mabouia* is absent. Thus, under the presence of a native species with similar characteristics as the exotic *H. mabouia* (similar body size, diel activity, use of microhabitat), the latter seems to face challenges in colonizing the town (Cacciali et al. 2016). Nevertheless, Norman (2020) reports the existence of *H. mabouia* in the southernmost part of the Paraguayan Chaco and could be even present in areas of Central Chaco (Jacob Unger, *pers. com.*) where the native gecko *P. przewalskii* is present and deserves a deeper further analysis. Recently, Cacciali (2024) reported the case of local extinction of the endemic species *Phyllopezus heuteri* Cacciali, Lotzkat, Gamble & Köhler, 2018 and the presence of *H. mabouia* instead, but still, it is not clear if the native gecko was first displaced by human activities and then the exotic gecko colonized the area, or if it was an effective case of direct competition.

We provide here the first contribution towards the assessment of competition between a gecko and a skink. This is the first step towards understanding the effect of this exotic lizard in Paraguay. Although financial restrictions are always a major limitation, continuous monitoring is necessary to provide early warning in case of invasion of natural areas by *H. mabouia* in Paraguay and to assess potential competition with other native lizards. To overcome financial constraints, engaging citizen participation through platforms like iNaturalist could be a proactive way to support ongoing monitoring efforts in natural areas. With this respect, it is important to note that species like *H. mabouia* are more frequently recorded in databases, likely because they are easier to observe and photograph, especially when found on walls or near light sources where they feed. This ease of recording may introduce a bias, as more conspicuous species tend to be documented more often. Although this influence on the data cannot be fully mitigated, it should be acknowledged when interpreting the frequency of species in such records. The use of citizen science and platforms like iNaturalist are valuable tools that significantly contribute to the conservation of local biodiversity. These tools facilitate the collection of extensive ecological data, enhance public engagement in scientific research, and support the monitoring and protection of diverse species within their natural habitats.

Acknowledgements

We thank John Kochalka and Bolívar Garcete for their assistance in the identification of stomach content items. Thanks to Martha Motte (Museo Nacional de Historia Natural del Paraguay) for allowing access to the specimens under her care. We are indebted to our families and spouses for unconditional support. A special thanks to Paul Freed for language corrections. The Consejo Nacional de Ciencia y Tecnología (CONACYT) from Paraguay, through the "Programa Nacional de Incentivo a los Investigadores" (PRONII) program, funded PC for research activities. Also, we would like to express our gratitude to the reviewers for their valuable time, constructive feedback, and insightful suggestions, which greatly contributed to the improvement of this manuscript.

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Supplementary material 1

Detailed explanation of Methods based on the PRISMA statement

Authors: Pier Cacciali, Raúl Maneyro

Data type: docx

Link: https://doi.org/10.3897/herpetozoa.37.e133456.suppl1

Supplementary material 2

Literature references are used for stomach content identification

Authors: Pier Cacciali, Raúl Maneyro

Data type: docx

Link: https://doi.org/10.3897/herpetozoa.37.e133456.suppl2

Supplementary material 3

Frequency of prey items consumed by *Notomabuya frenata* and *Hemidactylus mabouia* based on Vrcibradic and Rocha (1998a) and Rocha and Anjos (2007)

Authors: Pier Cacciali, Raúl Maneyro

Data type: docx

Link: https://doi.org/10.3897/herpetozoa.37.e133456.suppl3

OGH Herpetozoa
Austrian Herpetological Society

Herpetozoa 37: 327–338 (2024)
DOI 10.3897/herpetozoa.37.e135222

Description of a new species of the Asian newt genus *Tylototriton* (Amphibia, Urodela, Salamandridae) from Hunan Province, China

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Academic editor: Ben Wielstra ♦ **Received** 21 August 2024 ♦ **Accepted** 14 October 2024 ♦ **Published** 30 October 2024

Abstract

The newt genus *Tylototriton* is widely distributed in east, southeast, and south Asia. Previous studies have shown that basic surveys of this genus are far from comprehensive, and unknown species still exist within the group. In this study, we described a new species of this genus, *Tylototriton gaowangjienensis* **sp. nov.**, from Gaowangjie National Nature Reserve in Hunan Province, China, based on evidence from molecular identification and morphological comparisons. Additionally, we conducted phylogenetic analysis of the genus to elucidate the taxonomic status of the new species. Based on combined mitochondrial 16S rRNA and ND2 gene sequences, the phylogenetic tree supported the new species as a member of the *T. wenxianensis* species subclade in the subgenus *Yaotriton*. It can be distinguished from its congeners by the combination of the following characteristics: (1) medium-sized body; (2) finger tips extending beyond the snout while forelimbs stretched forward; (3) tips of forelimbs and hindlimbs reaching and overlapping when folded towards the body; (4) snout edge round; (5) absence of small papillae in the male cloacal fissure; (6) dorsal ridges narrow, with width smaller than the eye diameter; (7) head length (HDL) greater than head width (HDW) (HDL/HDW = 1.04~1.22); (8) orange markings absence on the parotoid; (9) nodule-like warts on body sides continuous; (10) vomerine teeth not connected at the tip, extending to the occipital bone at the posterior end.

Key Words

morphology, new species, phylogeny, taxonomy

Introduction

The genus *Tylototriton*, within the family Salamandridae, is widely distributed in the eastern, southern, and southeastern regions of Asia (Fei and Ye 2016; Frost, 2020). It was established in 1871 based on the species known as *Tylototriton verrucosus*, collected from Longchuan County, Yunnan Province, China (Anderson 1871). Many phylogenetic studies have suggested that the genus *Tylototriton* is a sister group of *Echinotriton* Nussbaum & Brodie, 1982, and *Tylototriton* is monophyletic, while the previously recognized *Yaotriton* Dubois & Raffaëlli,

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2009, is considered a subgenus within it (Nishikawa et al. 2013a, 2013b; Phimmachak et al. 2015; Wang et al. 2018; Dufresnes and Hernandez 2023). The species of *Tylototriton* have been usually divided into two groups, the *T. verrucosus* and *T. asperrimus* species group (Fei et al. 2005), corresponding to the two subgenera, *Tylototriton* and *Yaotriton*, respectively (Dubois and Raffaëlli 2009).

With the increase in field investigations and the advancement of molecular technology, more and more species of *Tylototriton* have been discovered in recent years, indicating that the species diversity of this genus was previously underestimated. Currently, there are 40 known species of *Tylototriton* worldwide, with 22 species recorded in China. These species distributed in China include: *T. anhuiensis* Qian, Sun, Li, Guo, Pan, Kang, Wang, Jiang, Wu & Zhang, 2017, *T. asperrimus* Unterstein, 1930, *T. broadoridgus* Shen, Jiang & Mo, 2012, *T. dabienicus* Chen, Wang & Tao, 2010, *T. daloushanensis* Zhou, Xiao & Luo, 2022, *T. hainanensis* Fei, Ye & Yang, 1984, *T. joe* Rao, Zeng, Zhu & Ma, 2022, *T. kweichowensis* Fang & Chang, 1932, *T. liuyangensis* Yang, Jiang, Shen & Fei, 2014, *T. lizhenchangi* Hou, Zhang, Jiang, Li & Lu, 2012, *T. maolanensis* Li, Wei, Cheng, Zhang & Wang, 2020, *T. panwaensis* Grismer, Wood, Quah, Thura, Espinoza & Murdoch, 2019, *T. pseudoverrucosus* Hou, Gu, Zhang, Zeng & Lu, 2012, *T. pulcherrimus* Hou, Zhang, Li & Lu, 2012, *T. shanjing* Nussbaum, Brodie & Yang, 1995, *T. sini* Lyu, Wang, Zeng, Zhou, Qi, Wan & Wang, 2021, *T. taliangensis* Liu, 1950, *T. tongziensis* Li, Liu, Shi, Wei & Wang, 2022, *T. verrucosus* Anderson, 1871, *T. wenxianensis* Fei, Ye & Yang, 1984, *T. yangi* Hou, Zhang, Zhou, Li & Lu, 2012, and *T. ziegleri* Nishikawa, Matsui & Nguyen, 2013. According to the phylogenetic relationships of previous studies, *T. taliangensis*, *T. shanjing*, *T. kweichowensis*, *T. verrucosus*, and *T. yangi* belong to the *T. verrucosus* species group (Yuan et al. 2011; Phimmachak et al. 2015), while *T. asperrimus*, *T. broadoridgus*, *T. dabienicus*, *T. hainanensis*, *T. liuyangensis*, *T. lizhenchangi*, and *T. wenxianensis* belong to the *T. asperrimus* species group (Yuan et al. 2011; Shen et al. 2012; Phimmachak et al. 2015).

In addition to the high species diversity, recent molecular studies have indicated that our understanding of *Tylototriton* taxonomy is far from complete. For instance, Grismer et al. (2018) revealed several mysterious lineages that may represent undescribed species (Grismer et al. 2018). Wang et al. (2018) showed the existence of three cryptic species within *T. wenxianensis*, distributing in the Dabie Mountains in Anhui, Wufeng County in Hubei, and Libo County in Guizhou Province, respectively (Wang et al. 2018). Currently, putative cryptic species from the Dabie Mountains and Libo County have been identified and described as two new species, *T. anhuiensis* and *T. maolanensis*, respectively (Qian et al. 2017; Li et al. 2020). However, individuals from Wufeng County, Hubei Province, have not been thoroughly investigated and further examined. In addition to taxonomic aspects, scientists are also concerned with the biology, characteristics, distribution, reproduction, and conservation of *Tylototriton* in various studies (Sparreboom et al. 2014; Hernandez et al. 2016; Wang et al. 2017).

During an amphibian survey and monitoring project, several *Tylototriton* specimens were collected in the Hunan Gaowangjie National Natural Reserve, an area previously not known to harbor any salamander species. In this study, we evaluated the taxonomic status of these specimens using morphological and molecular methods and described the specimens herein as a new species, *Tylototriton gaowangjienensis* sp. nov.. Based on field observations, we also discussed the biological and ecological characteristics of the new species. Additionally, a mitochondrial genome analysis involving this species has been already conducted in another study (Wang et al. 2022).

Materials and methods

Sampling

In May 2021, thirteen individuals of the newt species were collected in Hunan Gaowangjie National Nature Reserve (Fig. 1). Of these, six were deposited as type specimens, and one was used for making a skeleton specimen, remaining individuals were measured for morphological data and molecular samples were collected before release. Additionally, three fertilized females were supplementary collected for reproduction and juvenile observations. All type specimens were fixed in formalin and then preserved in 80% alcohol, and deposited at the animal museum in Jishou University. The permissions for field surveys for scientific purposes were approved by the local Bureau of the National Nature Reserve, and the sample collections and experiment protocols were approved by the Biomedical Ethics Committee of Jishou University (No: JSDX-2024-0083) adhered to the relevant laws and guidelines of China.

Morphological examination

Morphological studies were conducted by descriptions and measurements following the methods outlined by Fei et al. (2006), Shen (2014), and Li et al. (2020). Measurements were taken point-to-point with a vernier caliper by a single measurer (to the nearest 0.01 mm). Abbreviations of morphological characteristics used in this study are shown in Table 1. The methods for skull specimens followed the procedures of Tang et al. (1985). For several morphometric characters, Kruskal-Wallis test were conducted to test the significance of differences between various species in the *T. asperrimus* species group (at a significance level of P = 0.05, see more in Suppl. material 1: table S1) (Li et al. 2020). Furthermore, principal component analyses (PCA) were conducted to determine whether the different species were separated in morphometric combinations (see more in Suppl. material 1: table S2), using the R software (R Development Core Team).

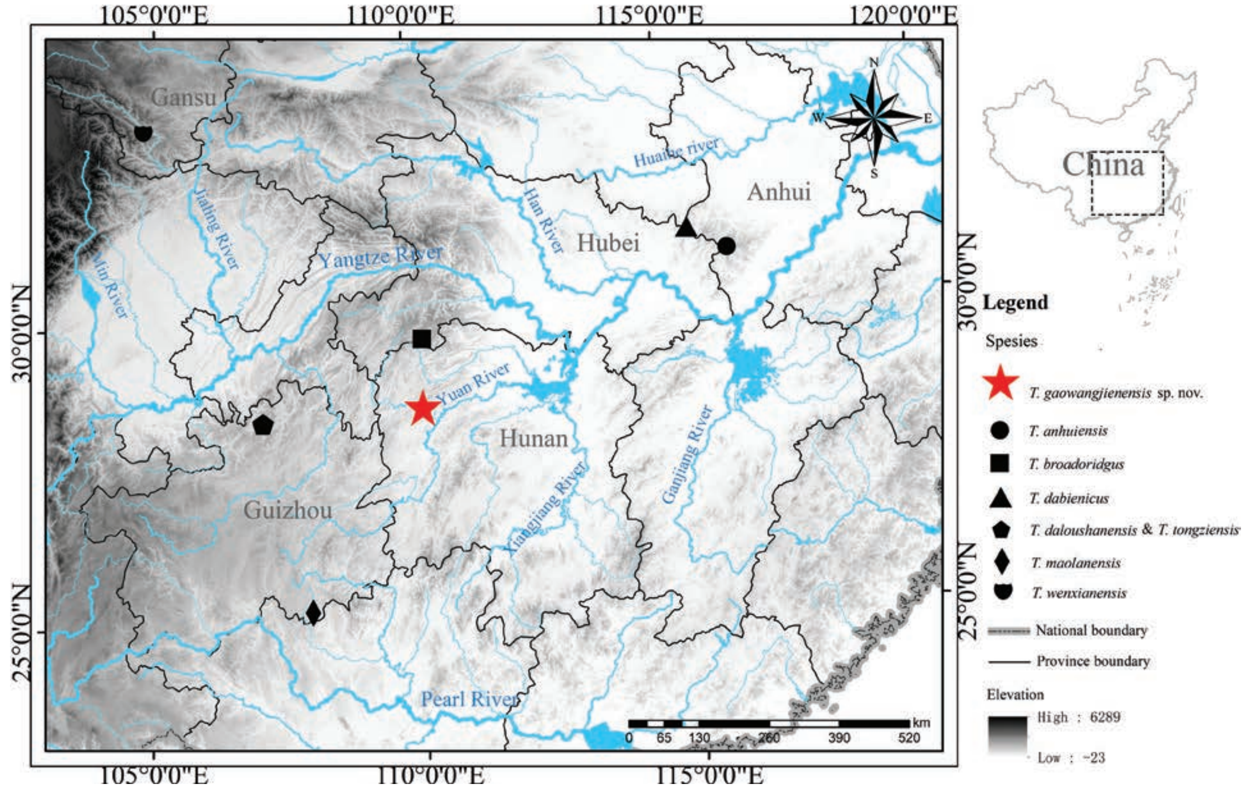

Figure 1. Type locality of species of *T. wenxianensis* species group, Red star: *T. gaowangjienensis* sp. nov.: Gaowangjie National Nature Reserve, Hunan Province, China; Black circle: *T. anhuiensis*: Yaoluoping National Nature Reserve, Anhui Province, China; Black square: *T. broadoridgus*: Badagongshan National Nature Reserve, Hunan Province, China; Black triangle: *T. dabienicus*: Dabie Mountain National Nature Reserve, Hubei Province, China; Black pentagon: *T. daloushanensis*: Huoqiuba Nature Reserve, Guizhou Province, China and *T. tongziensis*: Huanglian Nature Reserve, Guizhou Province, China; Black diamond: *T. maolanensis*: Maolan National Nature Reserve, Guizhou Province, China; Black peltate: *T. wenxianensis*: Wenxian County, Gansu Province, China.

Table 1. Abbreviations for morphological characteristics.

Abbreviation	Morphological characteristics	Explanation
TOL	Total length	Distance from tip of snout to tip of tail
SVL	Snout-vent length	Distance from tip of snout to posterior edge of vent
HDL	Head length	Distance from jugular fold to snout tip
HDW	Head width	Maximum head width
SL	Snout length	Distance from tip of snout to the anterior corner of eye
ED	Eye diameter	Distance from the anterior corner to the posterior corner of the eye
INS	Internasal space	Minimum distance between the external nares
TAL	Tail length	From anterior tip of cloaca to tip of tail
TH	Tail height	Maximum tail height
TRL	Trunk length	From gular fold of throat to anterior tip of vent
IOD	Interorbital distance	Minimum distance between the eyes
HL	Hand length	Wrist from elbow to longest finger
LLA	Length of lower arm	Distance from elbow to tip of wrist
FIIIL	The third finger length	Distance from base to tip of finger III
TLH	Thigh length	Distance from groin to knee
TL	Tibia length	Distance from knee to tarsus
IND	Internasal distance	Minimum distance between the external nares
TIIIL	The third toe length	Distance from base to tip of toe III

After preliminary diagnosis, the species in Gaowangjie National Nature Reserve showed similar features to the *T. asperrimus* species group. Therefore, *T. wenxianensis*, *T. dabienicus*, *T. broadoridgus*, *T. anhuiensis*, *T. maolanensis*, *T. daloushanensis*, and *T. tongziensis*, which belong to the *T. asperrimus* species group in the surrounding areas, were selected for in-depth morphological comparison and statistical analysis. The data of other species of *Tylototriton* were obtained from available literature (Anderson 1871; Fei et al. 1984; Liu et al. 1950; Nussbaum et al. 1995; Böhme et al. 2005; Fei et al. 2006; Chen et al. 2010; Stuart et al. 2010; Hou et al. 2012; Shen et al. 2012; Nishikawa et al. 2013a, 2013b, 2014; Yang et al. 2014; Khatiwada et al. 2015; Le et al. 2015; Phimmachak et al. 2015; Fei and Ye 2016; Qian et al. 2017; Grismer et al. 2018, 2019; Zaw et al. 2019; Bernardes et al. 2020; Pomchote et al. 2020; Lyu et al. 2021; Pomchote et al. 2021; Poyarkov et al. 2021; Luo et al. 2022).

Phylogenetic analysis

Five tissue samples were utilized for molecular phylogenetic analysis. DNA extraction was carried out using the Animal Genome DNA Extraction Kit (Sangon Biotech (Shanghai) Co., Ltd.). The mitochondrial 16S rRNA and NADH dehydrogenase subunit 2 (ND2) genes were amplified using the primer pairs P7 (5'- CGC CTG TTT ACC AAA AAC AT - 3') and P8 (5' - CCG GT CTG AAC TCA GAT CAC GT - 3'), and ND2-4F (5' - TAT GAG TAC GAG CAT CAT ACC C - 3') and ND2-4R (5' - CTT CTG CTT AAG ACT TTG AAG GTC - 3'), respectively. The PCR experiments were conducted following the protocols outlined by Li et al. (2020) and Kyu et al. (2021), respectively. Subsequently, the PCR products were detected by 1% agarose gel electrophoresis and then sequenced. All sequences obtained have been deposited in GenBank (Suppl. material 1: table S3).

For phylogenetic analysis, 94 sequences of 53 *Tylototriton* species (including the new species) were downloaded and incorporated as ingroup datasets, while 6 sequences of 3 species in Salamandridae (*Echinotriton chinhaiensis* Chang 1932, *Pachytriton granulosus* Chang, 1933, and *Pleurodeles waltl* Michahelles, 1830) were selected and downloaded as outgroups (Suppl. material 1: table S3). Both the 16S rRNA and ND2 genes were aligned using MAFFT and manually checked for accuracy. Subsequently, the aligned sequences were combined to construct a concatenated sequence. The uncorrected pairwise distances (p-distance) were calculated using MEGA11.

IQ-TREE 1.6.12 was utilized to conduct the maximum likelihood (ML) analysis (Nguyen et al. 2015), under the best-fit model TN+F+I+G4 that was selected by Modelfinder according to the Bayesian Information Criterion (BIC) (Kalyaanamoorthy et al. 2017). Ultrafast bootstrap approximation (UFB) was assessed using 20,000 ultrafast bootstrap replicates, and nodes with UFB values ≥ 95 were considered significantly supported (Hoang et al. 2018). For Bayesian inference (BI) analysis, the best-fitting model GTR+F+G4 was selected by Modelfinder according to the BIC (Kalyaanamoorthy et al. 2017). The Markov Chain Monte Carlo (MCMC) method was applied in MRBAYES 3.2.2., with four chains run for 20 million generations, sampled every 1000 generations, and the first 25% of samples discarded as burn-in. Nodes with Bayesian posterior probabilities (BPP) ≥ 0.95 were considered well supported.

Results

Taxonomic description

Tylototriton gaowangjienensis J. Huang, Y. Xiang, Y.-X. Zhang, T. Wu & W.-S. Jiang, sp. nov.

https://zoobank.org/15A7EEED-B7DA-4C27-BA9F-CE0C53491BA2

Fig. 2

Type materials. ***Holotype*** • 2021051701, adult, male; China, Hunan Province, Xiangxi Tujia and Miao Autonomous Prefecture, Guzhang County, Gaowangjie National Nature Reserve; 28°40'39.80"N, 110°07'8.54"E, alt. 677 m; collected on 17 May, 2023 by You-Xiang Zhang. ***Allotype*** • 2021051702, adult, female; same locality and date as holotype. ***Paratypes*** • 2021051703~2021051704, adult, male, 2021051705~2021051706, adult, female; same locality and date as holotype.

Etymology. The new species is named after its type locality. We suggest the Chinese name "高望界疣螈" (pinyin: Gāo Wàng Jiè Yóu Yuán), and the English name Gaowangjie crocodile newt.

Diagnosis. The new species can be distinguished from other members of *Tylototriton* by the following combination of characteristics: (1) medium-sized body; (2) finger tips extending beyond the snout while forelimbs stretched forward; (3) tips of forelimbs and hindlimbs reaching and overlapping when folded towards the body; (4) snout edge round; (5) absence of small papillae in the male cloacal fissure; (6) dorsal ridges narrow, with width smaller than the eye diameter; (7) head length (HDL) greater than head width (HDW) (HDL/HDW=1.04~1.22); (8) orange markings absence on the parotoid; (9) nodule-like warts on body sides continuous; (10) vomerine teeth not connected at the tip, extending to the occipital bone at the posterior end.

Description of holotype (Table 2, Figs 2, 3). Adult male, medium-sized, measures 123.69 mm TL, 67.20 mm SVL, and 56.06 mm TAL. Head length greater than head width (HDL/HDW = 1.13). Snout short and blunt, longer than

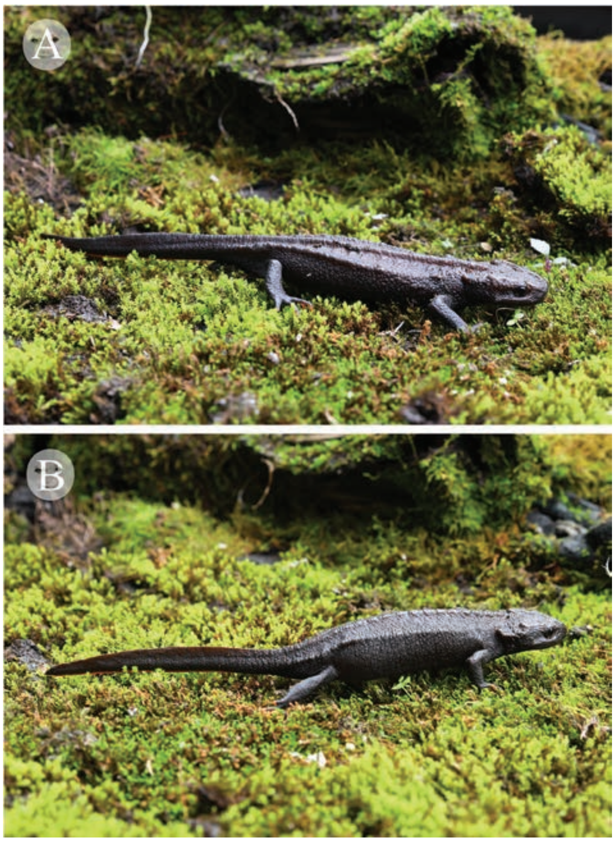

Figure 2. Photos of *T. gaowangjienensis* sp. nov. in live specimens. **A.** Female (2021051702); **B.** Male (2021051701). Photos by You-Xiang Zhang.

eye diameter, extends beyond lower lip from dorsal view. Eyes relatively large (3.8 mm), laterally oriented but visible in dorsal view, with nearly round eyelids. Mouth crack straight, exceeds half head, extending to posterior eyelids; jawbone with teeth. Vomerine teeth arranged linearly in "∧" shape, separated at anterior end, located between choanas, extending to posterior edge of orbit. Tongue oval-shaped, slightly concave in center, with bottom fixed and side edges free. Head ridges prominent, extending from snout to back head, forming longitudinal approximation "π" shape. Bone ridges form weak "v" shape at head; edge relatively smooth, and basically connected to middle ridge.

Table 2 Description of the morphology associated with the holotype, allotype and paratypes.

Item (mm)	Holotype (♂)	%	Allotype (♀)	%	Holotype+Paratype (♂) *2		%	Allotype (♀)+Paratype (♀) *2		%
					Means ± SD	Range		Means ± SD	Range	
SVL	67.20	100%	78.50	100%	69.15 ± 1.955	67.20~71.11	100%	74.01 ± 4.122	70.40~78.50	100%
TOL	123.69	184.06%	139.62	177.86%	134.2 ± 9.672	123.69~142.71	194.07%	135.5 ± 3.715	132.48~139.62	183.08%
HDL	15.93	23.71%	18.94	24.13%	18.26 ± 2.825	15.93~21.40	26.41%	17.09 ± 2.08	14.84~18.94	23.09%
HDW	14.06	20.92%	15.98	20.36%	14.91 ± 0.7903	14.06~15.62	21.56%	16.39 ± 1.066	15.59~17.6	22.15%
SL	4.38	6.52%	4.93	6.28%	5.013 ± 0.5594	4.38~5.44	7.25%	5.727 ± 0.69	4.93~6.13	7.74%
ED	3.80	5.65%	4.05	5.16%	4.14 ± 0.3027	3.80~4.38	5.99%	4.113 ± 0.5478	3.60~4.69	5.56%
TAL	56.06	83.42%	59.97	76.39%	61.57 ± 4.778	56.06~64.61	89.04%	61.4 ± 1.24	59.97~62.18	82.96%
TH	6.99	10.40%	6.59	8.39%	7.85 ± 0.7454	6.99~8.31	11.35%	7.52 ± 0.8843	6.59~8.35	10.16%
TRL	40.39	60.10%	52.2	66.50%	47.63 ± 7.039	40.39~54.45	68.88%	46.45 ± 5.082	42.57~52.2	62.76%
IOD	7.93	11.80%	8.96	11.41%	8.41 ± 0.4176	7.93~8.69	12.16%	8.197 ± 0.6643	7.75~8.96	11.08%
HL	8.50	12.65%	8.12	10.34%	8.343 ± 0.3066	7.99~8.54	12.07%	7.673 ± 0.395	7.37~8.12	10.37%
LLA	8.69	12.93%	7.57	9.64%	8.137 ± 1.339	6.61~9.11	11.77%	7.33 ± 0.2227	7.13~7.57	9.90%
FIIIL	3.93	5.85%	4.46	5.68%	4.51 ± 1.022	3.91~5.69	6.52%	4.04 ± 0.3897	3.69~4.46	5.46%
TLH	7.12	10.60%	8.25	10.51%	7.76 ± 1.358	6.84~9.32	11.22%	7.447 ± 0.6962	7.02~8.25	10.06%
TL	6.55	9.75%	5.98	7.62%	5.883 ± 0.6178	5.33~6.55	8.51%	6.67 ± 0.6951	5.98~7.37	9.01%
IND	4.34	6.46%	6.22	7.92%	4.9 ± 0.8931	4.34~5.93	7.09%	6.08 ± 0.1217	6~6.22	8.22%
TIIIL	5.91	8.79%	4.46	5.68%	5.957 ± 0.5615	5.42~6.54	8.61%	5.347 ± 0.7679	4.46~5.8	7.22%

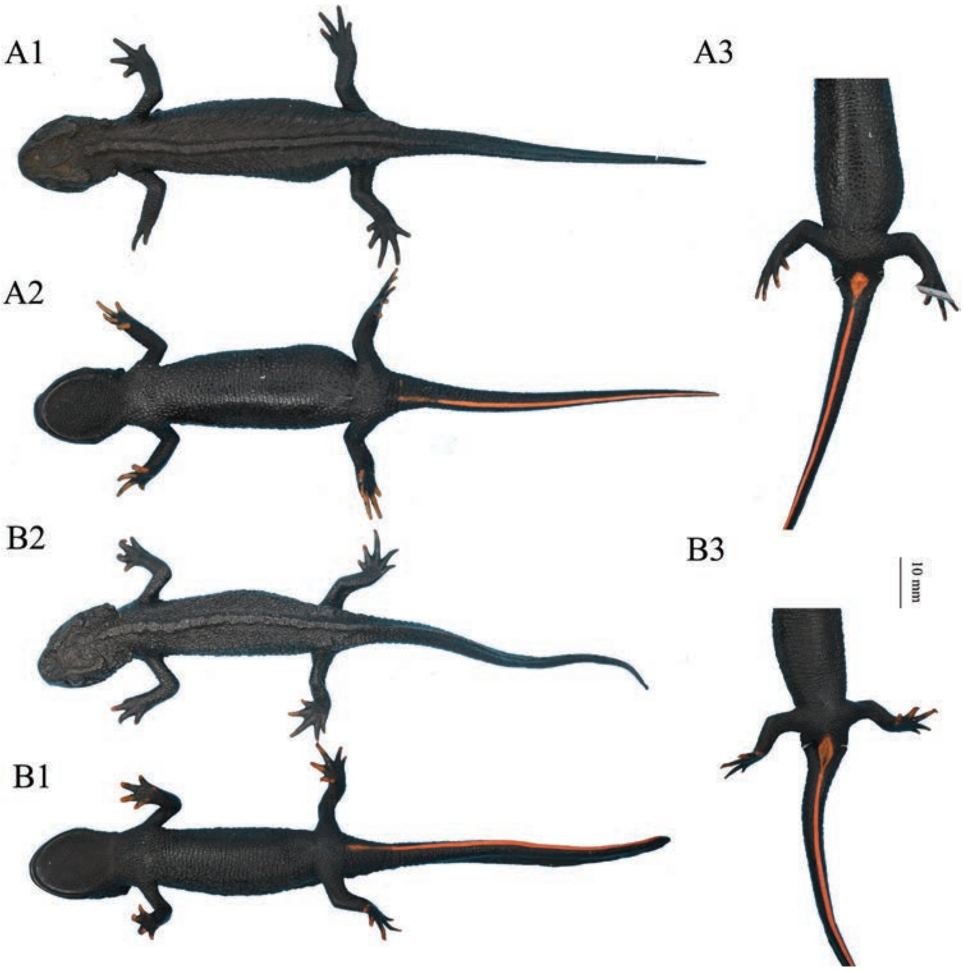

Figure 3. Photos showing color variation in *T. gaowangjienensis* sp. nov. in live specimens. **A.** Female (2021051702); **B.** Male (2021051701). Photos by You-Xiang Zhang.

Skin rough, small granules present on dorsal surfaces of head and dorsum, lateral sides of body and tail. On both sides of the trunk, a row of rib nodules composed of numerous slightly thick warts, slightly protruding outward but relatively flat; the labial margin, distal limbs, ventral limbs and the ventral edge of the tail smooth. Limbs relatively slender, with the hind limbs slightly longer than the forelimbs; tips of forelimbs and hindlimbs reached and overlapped when folding towards; finger tips extending beyond the snout while forelimbs stretched forward. Four fingers, comparative finger length: III > II > IV > I; five toes, comparative toes length: III > IV > II > V > I; all fingers and toes slightly flattened, with blunt ends and no fins.

The tail height exceeds the width at the tail base, with thin and lower dorsal fin folds. Caudal dorsal fin originates from the upper middle section of the tail, connected to the middle ridge of the back, without obvious segmentation. Ventral tail fin folds short and thick, starting from the tail base, with a small gap from the cloacal fissure's posterior edge. Cloacal region notably swollen, vent as longitudinal slit. Skin mostly black or dark gray. Ventral surface, distal ends of digits, peripheral area of cloaca, and ventral edge of tail orange-red; the orange-red region between tail's ventral edge and cloaca's periphery connected. After alcohol preservation, orange-red fades to milky white.

Description of skull (Fig. 4). Back of the skull rough, covered with numerous sculpture-shaped ridges. Skull wider than length, with obvious globular protrusions at the lateral posterior side of the squamosal. Square protrusions evident on the posterior lateral side of squamosal, extending to the base of the forelimbs. Ventral surface of the skull "∧" shaped. Left and right dentitions extend posteriorly along the inner wall of the eye socket to the base of the orbit and reach the occipital bone.

Secondary sexual characteristics and reproduction (Figs 3, 6). There was a remarkable difference in appearance between sexually mature females and males during the reproductive period: females exhibited a relatively thicker body compared to males, with a particularly prominent abdomen. In females, the cloacal fissure in the cloaca was short but significantly expanded outward, and the grooves on the inner wall protruded radially from the center to the surrounding areas. In contrast, males had a relatively long cloacal fissure but with moderate protrusion, smooth folds, and no grooves, which were less remarkable than those in females. However, both males and females showed no papilla within their cloacal fissures during the reproductive period.

The breeding season of *T. gaowangjienensis* lasted from April to June, with a peak period from late April to early May. In late March, the population gradually ended hibernation, and in early April, the males began to migrate to the breeding ground, usually pools or shallow waters, to await breeding opportunities. The females then entered the breeding ground about a week later. In late May, the females usually left the breeding ground after laying eggs, showing no protective behavior. After the females laid eggs and left, the males still stayed in the breeding ground for a period of time, and then left in mid to early June. With that, the breeding season ended, and the newts returned to a terrestrial preferred life in the forest.

Description of larva (Fig. 5). The juvenile's body is slender. Head length exceeds width. Eyes are large, rounded, laterally oriented but still visible in dorsal view. The front end of the three outer gills is obtuse. Skin is smooth, fingers and toes are light yellow, body color ranges from light black to brown. Dorsal fin folds are relatively high, occurring from about 35% of the total body length. Ventral fin folds are short and thick, extending from the cloaca to the end of the tail.

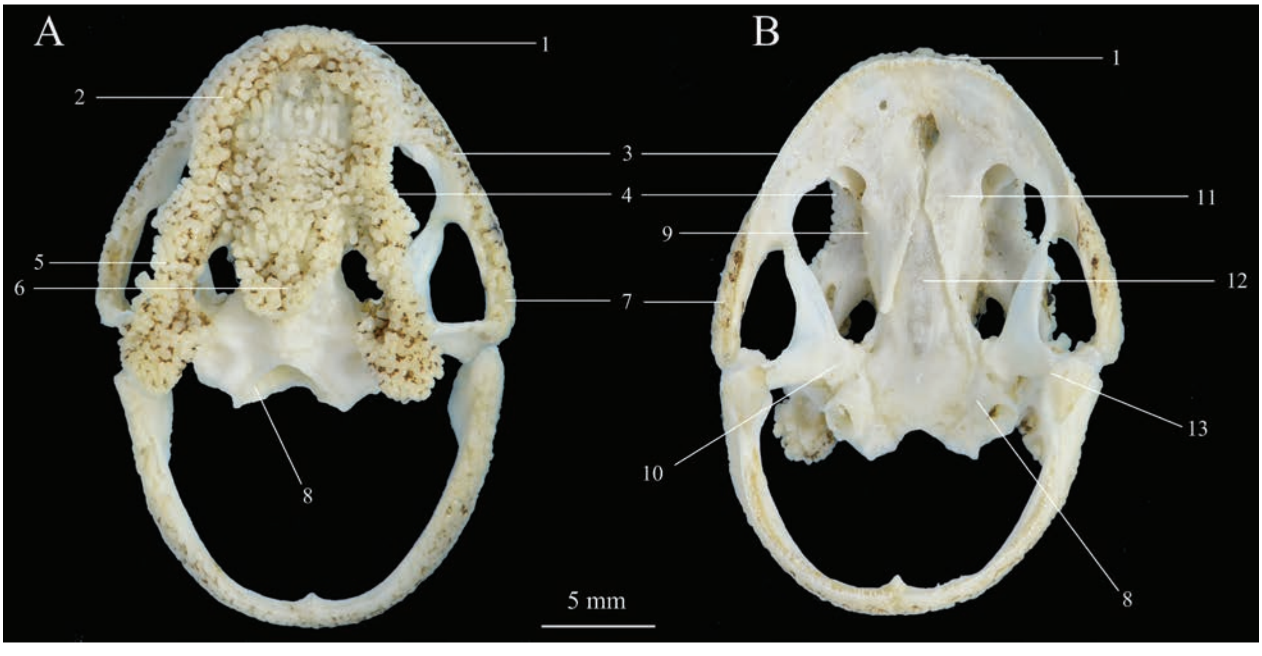

Figure 4. Skull specimen (JSUYY01) of *T. gaowangjienensis* sp. nov. **A.** Dorsal view; **B.** Ventral view. (1) premaxilla; (2) nasal; (3) maxilla; (4) prefrontal ; (5) squamosal; (6) parietal; (7) quadrate frontal; (8) prootic-exoccipital complex; (9) orbitosphenoid; (10) pterygoid ceratohyal; (11) vomer; (12) parasphenoid; (13) prearticular-angular. Photos by You-Xiang Zhang.

Distribution and habits (Figs 1, 6). *T. gaowangjienensis* is only known from Gaowangjie National Nature Reserve, Hunan Province, China. It was primarily distributed in natural broad-leaved or coniferous broad-leaved mixed forests at altitudes of 500–700 meters. During the breeding season, the habitats featured water and could be categorized into three types: seasonal roadside ponds, small creek ponds, and rice fields. The main vegetation types in the breeding areas were evergreen broad-leaved forests, evergreen coniferous broad-leaved mixed forests, and bamboo forests. The environment was lush with high humidity, and the ground was covered with a thick layer of fallen leaves and humus.

Figure 5. Photos of larva (JSUYY02) of *T. gaowangjienensis* sp. nov. in live specimens. **A.** Lateral view; **B.** Dorsal view. Photos by You-Xiang Zhang.

Figure 6. Breeding ground of *T. gaowangjienensis* sp. nov. in the Gaowangjie National Nature Reserve. Photos by You-Xiang Zhang.

Morphological comparisons

In PCA, the total variations of the first principal component was 67.7% and the second was 15.8% which, when combined, explained how the variations exceed 80%. The two-dimensional plots of PC1 vs. PC2 showed that the new species could be distinguished from its closely related species *T. dabienicus* and *T. tongziensis* (Fig. 7). Results from Kruskal-Wallis test also indicated that the new species was significantly different from *T. tongziensis*, *T. maolanensis*, *T. daloushanensis*, *T. anhuiensis*, *T. dabienicus*, and *T. wenxianensis* in specific morphometric characters, especially in males (P-values < 0.05, see more in Suppl. material 1: table S4). These findings demonstrated that the new species is morphologically distinct from all other known congeners.

Molecular analysis

The final alignment of the combined 16S rRNA and ND2 sequence dataset was 1925 bp in length. Both the ML and BI analyses yielded essentially identical topologies and ML tree was presented as Fig. 8. The monophyly of *Tylototriton* was strongly supported, with the 53 nominal species (or populations) mainly divided into two clades, corresponding to the subgenus *Tylototriton* (BPP/UFB = 1.00/99) and *Yaotriton* (BPP/UFB = 1.00/84), respectively. The subgenus *Yaotriton* further divided into two subclades, namely, the *T. wenxianensis* species subclade (BPP/UFB = 1.00/84) and the *T. asperrimus* species subclade (BPP/UFB = 1.00/77). The four individuals of our new species formed a monophyletic group, showing as a sister species to *T. dabienicus* (BPP/UFB = 1.00/96).

The p-distance values of 16S rRNA between the new species and other congeners ranged from 1.57% to 13.55% (see more in Suppl. material 1: table S5). The lowest value (1.57%) was observed between it and *T. dabienicus*, while the values between it and other species in the *T. asperrimus* species group were 2.69%, 3.65%, 3.85%, 3.85%, 3.85% and 4.81% relative to *T. tongziensis*, *T. anhuiensis*, *T. broadoridgus*, *T. maolanensis*, *T. daloushanensis*, and *T. wenxianensis* respectively. The p-distance values of ND2 sequence between the new species and other congeners ranged from 1.7% to 13.27% (see more in Suppl. material 1: table S6). The lowest value (1.7%) was still observed between it and *T. dabienicus*. The p-distance values indicated the degrees of differentiation between the new species and its sister species *T. dabienicus* were larger than some other known sister species pairs, such as these p-distances between *T. sparreboomi* and *T. pasmansi* (0.48 in 16S rRNA and 0.76 in ND2 gene) and between *T. verrucosus* and *T. shanjing* (0.98 in 16S rRNA and 0.92 in ND2 gene). It is of similar differentiations to that between *T. shanorum* and *T. ngarsuensis* (1.76 in 16S rRNA and 1.83 in ND2 gene).

Discussion

The new species described here, *T. gaowangjienensis* sp. nov., represents a distinct species based on both morphological and molecular analyses in this study. Based on the phylogenetic tree (Fig. 8) , *T. gaowangjienensis* sp. nov. is a new member in the *T. wenxianensis* species subclade that in the *T. asperrimus* species group (equals to the subgenus *Yaotriton*). The new species can be distinguished from other congeners in the genus *Tylototriton* by the following specific morphological characteristics (see more in Suppl. material 1: table S4).

T. gaowangjienensis sp. nov. can be distinguished from its sister group *T. dabienicus* by having the tip of the vom-

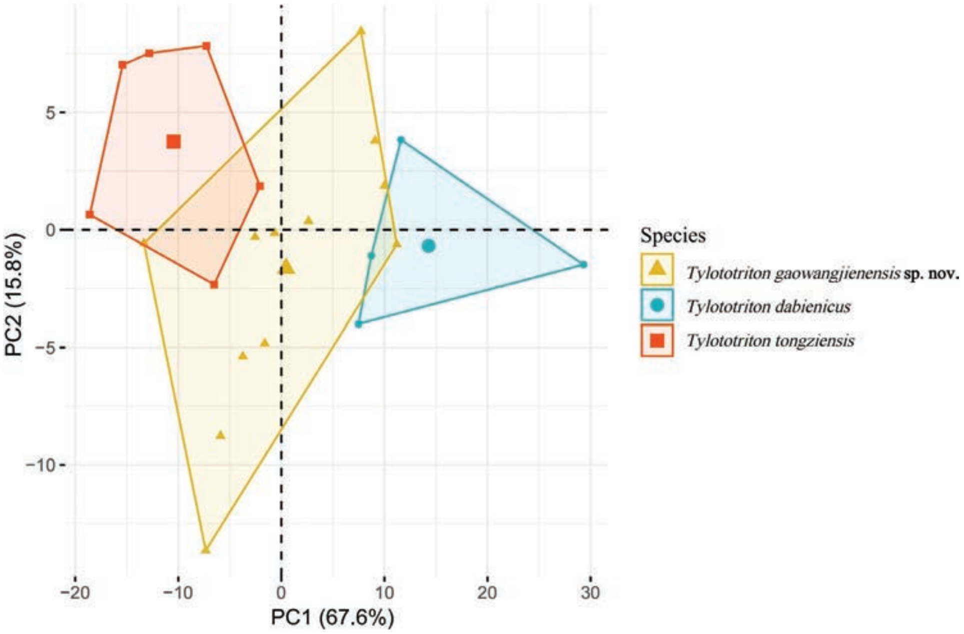

Figure 7. Plots of principal component analyses for *T. gaowangjienensis* sp. nov. and its closely related species according to morphological characteristics. PC1, the first principal component; PC2, the second principal component.

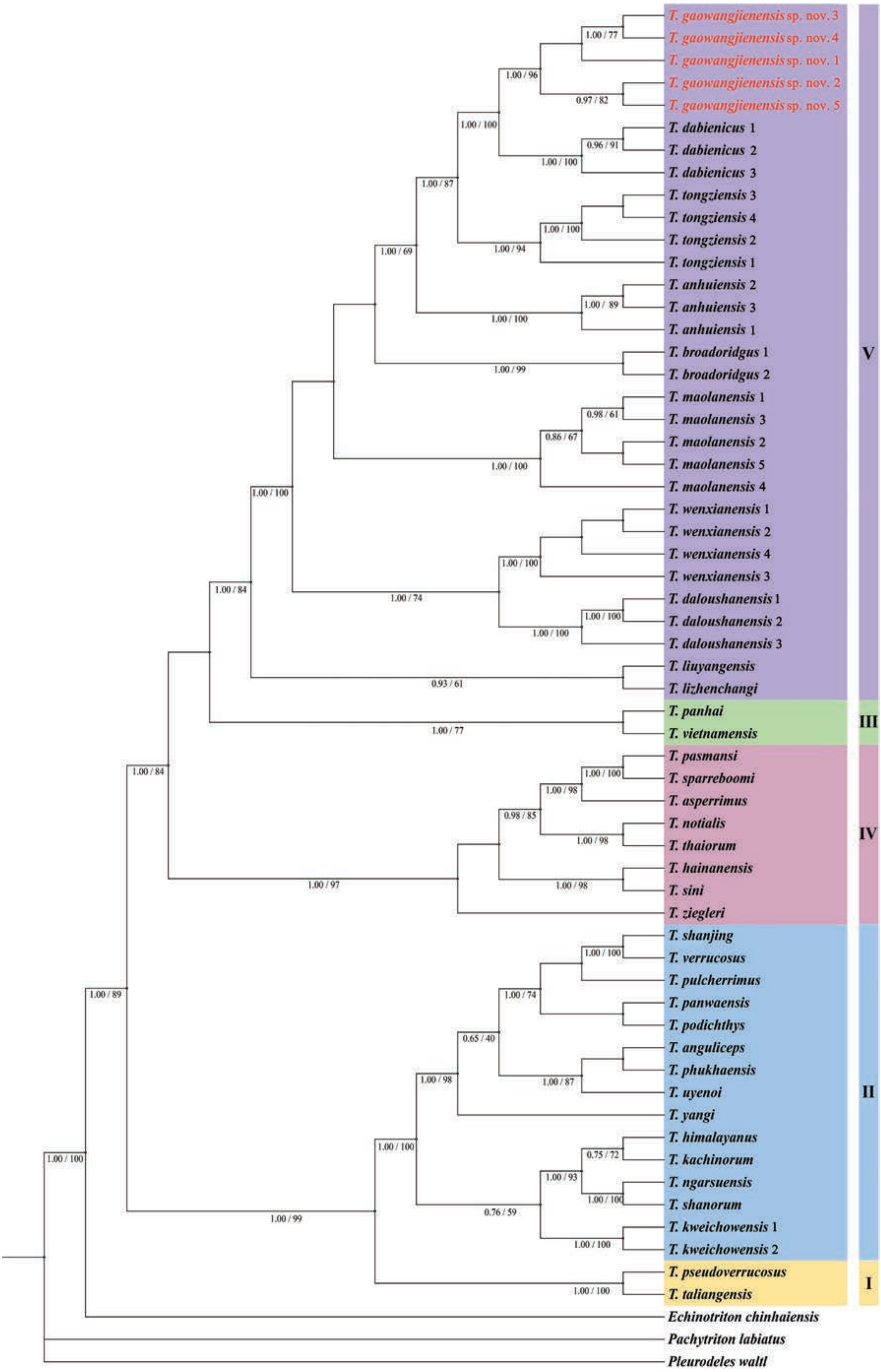

Figure 8. Phylogenetic tree of the genus *Tylototriton* reconstructed based on the mitochondrial 16S and ND2 gene sequences. I: *T. taliangensis* species group; II: *T. verrucosus* species group; III: *T. vietnamensis* species group; IV: *T. asperrimus* species group; V: *T. wenxianensis* species group.

erine teeth separated (vs. connected), and the fact that the posterior end extends to the occipital bone (vs. behind the eye socket); finger tips of forelimbs extending beyond the snout while forelimbs stretched forward (vs. to anterior corner of eyes); tips of forelimbs and hindlimbs reached and overlapped (vs. only slightly reached) when folding towards. In addition, the Kruskal-Wallis test showed that the two species were significantly different in the characters of TL and TAL/SVL of males, and significantly different in the characters of FIIIL and TL of females.

T. gaowangjienensis sp. nov. can be distinguished from *T. maolanensis* by having the nose end rounded (vs. truncated); the male cloacal wall has no papilla (vs. with papilla). The Kruskal-Wallis test showed that the males of two species were significantly different in the characters of SVL, TOL, HDW, TRL, SL, TLH, ED, TH, IND, LLA, HDL/SVL, TAL/SVL, and HDL/HDW.

T. gaowangjienensis sp. nov. can be distinguished from *T. broadoridgus* by having the tip of the vomerine teeth separated (vs. connected), and the posterior end extends to the occipital bone (vs. extends behind the eye socket); tips of forelimbs and hindlimbs reached and overlapped when folding towards (vs. touched a little or not); the midline spine of the dorsal is narrower, with a width smaller than the eye diameter (vs. the midline spine is wider, with a width equal to the eye diameter). The Kruskal-Wallis test showed that the males of two species were significantly different in the characters of HDL/SVL.

T. gaowangjienensis sp. nov. can be distinguished from *T. anhuiensis* by having tips of forelimbs and hindlimbs reached and overlapped when folding towards (vs. touched a little or not); the male cloacal wall lacks papilla (vs. with papilla). The Kruskal-Wallis test showed significant differences in TIIIL,TLH, HDL/SVL, TRL/SVL, HDL/HDW, IOD/HDW between the males of *T. gaowangjienensis* sp. nov. and *T. anhuiensis*.

T. gaowangjienensis sp. nov. can be distinguished from *T. wenxianensis* by having the tip of the vomerine teeth separated (vs. connected); tips of forelimbs and hindlimbs reached and overlapped when folding towards (vs touched a little); HL > HW (vs. HL = HW); finger tips extending beyond the snout while forelimbs stretched forward (vs. not reach the snout). The Kruskal-Wallis test showed significant differences in TAL, TL, HDL/SVL, ED/SVL and IOD/HDW between females of *T. gaowangjienensis* sp. nov. and *T. wenxianensis*.

T. gaowangjienensis sp. nov. can be distinguished from *T. tongziensis* by the lengths of TOL in females (TOL 130.2~141.1 mm vs. TOL 123.5–127.6 mm). The male cloacal wall has no small papilla (vs with papilla). The Kruskal-Wallis test showed significant differences in TLH, TRL/SVL, HDL/HDW between males of *T. gaowangjienensis* sp. nov. and *T. tongziensis*.

T. gaowangjienensis sp. nov. can be distinguished from *T. daloushanensis* by having finger tips extending beyond the snout while forelimbs stretched forward (vs. the finger tips are between the eyes and nostrils); the male cloacal wall lacks papilla (vs. with papilla). The Kruskal-Wallis test showed significant differences in HL, LLA, TL and FIIIL between *T. gaowangjienensis* sp. nov. and *T. daloushanensis* in males and females.

T. gaowangjienensis sp. nov. can be distinguished from *T. liuyangensis* by having finger tips extending beyond the snout while forelimbs stretched forward (vs. reaching the level of eye); tips of forelimbs and hindlimbs reached and overlapped when folding towards (vs. not touched).

T. gaowangjienensis sp. nov. can be further distinguished from *T. panhai* by the absence of orange markings on the parotoid (vs. present); from *T. vietnamensis* by the presence of gular fold (vs. absent); from *T. sparreboomi* by finger tips beyond the snout much while forelimbs stretched forward (vs. reaching the level of nostril), and nodule-like warts on body sides continuous (vs. separated); from *T. asperrimus* by nodule-like warts on body sides continuous (vs. separated), and tips of forelimbs and hindlimbs overlapping much when addressed along body (vs. meeting or overlapping); from *T. thaiorum* by nodule-like warts on body sides continuous (vs. separated); from *T. notialis* by nodule-like warts on body sides continuous (vs. separated), and orange markings on the parotoid and the rib nodules absent (vs. present); from *T. sini* by having nodule-like warts on body sides continuous (vs. separated); from *T. hainanensis* by finger tips extending beyond the snout much while forelimbs stretched forward (vs. reaching the level of eye); from *T. ziegleri* by nodule-like warts on body sides continuous (vs. separated), and vertebral ridge not segmented (vs. segmented).

T. gaowangjienensis sp. nov. can be distinguished from species of the *T. verrucosus* species group (equals to the subgenus *Tylototriton*), except for *T. taliangensis* and *T. pseudoverrucosus*, by having a black body except for tips of fingers and toes, margin of vent, and ventral edge of tail (vs. having distinct yellow to reddish brown markings on head, trunk, and tail edges). *T. gaowangjienensis* sp. nov. can be further distinguished from *T. lizhenchangi*, *T. taliangensis* and *T. pseudoverrucosus* by having a shorter body length in males and by having TOL 123.69~142.71 mm (vs. 145.6~173.0 mm, 186.0~220.0 mm, and 156.2~173.0 mm for the other three species in subgenus *Tylototriton*, respectively).

Only three *Tylototriton* species had been reported in Hunan Province prior to this study, all belonging to the subgenus *Yaotriton*. Besides the aforementioned morphological variations among them, they also exhibited distinct distribution patterns. Among these, *T. broadoridgus* is predominantly found in the northern part of Wuling Mountains, whereas *T. liuyangensis* and *T. lizhenchangi* are located at the border of Dawei Mountain and Yizhang County near Guangdong Province, respectively. Despite the relatively short geographic distances between *T. gaowangjienensis* and the other species in Hunan Province, it displayed the closest genetic relationship with *T. dabienicus* in Anhui Province, then followed by *T. tongziensis* in Guizhou and *T. anhuiensis* in Anhui Province. It also showed a phylogenetic affinity with *T. wenxianensis* in Gansu Province, while exhibiting a relatively larger genetic distance from *T. liuyangensis* and *T. lizhenchangi* in Hunan Province. However, all of these species mentioned above were placed in the same subclade as the *T. wenxianensis* species subclade. Wang et al. (2018) have indicated that the origin and primary distribution of the *T. wenxianensis* species subclade were in central and southern China. Through ancestral tracing and divergence time estimation, it was revealed that *T. liuyangensis* diverged earlier than other remaining species, such as *T. wenxianensis, T. broadoridgus,* and *T. dabienicus*.

Based on speculation suggesting that the Dabie Mountains to the Huaihe River may serve as a secondary distribution and specialization center for most species (Yao et al. 2018), it is inferred that *Tylototriton* species within the *T. wenxianensis* species subclade primarily spread eastward along this water system. It is speculated that the ancestor of the *T. wenxianensis* species subclade initially dispersed from the southwestern mountains and the eastern Himalayas into the Qinling Mountains along the Hengduan Mountains, traversing along the Jialing River in the upper reaches of the Yangtze River and its tributary, the Bailong River, to their current distribution area in southern China. However, owing to the geographical barrier between the Qinling Mountains and the Dabie Mountains-Huaihe River basin, the species in this secondary distribution and specialization center underwent separation and rapid evolution. This led to one subclade spreading eastward along the rivers to the Dabie Mountains, while another subclade dispersed to the Dongting Lake area southeast of the Qinling Mountains. The abundant water systems in the lake area provided conducive conditions for the dispersal of these newts thereafter. However, as of this study, more lineages and new species have been discovered, including monophyletic groups composed of several lineages such as *T. anhuiensis*, *T. dabienicus*, T. *tongziensis*, and *T. gaowangjienensis*, which are distributed in the Dabie Mountains to Wuling Mountains region. This may be the result of secondary or even multiple dispersal.

Based on field observations, one of the primary breeding areas of *T. gaowangjienensis* identified was that in the rice field, yet this habitat is increasingly facing challenges due to human activities. While it remains unclear whether this habitat selection was actively or passively chosen by the species in response to rapid environmental disturbances, it serves as a reminder to prioritize attention to these special habitats of such amphibians. Therefore, it is imperative to conduct more investigations and monitoring in the future to better understand and protect the species in the face of the ongoing environmental changes.

Acknowledgements

This study was funded by the “Observation and study on the reproductive behavior of crocodile newt in Hunan Gaowangjie National Nature Reserve” (No. S202210531056).

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Supplementary material 1

Supplementary information

Author: Jie Huang
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Austrian Herpetological Society

Herpetozoa 37: 339–346 (2024)
DOI 10.3897/herpetozoa.37.e129656

Update on the herpetofauna of Syros (Cyclades, Greece) and current land-use and climate change threats

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https://zoobank.org/06AB3DE5-EE70-417A-9C74-C5FB7F625170

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Academic editor: Günter Gollmann ♦ **Received** 13 June 2024 ♦ **Accepted** 17 October 2024 ♦ **Published** 30 October 2024

Abstract

The current knowledge on the herpetofauna of Syros is updated, whereby no publications on it have been made since 1975. I provide the results from a field trip in 2024 that focused on the hygrophilous species. The Balkan Water Frog and the Balkan Terrapin, mentioned for Syros in the 19th century, meanwhile have become extinct. The Green Toad is threatened with extinction, the status of the Grass Snake is unclear. The Green Toad has benefited previously from anthropogenic spawning grounds in the form of open cisterns, which have fallen into disuse and thus are no longer available to the amphibians. The ongoing climate change in the Aegean region is reflected in drier winters, which dry out water bodies. The situation for the wetlands and their fauna and flora on Syros is alarming.

Key Words

conservation, hygrophilic herpetofauna, status, wetlands

Island portrait

Syros, also known as Syra in older spellings, lies in the middle of the Cyclades Archipelago. Its area is 84 km^2, with Pygros peak reaching 442 metres above sea level. With its 21,500 inhabitants (2011), it is the most populous island in the Cyclades, and includes Ermoupoli, the administrative centre for the southern Aegean region. The island is divided into a largely uninhabited, treeless northern half (Fig. 1) and a more intensively used and urbanised southern half.

Historical and subrecent data on the herpetofauna of Syros

There has been no dedicated study on the amphibians and reptiles of Syros, and no field herpetological results have been published on this island for almost 50 years. Bedriaga (1882) mentions the occurrence of the Balkan Terrapin (*Mauremys rivulata*) and the Balkan Water Frog (*Pelophylax kurtmuelleri*) on Syros. These were my incentives to visit the island and check for possible occurrences.

The first herpetological references to Syros go back to Theodor Erhard's Fauna of the Cyclades (Erhard 1858). Bedriaga (1882, p.7, 14) describes Erhard's contribution as erroneous; he is said to have confused species and islands in his work.

Fifty-six years later, Werner (1938) recorded eight species of amphibians and reptiles on Syros. He also adopts "*Clemmys caspica*" and refers to Bedriaga (1882). The most intensive herpetological work on Syros was done by Frör and Beutler (1980). The authors visited Syros in 1974 and 1977 and were able to evaluate additional herpetological collections (especially parts of Buchholz and Gruber and Fuchs collected materials, kept in the museums of Bonn, München and Vienna). Beutler and Frör (1980) provide an overview of the amphibians and reptiles found on the northern Cycladic islands (table 2 in their work). They consider the Syros herpetofauna to belong to the Central Cyclades. Overall, 13 species are listed in Beutler and Frör (1980) for Syros.

Figure 1. Largely uninhabited north of the island Syros with Phrygana and very dry.

Unpublished personal data

There were three of us on our daily excursions. We visited Syros from 3–16 April 2024 with an overnight stay in Kini (former Kinion) on the west coast. During our stay on the island, the weather was mostly sunny, with air temperatures around 18–21 °C in the daytime and around 8 °C at night, mostly windy but occasionally stormy. We visited the whole island by car and on foot. In Kini, the area was also searched at night for the Green Toad. Of the 13 species recorded on Syros, we were only able to identify six. Below I provide a species list, including references to published data and specific data from this field sampling.

Bufotes viridis (Laurenti, 1768) European Green Toad

Bedriaga (1882, p. 65) writes: "In mid-March 1880, despite the cold weather, I already found adult tadpoles of *Bufo variabilis* on Syra". It was confirmed by Beutler and Frör (1980) for the areas of Kinion, Posidonia, Hermoupolis. We found only tadpoles on 10 April in Posidonia (37.38611°N, 24.88917°E). Further occurrences could not be detected (see details for this species later).

Pelophylax kurtmuelleri (Gaida, 1940) Balkan Frog

Bedriaga (1882, p. 55) states: "*Rana esculenta* is not missing anywhere on all Greek islands"; the water frog is also mentioned for Syra but without locality information. Beutler and Frör (1980) note: "Whether the species still occurs on Syros today must appear questionable, in any case we have not been able to find it there". We did not observe this species. According to our investigations, the species must be considered locally extinct.

Mauremys rivulata (Valenciennes, 1833) Balkan Terrapin

For the Balkan Terrapin (*Mauremys rivulata*), Bedriaga (1882, p. 188) notes: "I have encountered large numbers of this turtle species everywhere in the Cyclades". It is just mentioned for Syra. Beutler and Frör (1980) mentioned: "*M. rivulata* may already be extinct on Syros. At least it has not been observed for a hundred of years", but still included it on their list. Our lack of observations suggests that *M. rivulata* is locally extinct.

Mediodactylus kotschyi (Steindachner, 1870) Kotschy's Gecko

Bedriaga (1882, p.86), Erber (1866), von Oertzen after Boettger (1888), and Werner (1937) all list it without specific locations. Beutler and Frör (1980) observed it in a dry stream 1 km north of Kinion and in the surroundings of Hermoupolis. During our survey it was widespread, especially in the many stone walls, under stones and in fountains, where we found *M. kotschyi* especially in the north more than 20 times. I had the impression of seeing this species more often on smaller Aegean islands than on larger ones.

Hemidactylus turcicus (Linnaeus, 1758) Mediterranean House Gecko

Bedriaga (1882), Gruber and Fuchs in Gruber (1974) as well as Beutler and Frör (1980) note its occurrence in the surroundings of Hermoupolis and 1 km north of Kinion. We did not encounter it, despite searching in many wells and stone walls and on houses.

Lacerta trilineata (Lantz & Cyren, 1920) Balkan Green Lizard

Bedriaga (1882, p. 101), Boulanger after Boettger (1888) and Beutler and Frör (1980) list it occurring between Kinion and Hermoupolis and Posidonia, as a confirmation of Bedriaga. Beutler and Frör (1980) mentioned: “It is rare on Syros, which is probably due to the intensive agriculture use and the high volume of traffic”. Our search was unsuccessful at all the stone walls. Only once, on 15 April 2024, an individual crossed the road near the top of the pass between Ermoupoli and Kini (37.44528°N, 24.92167°E).

Podarcis erhardii (Bedriaga, 1882) Aegean Wall Lizard

Bedriaga (1882, p.124) and Werner (1937) provided no specific locations; Beutler and Frör (1980) mention it from a dry stream at Hermoupolis and from Kinion, Posidonia, and Galissas. According to our observations this lizard was omnipresent on the island during our survey, making it the most common reptile.

Ablepharus kitaibelli (Bibron & Bory St. Vincent, 1833) European Snake-eyed Skink

Bedriaga (1882, p.73), Erber (1866), Beutler and Frör (1980) all mention its presence south of Hermoupolis. We found it several times in the leaf litter of the Phrygana in the north of the island. We encountered it on 4 April 2024 in the southern part only once in Dolphini Bay and on 5 April on the footpath to the cave church of Agios Stephanus near Galissas (37.41361°N, 24.87667°E).

Dolichophis caspius (Gmelin, 1789) Large Whip Snake

Clark (1969) included it without location, it was not seen by Beutler and Frör (1980). We encountered subadults on 6 April 2024 on the footpath from Kambos to the northern tip of the island near the Kanavoria field not far from the old marble quarry and also in the north near Syrigas on 8 April 2024. This was a confirmation of its initial discovery by Clark (1969).

Zamenis situla (Linnaeus, 1758) Leopard Snake

Erber (1866) and Clark (1969) observed it, but did not provide locations. Beutler and Frör (1980) mentioned its presence in a collection (Material Buchholz ZFMK, Museum Alexander König, Bonn), but it was not seen by them or us.

Natrix natrix persa (Pallas, 1814) Eastern Grass Snake

Von Oertzen after Boettger (1888) observed it but provided no location, Beutler and Frör (1980) recorded it in Posidonia, Kinion, Galissas, and state “In the North Cyclades *N. natrix* has only been observed near the water. The populations are now highly endangered due to ongoing habitat destruction”. We did not find any *N. natrix* on the island. A spring is marked on the Skay map Nr. 305 north of Ermoupoli in the gorge area near Aghios Athanasios, which we identified as an older spring catchment. The landowner Nikos Monoryios confirmed to us that water used to flow there in the spring and he had seen snakes in the water there 10–15 years ago. This report supports a possible former occurrence of the Grass Snake. The current situation is unknown, the concerned biotopes of wetlands or streams are missing today on the island.

Telescopus fallax (Fleischmann, 1831) European Cat Snake

Clark (1969) observed it but provided no locations, it was not seen by Beutler and Frör (1980). We did not encounter it.

Vipera ammodytes (Linnaeus, 1758) Nose-horned Viper

Beutler and Frör (1980) mentioned for Syros Oertzen after Boettger (1888), material of Buchholz and Clark (1969) without locations. Beutler and Frör (1980) saw *Vipera ammodytes* in the surroundings of Hermoupolis. We encountered four individuals, all relatively small, i.e. 35–45 cm long. The first approx. 35 cm long and light brown coloured specimen was observed on 5 April on a rocky embankment from Chroussa to Agios Paghos near the church of Panaghia Faneromeni in the southern half of the island (37.41056°N, 24.92028°E). On 6 April, we found a fresh roadkill in the north of San Michaelis (37.49000°N, 24.91833°E). Another live specimen was seen near Kambos on 8 April (37.49639°N, 24.91694°E). Finally, a live individual about 35 cm long was found on 14 April on the roadside at the top of the pass from Ermoupoli to Kini (37.44528°N, 24.92167°E).

Unsuccessful search for wetlands

A particular focus of our visit was the search for wetlands. Beutler and Frör (1980, p. 257) found no large wetlands on Syros. They found smaller bodies of water at Posidonia, Ghalassas and Ermopoulis. In the inventory of wetlands on Greek islands (WWF 2014), only two inventory objects are designated for Syros (Fig. 2). SYR 001 "Estuary of Varvaroussa Beach" is located nearby Kini (37.46861°N, 24.89694°E). On 12 April, only a remnant of a brackish backwater measuring approx. 10 m^2 was still present there (Fig. 3). SYR 002 "Ghalissas Bay" is located west-north-west of Ghalissas and should also include a brackish backwater on 1.18 ha (37.42083°N, 24.87777°E). During the site visit on 5 April, there was no surface water at all, only remnants of a reed bed were still present (Fig. 4). The wetland inventory protocols do not contain any further natural history information including about amphibians.

Figure 2. Map of Syros with indication of WWF objects wetlands and former and present occurrence of the Green Toad. Map source: WWF Greece 2014.

Figure 3. Estuary of Varvarousa Bay in WWF-Greece wetland inventory.

Figure 4. The former wetland in Ghalissas Bay as indicated in WWF-Greece wetland inventory.

The estuaries of somewhat larger catchment areas into the sea were specifically investigated, as were all the spring locations included in the Skai map (no. 305 Syros; scale 1:20 000). These were either captured or have dried up. We saw flowing water (a short open sewer that flowed into the bay) only in the southern industrial area of Ermoupoli

Water scarcity and the effects of climate change

In the southern half of the island, the demand for water is high due to the intensive vegetable and horticultural plantations. No other Cyclades island has had such a dense network of cisterns and so many groundwater wells. However, many of the open cisterns are no longer in use. The groundwater in the wells, which are also no longer active, can be found at an estimated depth of 8–10 m.

In the summer months, the ecological system suffers under the drought. The influx of tourists temporarily doubles the population. Water consumption on Tinos increases from 1,500 cubic metres to an average of 2,700 cubic metres per person per day; the situation is similar on Syros. Drinking and processed water comes from the sea, 95% of which is treated in desalination plants. There is also a lack of infrastructure, such as piping, to utilise the purified water (Papasozomenou 2017).

Climate change effects are not manifested uniformly worldwide. In the Mediterranean, an increase in dry winters is noticeable (data from ERAS, fifth generation atmospheric analysis of global data, covering 1979–2021 with a spatial resolution of 30 km (www.meteoblue.com/de/wetter/archive/export/syros_griechenland2537; accessed on 9 Oct 2024).

The average temperature has increased by 1.6 °C. since 1979 (Fig. 5). The average annual precipitation in the period 1979–2023 was 289 mm. The years 1981, 2003 and 2019 were wetter but since 2020 it has remained consistently dryer (Fig. 6). During our stay on the island, we did not find any surface water in the dry streams that carry water during heavy winter rainfall. Water can usually be found in a normal year in siphons in rocky pools or as backwater at the mouths of the streams. These changes in weather have a massive impact on the hygrophilic herpetofauna, as has already been described for the island of Ios (Broggi 2023). The impacts are described below for two species of herpetofauna for Syros.

On the occurrence of the Green Toad (*Bufotes viridis*)

It has been very dry in recent years, as confirmed by our own observations in 2024. We could not find available natural biotopes on Syros as spawning sites for Green Toads. Open cisterns are sometimes accessible for the reproduction of the Green Toad, as was shown for the Cycladic island of Kythnos (Broggi 2021). The numerous open cisterns on Syros have also been used historically as alternative spawning grounds by the toads

The three locations of the Green Toad in Kini, Ghalissas and Ermopouli mentioned by Beutler and Frör (1980) were searched (Fig. 2) intensively, especially during the overnight stay in Kini. At Kini only two open cisterns

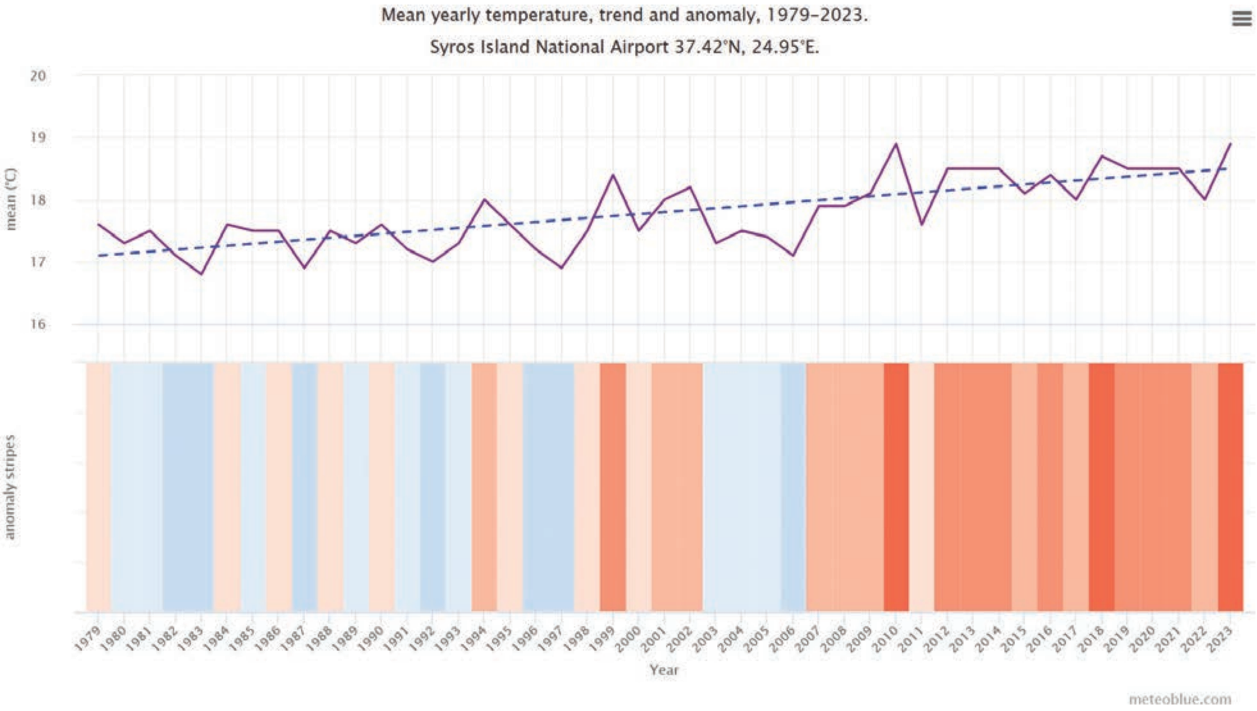

Figure 5. Mean annual temperature in Syros 1979–2021. The dashed blue line is the linear trend of temperature change. The stripes in the lower part of the graph indicate deviations from the mean, with blue for colder and red for warmer years (Data from: www.meteoblue.com/de/wetter/archive/export/syros_griechenland2537; accessed on 9 Oct 2024).

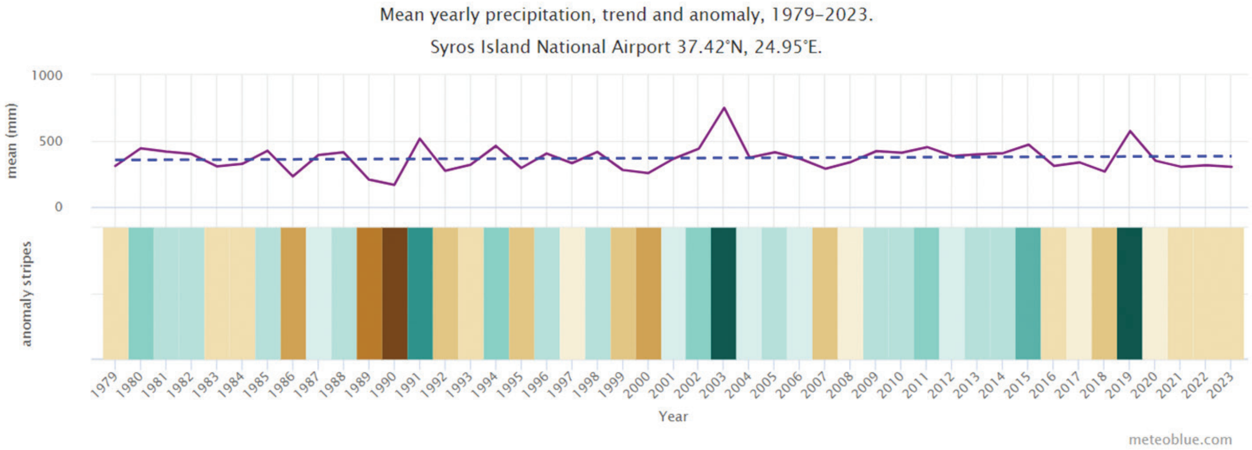

Figure 6. Annual precipitation in Syros 1979–2021. The stripes in the lower part of the graph indicate deviations from the mean, with green for wet and brown for dry years (Data from: www.meteoblue.com/de/wetter/archive/export/syros_griechenland2537; accessed on 9 Oct 2024).

filled with water were found to be possible spawning sites for the Green Toad. However, Goldfish (*Carassius auratus*) were found in these tanks. The spawn of amphibians is eaten by the Goldfish, especially if there is no protection by vegetation. Goldfish were also found in several other cistern sites in the south of the island (Fig. 7). Benefits for people from these Goldfish are hard to conceive, whereas the harm to the amphibians is immense. Coexistence between fish and amphibians is possible, but depends on the presence of shallow water zones with riparian vegetation (Laufer and Wollenzin 2011). This is not the case with cisterns.

The intensive search for the Green Toad on 10 April led to an open cistern in the middle of Posidonia with a basin size of approx. 12 × 12 metres (37.38611°N, 24.88917°E). Well-developed tadpoles of the Green Toad were found in it (Fig. 8). This remained the only island record. Evidently, the Green Toad on Syros is threatened with extinction.

These general conditions also indicate why the Balkan Frog (*Pelophylax kurtmuelleri*), mentioned by Bedriaga (1882) as occurring on Syros, can no longer be found on the island. Beutler and Frör (1980) described the then current occurrence of the water frog as “questionable”.

Figure 7. Open cistern with water but also Goldfish.

Figure 8. Cistern in Posidonia with tadpoles of *Bufotes viridis* (37.38611°N, 24.88917°E).

On the possible occurrence of the Balkan Terrapin (*Mauremys rivulata*)

There is only a reference by Bedriaga (1882) for the occurrence of the Balkan Terrapin on Syros, unfortunately, without providing a location. Beutler and Frör (1980) stated that the Balkan Terrapin “may already be extinct”. In the village of Hartiana in the north of the island on 7 April, we met two young Greeks with whom we struck up a conversation. They asked about our reason for visiting the island. When we asked about the possible presence of turtles, one of them surprised us by telling us that a friend had told him that he had seen turtles in a body of water in Sykaminia Bay below the hamlet of Kambos in the north of the island. He marked the possible location on the map, which was a few hundred metres above the bay. This made it impossible to confuse it with sea turtles. A search of the indicated location the following day gave no indication of such a possible habitat. Geologists encountered in the area confirmed that they had not seen any water surface in this area. How should this observation be interpreted? Was there still a relict population on the island in recent times? We cannot judge this, but believe that the location given is hardly possible for a long-standing water surface. From today’s perspective, based on our own research, there is a lack of suitable habitats for the terrapin on the island. Still, the extinction of the Balkan Terrapin must be confirmed for Syros.

Figure 9. Abandoned cistern.

Conclusions

Four conclusions can be drawn for the current state of the hygrophilic herpetofauna on Syros:

1. Wetlands are naturally rare on the small Cyclades islands of less than 100 km^2 in size, as they usually lack larger hydrological catchment areas with water retention. Such habitats are usually only possible on a small scale, they are therefore vulnerable, very sensitive to external influences and the animal and plant species living in them are relictual in nature. Genetic exchange is hardly possible anywhere.
2. Due to their fragility, aquatic habitats are particularly at risk. For island wetlands, there are conflicts of use with agriculture and tourism. Agriculture is interested in suitable agricultural land where watercourses flow into the sea and has therefore cultivated wetlands. Agriculture development has utilised the watercourses that flow in winter and channeled the water through hoses into reservoirs and cisterns for irrigation of crops. Pumping also lowers the groundwater level. There is also a conflict of use in the estuary area of the dry streams with tourism, which is interested in utilising suitable sandy beaches.
3. While the natural habitats were impaired by increasing utilisation pressure, the hygrophilic herpetofauna was able to benefit in the past in part from the anthropogenic structures with the open cisterns. In the course of the ongoing structural change in agriculture, the maintenance of this complex irrigation regime is increasingly being dispensed with (Fig. 9). Groundwater pumps are being used or agricultural practices are being abandoned (Fig. 10). As a result,

Figure 10. Historic groundwater well out of operation.

these sub-optimal habitats are no longer available. Goldfish stocking in cisterns presents a further threat.

4. Climate change is noticeable through marked increases in temperature, but also manifests itself in drier winter months with a lack of the usual heavy rainfall. This contributes to a massive reduction in suitable habitats and prevents amphibians from reproducing. In the Cyclades elsewhere, amphibians such as the Balkan Water Frog (*Pelophylax kurtmuelleri*), the Green Toad (*Bufotes viridis*), the Common Toad (*Bufo bufo*) and the Tree Frog (*Hyla arborea*) are particularly affected. Among the reptiles, this also applies to the Balkan Terrapin (*Mauremys rivulata*), the Grass and Dice Snakes (*Natrix natrix* and *N. tessellata*) and, to a lesser extent, the Nose-horned Viper (*Vipera ammodytes*) and the Giant Green Lizard (*Lacerta trilineata*), which also prefer more humid structures.

Acknowledgments

I thank my two travelling companions Dr. Peter Goop (Vaduz), and Mag. Günter Stadler (Frastanz), for their valuable assistance during the natural history excursions. A big thank you also goes to the reviewers of this contribution.

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ÖGH Herpetozoa
Austrian Herpetological Society

Herpetozoa 37: 347–358 (2024)
DOI 10.3897/herpetozoa.37.e130028

Water bodies created by peatland restoration are potential habitats for amphibians and reptiles

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Academic editor: Andreas Maletzky ♦ **Received** 19 June 2024 ♦ **Accepted** 27 September 2024 ♦ **Published** 11 November 2024

Abstract

In Lower Austria's Waldviertel region, artificial drainage ditches were constructed in the last century in order to use peatlands for forestry, agriculture, and peat extraction. By now, many of these peatlands are part of the Natura 2000 network and therefore gradually undergo restoration measures, which aim to rewet the peatlands. For this, the ditches are blocked with wooden dams, leading to a water runback, which in turn fills the ditches and peat pools. Such artificial water bodies generally depict secondary habitats for many species. Here, we investigated the amphibian fauna in four restored peatlands in the Waldviertel region and measured abiotic factors of the aquatic habitats to answer the question whether blocked ditches and peat pools are valuable secondary habitats for amphibians. We characterized the microhabitats of amphibians based on various structures and vegetation. Additionally, this study provides a basic assessment of reptile species in the investigated peatlands. During our assessment, we observed 1520 individuals of eight amphibian species, 64 individuals of four reptile species, and characterized 12 different microhabitats. Despite the low pH values of 3.2–4.2, four amphibian species and amphibian spawn were detected in Schwarzes Moos. Our results indicate that peat pools, drainage ditches, and open moorlands are potential habitats for amphibians and reptiles, making their conservation and management an important factor in the protection of amphibian and reptile species.

Key Words

acid tolerance, Austria, blocked drainage ditch, conservation, microhabitats, Natura 2000, peat pools, reptiles, Waldviertel

Introduction

Worldwide, peatlands suffer from destruction, pollution, and climate change (Holden et al. 2007; Joosten 2009; Davidson 2014; Bonn et al. 2016). These ecosystems are highly important for ecology, storing water, carbon, and nutrients in high amounts and representing essential habitats for many species (Lamers et al. 2015). In Austria, more than 90% of the peatlands were destroyed (Dewitz 2023). The particularities of this ecosystem are the acidity of the substrate, the water regime, the fast-changing microclimate, and the low availability of nutrients, which lead to the presence of many highly specialized plant and animal species (Jungmeier et al. 2004; Dierßen and Dierßen 2008).

In the past, artificial drainage ditches were created to dry the soil so that the peatlands could be used for forestry and agriculture and to extract peat. This drains the water directly and lowers the water level (Dierßen and Dierßen 2008). These artificial ditches destroy peat formation and alter soils, vegetation, hydrology, hydrochemistry, and biodiversity (Suislepp et al. 2011; Lõhmus et al. 2015). Along with this, the growing tree coverage in peatlands increases transpiration, which leads to even more desiccation (Succow and Joosten 2001; Dierßen and Dierßen 2008). At the same time, natural water bodies are being converted into linear water networks, leading to a restructuring of the species composition (Vaikre et al. 2020).

In Lower Austria's Waldviertel region, many remaining peatlands are part of the Natura 2000 Network and therefore gradually undergo restoration measures (Provincial Government of Lower Austria 2009). The main aim of the restoration measures is to rewet the peatlands. For this purpose, the artificial ditches are blocked with wooden dams, leading to a water runback, which in turn fills the ditches and the peat pools. Such artificial water bodies generally depict secondary habitats for many animal species (Babik and Rafinski 2001; Tichanek and Tropek 2015; Vaikre et al. 2020). Secondary habitats such as blocked ditches can play an important role for amphibians in protected peatland areas (Hartel et al. 2011; Remm et al. 2018). Water-depth increases with ditch-blocking, which in turn enhances amphibian breeding in the long term (Soomets et al. 2023). However, Remm et al. (2018) found that ditches left to natural succession are less valuable habitats for brown frogs (*Rana arvalis* and *R. temporaria*). Shading by dense tree canopies and increased growth of sphagnum mosses (which results in siltation) negatively affect amphibian reproduction (Soomets et al. 2017; Remm et al. 2018), whereas a decrease in canopy shade increased amphibian breeding and the number of amphibian species in the year following the removal of brushwood (Soomets et al. 2017).

Natural peatlands provide important habitats for amphibians (Desrochers and Van Duinen 2006), which is especially important as amphibians are highly endangered across the world (Gibbons et al. 2000; Stuart et al. 2004). Due to their semi-permeable skin and their aquatic phase, amphibians are particularly susceptible to the acidity (pH value) of the water (Sparling et al. 2000). Although peatlands are acidic, several amphibian species are known to inhabit peatlands, e.g., Green Frogs (*Aquarana clamitans*) use peatlands as summer habitats (Mazerolle 2005), and the spawn and larvae of the Moor Frog (*R. arvalis*) are resistant to acids and therefore able to inhabit acidic-mesotrophic habitats (Räsänen et al. 2003; Glandt 2008; Burmeister 2015).

In this study, we explore whether blocked ditches and peat pools are valuable secondary habitats for amphibians in the Waldviertel region. We characterized microhabitats and measured abiotic factors (including pH values) to find out whether the restored peatlands provide habitats for amphibians. Additionally, as there was only limited data available for peatlands (especially for reptile species), this study will serve as a baseline inventory of amphibians and reptiles for future studies in this region.

Methods

We investigated the amphibian and reptile fauna in four peatlands in the Waldviertel region and measured abiotic factors of the aquatic habitats. We chose four peatlands (Haslauer Moor, Heidenreichsteiner Moor, Schremser Hochmoor, and Schwarzes Moos) due to the presence of water bodies (peat pools and/or blocked ditches), open moorlands, and accessibility. Additionally, we characterized the microhabitats of amphibians and reptiles based on various structures and vegetation.

Study site

The study took place in the Natura 2000 site 'Waldviertler Teich-, Heide- und Moorlandschaft' (Flora-Fauna-Habitat Directive (FFH Directive)) and "Waldviertel" (Birds Directive), in the Lower Austrian Waldviertel region (district Gmünd). The Natura 2000 site includes different priority habitat types defined by the FFH Directive (Ellmauer et al. 1999). The Waldviertel region is at the border of subcontinental and suboceanic climates, characterized by cold temperatures and moderate rainfalls (Jungmeier et al. 2004). The nearest weather station in Litschau (sea level: 558 m) records a mean annual air temperature of 7.1 °C and a mean annual rainfall of 844 mm (ZAMG 2017). The Waldviertel region in the northwest of Lower Austria is part of the Bohemian Mass, characterized by many large acidic-mesotrophic forest peat bogs, dominated by *Pinus sylvestris* (Steiner 1992; Pfundner 2021). The carbonate-free bedrock is impermeable, which, in addition to the low temperatures typical for this region, forms a low-nutrient and highly acidic soil (Pruckner 2002; Pranjič et al. 2006).

Peatlands

Each of the four investigated peatlands contains water bodies (water-filled peat pools and/or blocked drainage ditches) (Fig. 1). The four sites are acidic, low in nutrients, and supplied by rainwater (Steiner 1992; Pranjič et al. 2006). The main habitats are bog forests with red pine (*P. sylvestris*) and wild rosemary (*Ledum palustre*), upland bogs with bog pine (*P. mugo*) and wild rosemary, and silted-up peat pools.

All four sites are medium to heavily damaged from past peat extraction, and large parts of the Waldviertler peatlands were destroyed by forestry, which, along with neophytes and tourism, continues to threaten them today (Penz 2000; Pfundner 2021). Different restoration projects took place in the Natura 2000 site, which aimed to rewet the peatlands and stabilize the water levels by damming/blocking drainage ditches (Pfunder 2021). From 2017–2021, the four sites, among others, were part of the cross-border INTERREG project "Crossborder Habitat Network and Management," which aims to restore the peatlands (Pfundner 2021). From 1991–2000 the World-wide Fund for Nature (WWF) and a LIFE project from 1996–1999 took place at the Haslauer Moor and Heidenreichstein in which dam constructions and clearing of the central peatland areas were conducted to rewet the peatlands (Egger 2000; Seehofer et al. 2003; Ebhart 2011).

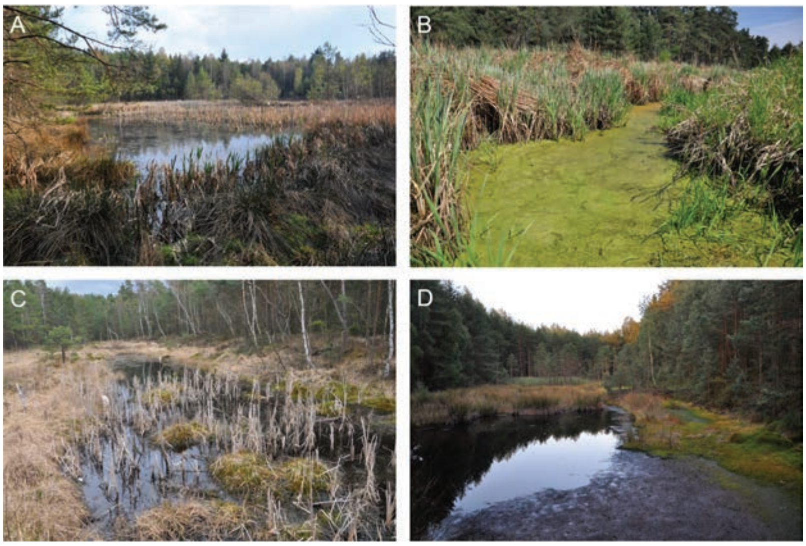

Figure 1. The four investigated peatlands in the Waldviertel region. **A.** A large peat pool in the Haslauer Moor; **B.** A blocked, water-filled drainage ditch in Heidenreichstein; **C.** A silting peat pool in Schrems; and **D.** A partly silting pool in the Schwarzes Moos.

In the Schremser Hochmoor, from 2003–2006, the project "Ländliche Entwicklung" conducted different restoration steps to rewet the peatland (Provincial Government of Lower Austria, 2009).

The Haslauer Moor (48.82472'N, 15.09889'E (WGS 84)) is located in the municipality Haslau. The peatland had an initial size of about 120 ha, of which only about 30 ha exist today (Egger 2000; Jungmeier et al. 2004). The Heidenreichsteiner Moor (48.85444'N, 15.14444'E (WGS 84)) in the municipality Heidenreichstein is part of the protected landscape (IUCN category V) "Naturpark Heidenreichsteiner Moor," which is approximately 30 ha large (http://www.moornaturpark.at, accessed on 31.07.2023). The Schremser Hochmoor (48.79861'N, 15.10000'E (WGS 84)), located in the municipality of Schrems and also part of a protected landscape ("Naturpark Hochmoor Schrems"), is the largest of the four peatlands. The peatland is 107 ha in size (Natura 2000 standard data form). The peatland Schwarzes Moos (municipality Brand-Altnagelberg; 48.871944'N, 14.980556'E (WGS 84)) is next to the Czech border. Originally, the peatland was 54 ha in size, but due to the former glass industry and strong forestry, only 1 ha of the peatland is preserved (Pranjič et al. 2006). The site consists of several water-filled peat pools and drainage ditches in different silting stages inside a private forest (Table 1).

According to the herpetofaunistic database of Austria (HFDÖ), six reptile and nine amphibian species are potentially located in the area of the study site: *B. bufo* (Common Toad), *Hyla arborea* (European Tree Frog), *Ichthyosaura alpestris* (Alpine Newt), *L. vulgaris* (Smooth Newt), *Pelophylax* kl. *esculentus* (Edible Frog), *P. lessonae* (Pool Frog), *R. arvalis* (Moor Frog), *R. dalmatina* (Agile Frog), *R. temporaria* (Common Frog), *Anguis fragilis* (Slow Worm), *Coronella austriaca* (Smooth Snake), *Lacerta agilis* (Sand Lizard), *Natrix natrix* (Grass Snake), *Vipera berus* (Adder), and *Zootoca vivipara* (Viviparous Lizard), of which five species are listed as "vulnerable" by the IUCN (*H. arborea*, P. *lessonae*, *R. arvalis*, *C. austriaca*, *V. berus*).

Table 1. Size and type of waterbodies of the investigated peatlands.

Number	Type of water body	Size of water body	Location
01	water-filled peat pools	~ 200 m^2	Haslauer Moor
02	water-filled peat pools	~ 250 m^2	Haslauer Moor
03	blocked drainage ditch	~ 100 m^2	Heidenreichstein
04	water-filled peat pools	~ 250 m^2	Schrems
05	water-filled peat pools	~ 2000 m^2	Schrems
06	water-filled peat pools	~ 80 m^2	Schwarzes Moos
07	water-filled peat pools	~ 100 m^2	Schwarzes Moos
08	water-filled peat pools	~ 120 m^2	Schwarzes Moos
09	water-filled peat pools	~ 800 m^2	Schwarzes Moos
10	water-filled peat pools	~ 1050 m^2	Schwarzes Moos

Amphibian assessment

The amphibian assessment took place eight times, mainly focusing on water bodies. As amphibians inhabit different habitats at different life stages, we combined several methods to assess the potential occurring species (Lüscher and Althaus 2009). The assessment started on 15 April 2018 and ended on 30 June 2018, consisting of six different methods: (1) acoustic mapping, (2) spawn mapping, (3) larvae mapping, (4) newt shining, (5) use of fish traps, and (6) visual mapping, which took place in all four study sites. All six mapping steps were planned to take place at the appropriate time, regarding their developmental stage (Cabela et al. 2001). The acoustic mapping took place once in April and once in May, i.e., during the reproductive season when male amphibians call to attract females (Glandt 2008). In each site, we spent a minimum of 30 min. To map the amphibian spawn, we slowly walked along the banks of the water bodies (for 15 min at each water body) at the end of April. The larvae mapping took place twice at the end of May, using a landing net with a mash size of approximately 2 mm. In small water bodies (< 10 m^2) we took five strokes, in medium water bodies (ca. 10–30 m^2) we took 10 strokes, in large water bodies (ca. 30–100 m^2) 15 strokes, and in very large water bodies (> 100 m^2) 20 strokes. To record the newts, we slowly walked along the banks of the water bodies after sunset and shone lights through the water for 15 min at each water body. In June, four fish traps were cast in each site to catch tadpoles and newts, which were emptied and dismantled the next morning. Visual mapping took place each time we were at the study site for the mapping steps described above.

In all six mapping steps, we determined the species directly at the study site. In doubtful cases, however, we recorded the amphibian calls with an iPhone SE 5 (acoustic mapping), took pictures with a camera (Nikon D90 or an iPhone SE 5; visual mapping), or took samples in alcohol (98% ethanol) for later determination with a binocular (ZEISS, Stemi SV11) and the identification key provided by Thiesmeier (2014). All people involved in the assessment used rubber boots, which were disinfected after each different study site to prevent spreading chytrid fungus or other transmissible diseases (Schmidt et al. 2009).

Reptile assessment

The reptile assessment took place from 4 June to 15 August 2018, along the banks of the water bodies, at the open parts of the peatlands (here referred to as moorlands) and their surrounding forests, with each site being visited four times during this period. The mapping consisted of a transect leading through the open moorlands and forests, approximately 400 m in length. The observer slowly moved along the previous determined path (transect) for one hour and mapped all visible reptile species along the transect and approximately 2 m right and left of it, following a zigzag pattern (Fitzgerald 2012). The mapping took place in the morning from 8:00 to 11:00 am or in the late afternoon, after 3:00 pm until 7:00 pm, and was interrupted during rainfall.

Abiotic factors and microhabitats

To characterize and verify the quality of the habitats, we (1) measured the pH value of each water body in the four sites using an HQd Portable Meter (Hach Lange GmbH, Düsseldorf, Germany) and (2) mapped the microhabitats and calculated an indicator species analysis (ISA). ISA is used to identify species characteristics of specific habitat types within the study site. The analysis was performed using the labdsv package in R Studios. Species with significant indicator values ($P < 0.05$) were considered strongly associated with specific habitat types, indicating their potential as habitat indicators. The results were interpreted to identify key species representing different habitat types in the study area. Microhabitats are small habitats with vertical and/or horizontal vegetation and landscape structure in a terrestrial and/or aquatic ecosystem (Ricklefs and Miller 1999). The scale for microhabitats used usually ranges from 25–100 m^2 (Baraloto and Couteron 2010). Microhabitats were defined based on the habitats provided by Cabela et al. (2001) and the results of Ebhart (2011). We drew polygons of each microhabitat based on the orthophotos of GEOLAND basemap (datasource: https://basemap.at/) and hand sketches of each peatland in ArcGIS 10.6.1 (ESRI) geographic information system (GIS) software to assemble microhabitats and the results of the amphibian and reptile assessments for each study site.

Authorizations

Two authorizations by the Lower Austrian government allowed us to catch and collect amphibians and reptiles (Provincial Government of Lower Austria, 3109: Stückler S.: BD1-N-200/021-2004; RU5-BE-64/019-2018; Schweiger S.: RU5-BE-64/018-2018; BD1-N-200/021-2004; NÖ-UA-V-30/004-2015).

Results

Amphibian assessment

In total, we located 1520 individuals (including larvae and spawn) of eight amphibian species in the study site: *B. bufo*, *H. arborea*, *P.* kl. *esculentus*/*lessonae* (hereafter grouped and called *Pelophylax* sp. as the distinction in the field is not reliable), *R. arvalis*, *R. dalmatina*, *R. temporaria*, *I. alpestris*, and *L. vulgaris* (Table 2). The species composition and the number of individuals differed in the two main aquatic habitats (peat pools and blocked ditches); in the peat pools, we located *B. bufo*, *Pelophylax* sp., *R. arvalis*, *R. dalmatina*, *R. temporaria*, and *L. vulgaris*. In total, we detected 725 individuals in the peat pools. The most abundant species was *Pelophylax* sp. (416 individuals; 46.3% of total individuals found), followed by *B. bufo* tadpoles (250 individuals; 27.8% of total found individuals). In the blocked ditches, *B. bufo*, *H. arborea*, *Pelophylax* sp., *R. arvalis*, *I. alpestris*, and *L. vulgaris* were identified. In total, we detected 174 individuals in the blocked ditches, again with *Pelophylax* sp. having the highest number of individuals (158 individuals, 17.6% of total found individuals) (see Table 2, Fig. 2).

Reptile assessment

We located 64 individuals of four reptile species in the study sites: *A. fragilis*, *N. natrix*, *V. berus*, and *Z. vivipara*. In the Schwarzes Moos and the Haslauer Moor, we located three reptile species: *A. fragilis*, *N. natrix*, and *Z. vivipara*. In Schrems we found three species, namely *N. natrix*, *V. berus*, and *Z. vivipara*. Only in Heidenreichstein could we detect all four species: *A. fragilis*, *N. natrix*, *V. berus*, and *Z. vivipara* (Table 3, Fig. 3).

Table 2. Results of the amphibian assessment in the four peatlands in the Waldviertel region. Given are the absolute numbers of found individuals. Note that species marked with an asterisk (*) are spawn or larvae.

Species	Haslauer Moor	Heidenreichstein	Schrems	Schwarzes Moos	Number of individuals
Ichthyosaura alpestris	0	5	0	0	5
Lissotrion vulgaris	0	1	4	0	5
Bufo bufo	210	8	18	51	287
* *Bufo bufo* larvae	300	0	0	0	300
Hyla arborea	0	4	0	0	4
Pelophylax sp.	73	205	477	91	846
* *Pelophylax* sp. larvae	6	1	20	0	27
Rana arvalis	1	1	2	1	5
Rana dalmatina	0	0	6	0	6
* *Rana dalmatina* spawn	1	6	0	4	11
* *Rana dalmatina* larvae	19	0	0	0	19
Rana temporaria	1	0	1	3	5
Number of species	5	7	6	5	1520

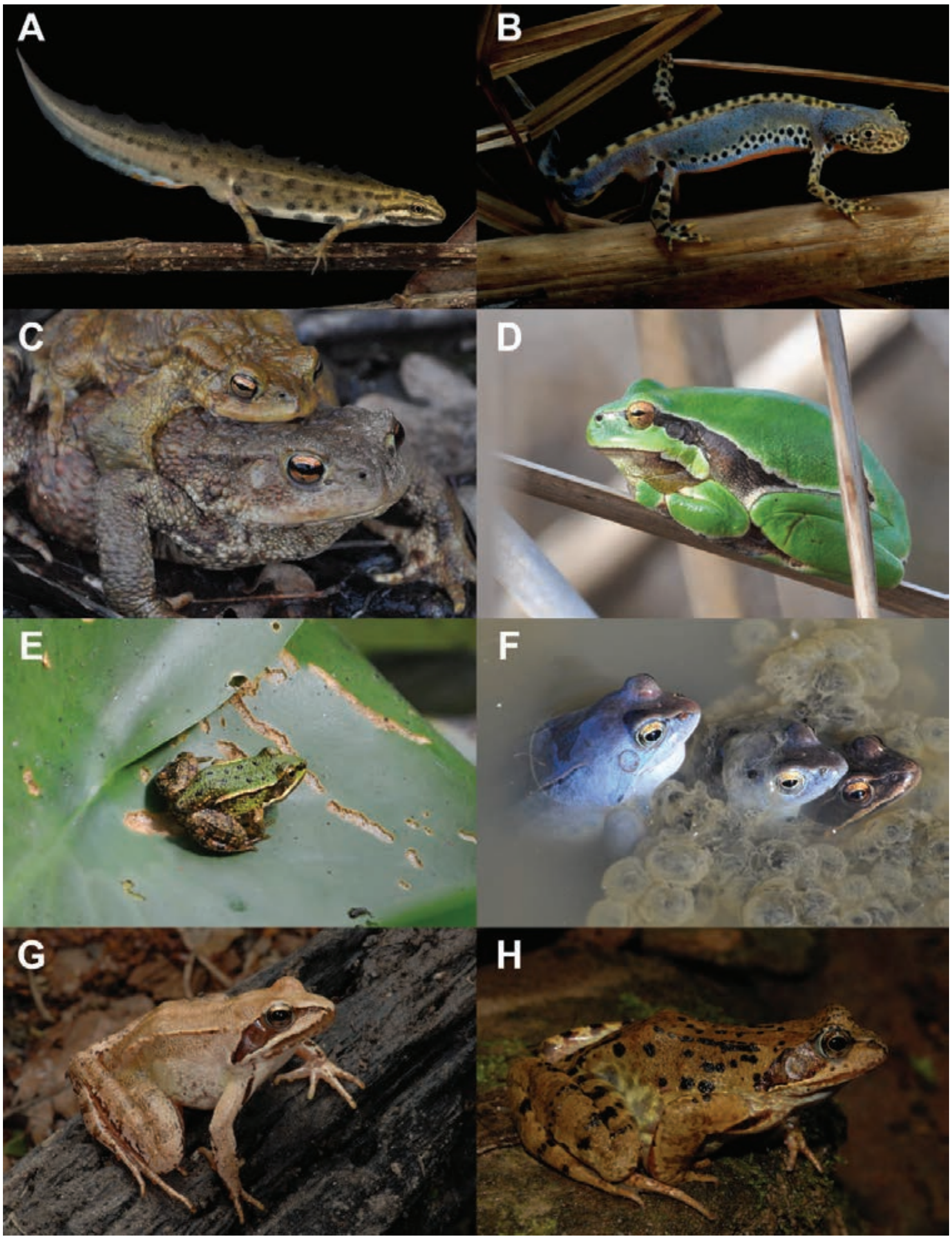

Figure 2. Amphibian species observed in the Waldviertler peatlands: **A.** *Lissotriton vulgaris*; **B.** *Ichthyosaura alpestris*; **C.** *Bufo bufo*; **D.** *Hyla arborea*; **E.** *Pelophylax* sp.; **F.** *Rana arvalis*; **G.** *R. dalmatina*, and **H.** *R. temporaria*. Pictures **A, B, G, H** provided by Michael Franzen.

Table 3. Results of the reptile assessment in the four peatlands in the Waldviertel region. Given are the absolute numbers of found individuals.

Species	Haslauer Moor	Heidenreichstein	Schrems	Schwarzes Moos	Number of individuals
Anguis fragilis	2	2	-	1	5
Natrix natrix	4	5	8	2	19
Vipera berus	-	3	7	-	10
Zootoca vivipara	6	7	7	10	30
Number of species	3	4	3	3	64

Figure 3. Reptile species observed in the peatlands of the Waldviertel region: **A.** *Anguis fragilis*; **B.** *Natrix natrix*; **C.** *Vipera berus*, and **D.** *Zootoca vivipara*. All pictures provided by Michael Franzen.

Abiotic factors

The lowest pH levels were found in the Schwarzes Moos, while the other three peatlands had higher pH levels (see Table 4, Fig. 4).

Table 4. Results of the measurements of the abiotic factors in the Waldviertler peatlands: Haslauer Moor (*N* = 4), Heidenreichstein (*N* = 3), Schrems (*N* = 6), and Schwarzes Moos (*N* = 5).

Abiotic factor	Haslauer Moor	Heidenreichstein	Schrems	Schwarzes Moos
pH (mean)	6.35	6.94	7.05	3.72
pH (max.)	6.66	7.39	9.14	4.22
pH (min.)	6.11	6.12	5.03	3.21

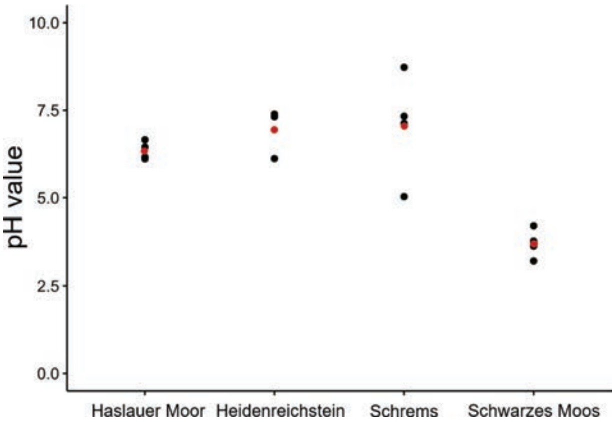

Figure 4. Comparison of pH values (visualized as strip charts). The peatlands included in the study site are Haslauer Moor (N = 4 pH measurements), Heidenreichstein (N = 3 pH measurements), Schrems (N = 6 pH measurements), and Schwarzes Moos (N = 5 pH measurements). Each point of the strip chart represents the pH level of a measurement point (black) and the mean value (red). For detailed results, see Table 4.

Microhabitats

We characterized twelve different microhabitats in the assessment area: open moorland with moss, open moorland with sedges, open moorland with bushes, reeds with water body, water-filled blocked drainage ditch, temporary pond, open water-filled peat pool, silting water-filled peat pool, sparse forest/moor forest, forest, margin, and path (Suppl. material 2). The numbers of amphibian and reptile species ranged from 1–9 per microhabitat type.

The ISA did not show any species with significant indicator values. Although species-specific indicator values were calculated, we did not find any significance for any habitat group, suggesting that no species demonstrated a strong preference for a specific habitat in our investigation. In general, silting water-filled peat pools were the microhabitat where we found the most species. We located most amphibian species in silting water-filled peat pools and water-filled blocked drainage ditches, and most reptile species in open moorland with moss, open moorland with bushes, and on paths. We observed *Pelophylax* sp. in nine, *N. natrix* in seven, and *Z. vivipara* in six different microhabitats, whereas *I. alpestris* only in water-filled blocked drainage ditches (Fig. 5A–C).

Discussion

Amphibian assessment

Our results show that blocked ditches and peat pools provide aquatic habitats for eight amphibian species. We found higher numbers of individuals in silting water-filled peat pools than in blocked ditches, which might be related to heavy siltation and desiccation of the ditches (Remm et al. 2018). We observed all expected amphibian species in the study site: *I. alpestris*, *L. vulgaris*, *B. bufo*, *H. arborea*, *R. arvalis*, *R. dalmatina*, *R. temporaria*, and *Pelophylax* sp., but we did not locate all expected species in each peatland. *Bufo bufo*, *Pelophylax* sp., and *R. arvalis* we recorded in each peatland. *Bufo bufo* and *Pelophylax* sp. are very common species with low habitat requirements and are widely distributed across Austria (Cabela et al. 2001). *Rana arvalis* occurs across the Eurasian subcontinent but is limited in Austria to the eastern and southern lowlands (Glandt 2008). Spawn and larvae of *R. arvalis* are more resistant to acids than other amphibian species and therefore able to inhabit acidic peatlands (Räsänen et al. 2003; Burmeister 2015). We found several amphibian species in low abundances: *I. alpestris*, *L. vulgaris*, *R. dalmatina*, *R. temporaria*, and *H. arborea*.

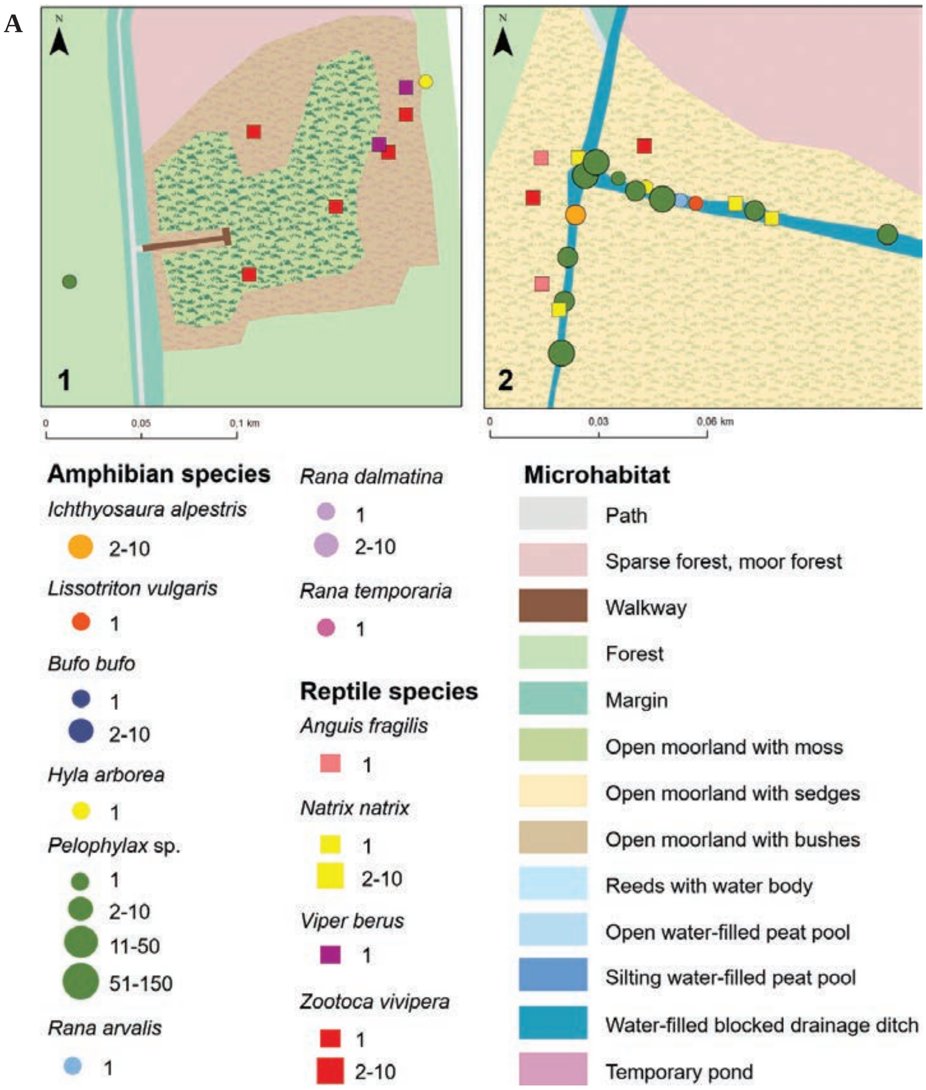

Figure 5. A. Microhabitats and amphibian and reptile species in Heidenreichstein (1: open moorland; 2: drainage ditch). Circles represent amphibian species; squares represent reptile species. Note the different scales; **B.** Microhabitats and amphibian and reptile species in 1: Haslauer Moor and 2: Schwarzes Moos. Circles represent amphibian species; squares represent reptile species; **C.** Microhabitats and amphibian and reptile species in Schremser Moor (1: large water-filled peat pool; 2: open moorland and a silting water-filled peat pool). Circles represent amphibian species; squares represent reptile species. Note the different scales.

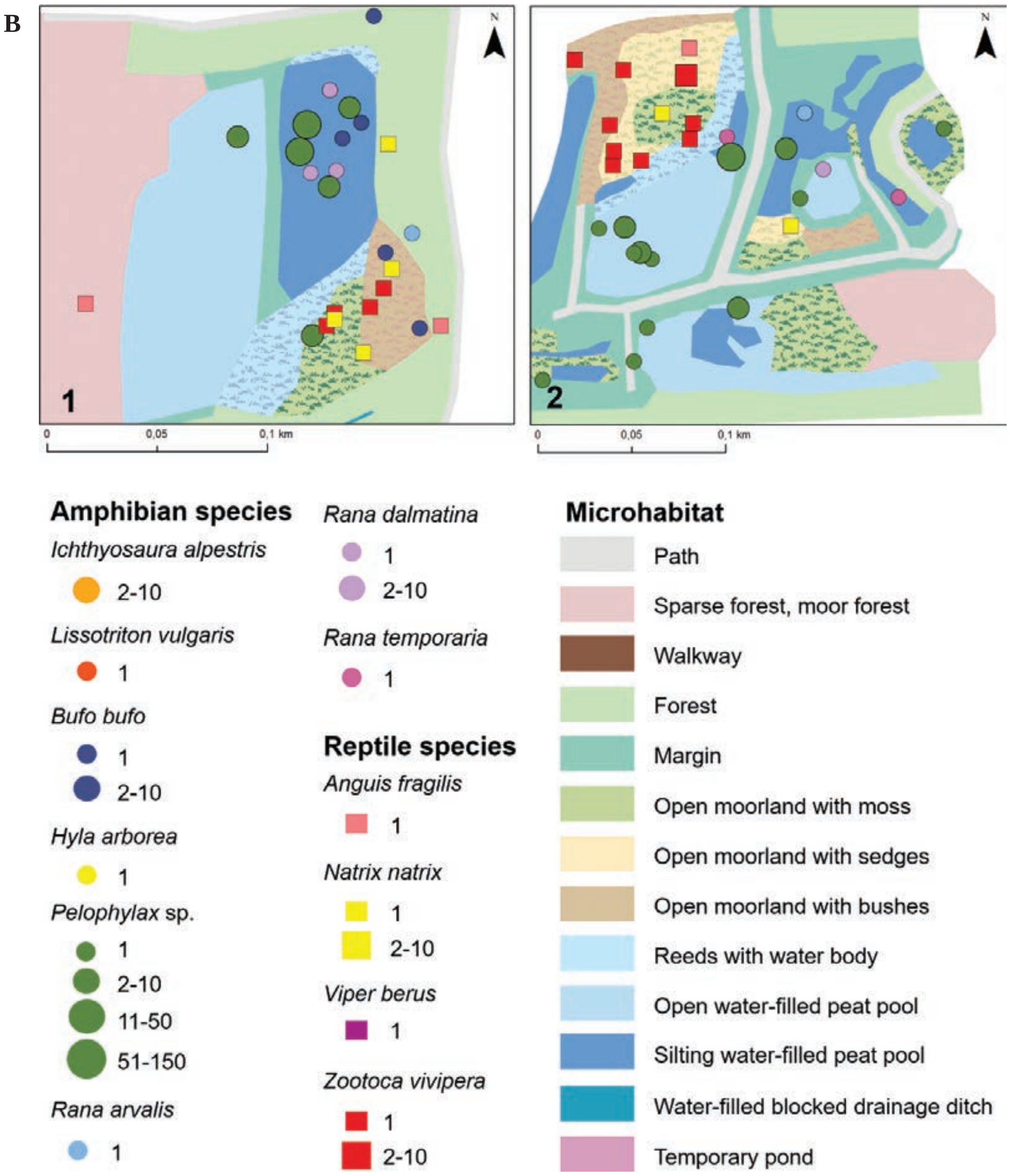

Figure 5. Continued.

In the Schwarzes Moos, we recorded the lowest pH levels ranging from 3.2–4.2 (Table 4); nevertheless, we observed calling *B. bufo*, *Pelophylax* sp., *R. arvalis*, and *R. temporaria* and located *R. dalmatina* spawn in peat pools. Mortality of many amphibian species increases when pH levels are below 4 (Pierce 1985). Dolmen et al. (2008) reported that low pH levels cause the absence and extinction of amphibians in Southern Norway: pH levels of 4.5–4.6 are critical for *R. temporaria*, 4.7 was the lowest pH level recorded for *B. bufo*, and 4.8 for *L. vulgaris*. Successful reproduction was not observed below 5.2 and 5.3 in *R. arvalis* and *Triturus cristatus* (Dolmen et al. 2008). However, pH levels of water bodies are subject to certain fluctuations in relation to, e.g., rainfall (Fraindová et al. 2022); therefore, a more comprehensive abiotic investigation of the water bodies on a long-term basis across the year is necessary to provide more detailed insights into the relationship between pH value and the amphibian fauna in the peatlands and its ecological role. Secondly, a more intensive assessment and an adjustment of monitoring methods might have been necessary to find species that are harder to detect. The two expected newt species (*I. alpestris* and *L. vulgaris*) do not call to attract females (Thiesmeier and Schulte 2010; Große 2011), which hampers localization. The use of more fish traps and additional newt-shining surveys could have helped to detect both species in higher numbers in all four peatlands.

Reptile assessment

Our study verified four reptile species in the Waldviertler peatlands, located mainly in open moorland habitats and on paths. During the reptile assessments that have been carried out at temperatures ranging from 18–27 °C, the most frequently observed reptile species was *Z. vivipara* (Table 3).

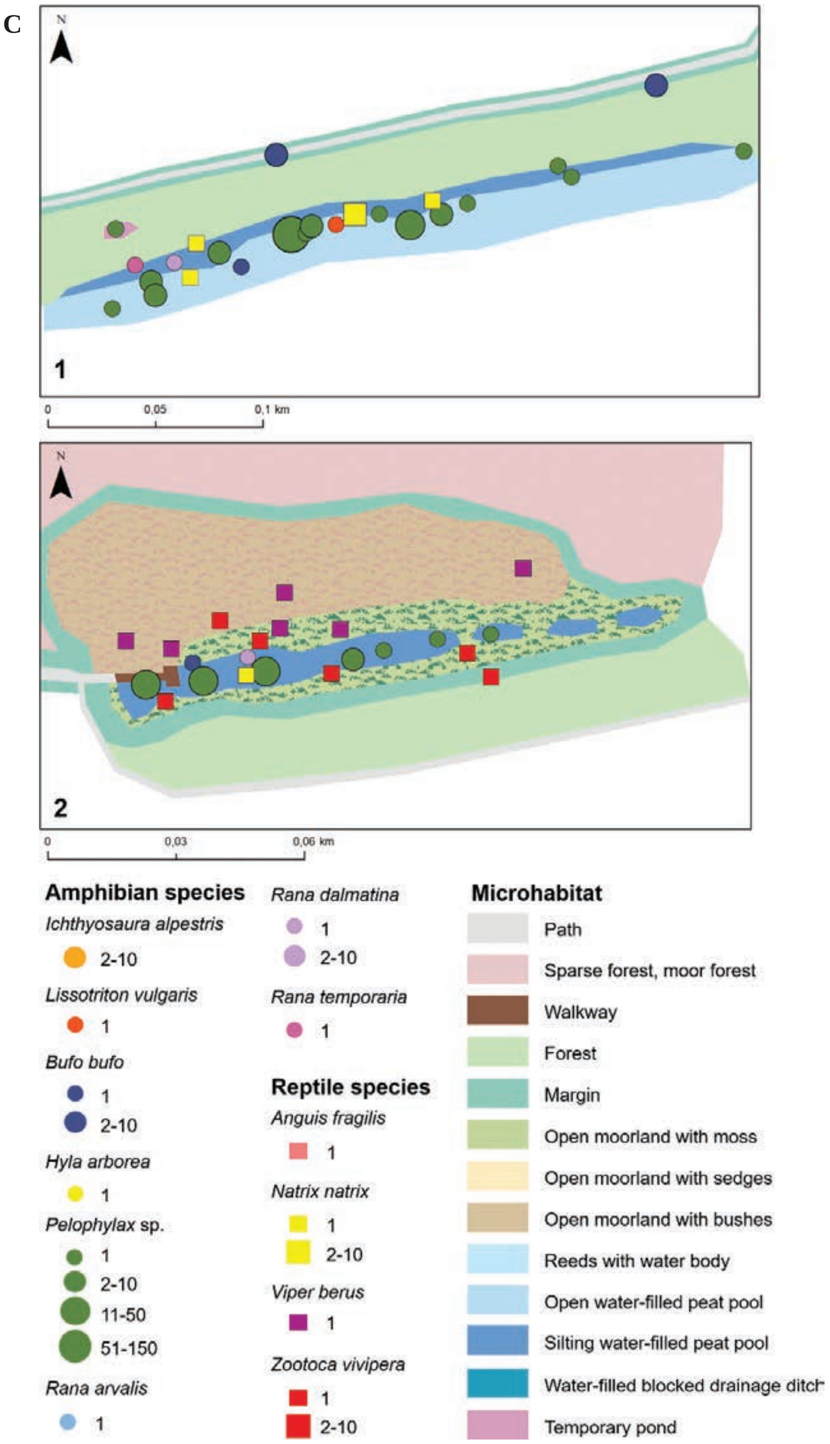

Figure 5. Continued.

We located *Z. vivipara* in all four peatlands, with the highest total number of individuals of all recorded reptiles. *Zootoca vivipara* also uses aquatic habitats, mainly to escape from enemies, and can submerge for up to 20 min (Thiesmeier 2013). On hot summer days, *Z. vivipara* relocates to wet and humid areas (e.g., dense understory) to prevent water loss through transpiration (Buschinger and Verbeek 1970). Similar to these observations, we also located most *Z. vivipara* between wet moss cushions in open moorlands.

The second most abundant species located in the study site was *N. natrix*, which we could verify in each peatland. The species is often found near water bodies, likely because *N. natrix* feeds mainly on frogs (*Pelophylax* sp., *B. bufo*, *Rana* sp.) (Filippi et al. 1996). The second snake species located in the study area was the Adder, *V. berus*. In Austria, *V. berus* occurs in the montane-alpine region and in the Bohemian Mass in the north of the country. In the latter area, *V. berus* mostly occurs in wet meadows and peatlands (Cabela et al. 2001). *Vipera berus* prefers high temperature variations between day and night, short vegetation periods, high precipitation and/or humidity, and structure-rich habitats (Völkl and Thiesmeier 2002).

In peatlands, *V. berus* needs structures that heat up quickly (Otte et al. 2020). Consistent with these findings, we mostly found *V. berus* next to dead wood, on moss pads, or between high grass in open moorlands. We did not assess all reptile species that were expected in the area. To estimate the total reptile diversity in the peatlands, further investigations are necessary.

Conclusion

This study provides a first amphibian and reptile assessment in the peatlands of the Natura 2000 area 'Waldviertler Teich-, Heide- und Moorlandschaft' in the Waldviertel region. Here, we show that peat pools, drainage ditches, and open moorlands are important habitats for amphibians and reptiles, species that are threatened all over the cultural landscape (Nöllert and Nöllert 1992). These microhabitats are uncommon in the cultural landscape of Austria, as they are created when former peat-worked peatlands are restored. In the future, peat pools and drainage ditches will silt up as a result of natural succession, which leads to the question of whether the peatland water bodies can persist in line with the aims of the peatland conservation. Clearing of drainage ditches and removal of bushes and shrubs along the ditches are effective conservation measures in drained peatlands, increasing brown frog colonization (Soomets et al. 2017; Remm et al. 2018), which at least partially could be considered in the conservation management plans of the Waldviertler region.

The conservation management in the Natura 2000 site mainly aims to rewet the peatlands, stabilize the water levels by damming the drainage ditches, and attain the natural climax stadium, which in the south of the Waldviertler region are moor forests (Ebhart 2011). The restoration of peatlands created habitats for many endangered amphibians and reptile species. Habitat loss, landscape fragmentation, over-exploitation, invasive neobiota, pollution, diseases, and climate change endanger amphibian and reptile species across the world (Gibbons et al. 2000; Stuart et al. 2004), demonstrating the importance of maintaining and creating near-natural aquatic and terrestrial habitats with low levels of disturbance to protect and preserve local amphibian and reptile fauna.

Funding

The study was financially supported by the Land Niederösterreich (TOP Stipendium to SST) and the Natural History Museum Vienna.

Author contributions

Conceptualization: SST, SS; Methodology: SST, SS; Data collection: SST, RS; Analysis: SST; Resources: SST, SS; Data Curation: SST; Writing—original draft: SST, RS, SS; Writing—review and editing: SST, RS, SS; Visualization: SST, RS, SS; Supervision: SS; Project administration: SS; Funding Acquisition: SST, SS.

Acknowledgements

We thank the Natural History Museum Vienna, especially the Herpetological Collection, for their support. We thank Walter Hödl, Axel Schmidt, Günther Wöss, and Harald Zechmeister for the valuable discussion. Further, we thank Marie-Therese Fischer, Gabriela Dangl, and Joachim Rigler for helping in the field.

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Supplementary material 1

Raw data

Authors: Susanne Stückler, Ria Sonnleitner, Silke Schweiger
Data type: xlsx

Link: https://doi.org/10.3897/herpetozoa.37.e130028.suppl1

Supplementary material 2

Description of the microhabitats defined in the Waldviertel region

Authors: Susanne Stückler, Ria Sonnleitner, Silke Schweiger
Data type: docx

Link: https://doi.org/10.3897/herpetozoa.37.e130028.suppl2

ÖGH Herpetozoa
Austrian Herpetological Society

Herpetozoa 37: 359–372 (2024)
DOI 10.3897/herpetozoa.37.e122825

Rediscovery and redescription of *Ixalus garo* Boulenger, 1919, and *Ixalus kempiae* Boulenger, 1919, with a reassessment of the taxonomic status of *Raorchestes cangyuanensis* Wu, Suwannapoom, Xu, Murphy & Che, 2019 and the description of a new species from the Garo Hills of Meghalaya

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https://zoobank.org/11B82D36-D464-429B-9C3B-D22423B8FBC6

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Academic editor: Günter Gollmann ♦ **Received** 12 March 2024 ♦ **Accepted** 19 October 2024 ♦ **Published** 18 November 2024

Abstract

Bush frogs from Garo and Khasi Hills were collected, and a thorough examination based on morphology and geographic distribution revealed that two of the populations sampled from Garo Hills during this study agree with the holotypes and descriptions of the taxa *Ixalus garo* and *I. kempiae*, described more than a century ago. The original description lacked information on several morphological characters, genetic material, and photographs of the species in life. The absence of such information posed challenges in field identification of these species. Our phylogeny shows them to be nested within the *Raorchestes* clade, and therefore, we allocate these two species to this genus and formally announce their rediscovery. We also redescribe these two species here based on additional adult vouchers and report geographic range extensions from new localities. Based on molecular and morphological analysis, we reassess the status of the recently described *Raorchestes cangyuanensis*, resulting in its placement under the subjective junior synonymy of *R. kempiae*. We also describe a morphologically distinct new species from this region. This paper bridges an important gap in the knowledge of the genus *Raorchestes* in this part of India and highlights the importance of systematic surveys in documenting and understanding amphibian diversity in the region.

Key Words

cryptic diversity, Indo-Burma hotspot, IUCN red list, Rhacophoridae, synonymy, systematics

Introduction

The rhacophorid frog genus *Raorchestes* Biju, Shouche, Dubois, Dutta & Bossuyt, 2010 represents one of the most diverse vertebrate radiations in the Indian subcontinent (Vijayakumar et al. 2016). Though much of its diversity occurs in the Western Ghats of peninsular India (Vijayakumar et al. 2014; Priti et al. 2016; Garg et al. 2021), three species also occur in the eastern parts of peninsular India (Ganesh and Arumugam 2016; Gowande et al. 2020), extending further eastwards through Northeast India and Indochina (Wu et al. 2019; Al-Razi et al. 2020; Lalronunga et al. 2021; Huang et al. 2023). Most of these Southeast Asian species belong to the *Raorchestes parvulus* group (Vijayakumar et al. 2016; Garg et al. 2021). Boulenger (1919) described two species, *Ixalus garo* and *I. kempiae*, from 'above Tura' in the erstwhile Assam (now Meghalaya). Ahl (1931) transferred *I. garo* and *I. kempiae* to the genus *Rhacophorus* Kuhl & Van Hasselt, 1822, under the subgenus *Philautus* Gistel, 1848. They were then moved to the genus *Philautus* by Bourret (1942) (also see Bossuyt and Dubois 2001). Though there have been recent reports and descriptions of *Raorchestes* species from near the Northeast Indian region (Wu et al. 2019; Al-Razi et al. 2020; Lalronunga et al. 2021), these two species have never been documented. To this day, they remain poorly known to such an extent that even their generic assignment after the partition of *Philautus* remains unclear (see Frost 2024). We collected specimens of bush frogs from the Garo Hills, whose identities and affinities are examined in this paper.

Materials and methods

Field survey

Field surveys were conducted between October 2020 and January 2022 across 13 sites in the Garo and Khasi Hills of Northeast India. Nocturnal visual encounter surveys were carried out to locate calling individuals of bush frogs. Once located, the frogs were captured, photographed, and euthanized using benzocaine gel following protocols highlighted by Torreilles et al. (2009) and fixed in 90% ethanol after extraction of liver tissues, transferred to 75% ethanol for long-term storage, and deposited in the herpetological collection facility at SACON.

Species distribution mapping

The coordinates of each sighting of individuals encountered during this study were recorded using a hand-held GARMIN 64s GPS device (WGS 84 datum). The geographic range of the new species was estimated by plotting the known occurrences of the species from the current study on a distribution map generated using QGIS 3.24.1. The area under the minimum convex hull was computed by connecting the outer most occurrence points to calculate the Extent of Occurrence (EOO) as defined by the IUCN (2001).

Morphometrics

The following measurements were recorded to the nearest 0.02 mm from the specimens with an INSIZE dial caliper: snout–vent length (SVL, from the tip of the snout to the anterior margin of the cloaca), axilla–groin distance (AG, from the posterior margin of the forelimb at its insertion point on the body to the anterior margin of the hind limb at its insertion point on the body), head length (HL, from the posterior edge of the mandible to the tip of the snout), head width (HW, the maximum width of the head at the angle of the jaws), head depth (HD, the maximum depth of the head), body width (BW, the maximum width of the body at the trunk), eye diameter (ED, the greatest horizontal diameter of the orbit), eye–nostril distance (EN, from the anterior border of the orbit to the middle of the nostril), eye–snout distance (ES, from the anterior border of the orbit to the tip of the snout), tympanum diameter (TYD, greatest diameter of the tympanum), upper eyelid width (UEW, the maximum width of the upper eyelid), interorbital distance (IO, distance between the upper eyelids), internarial distance (IN, distance between the nostrils), upper arm length (UAL, from the axilla to elbow), lower arm length (LAL, from the posterior margin of the elbow to the base of the outer metacarpal tubercle), palm length (PAL, from the posterior border of the outer metacarpal tubercle to tip of the 3rd finger), femur length (FEL, from the cloaca to the knee), tibia length (TBL, from knee to heel), foot length (FOL, from inner metatarsal tubercle to the tip of the 4th toe). Webbing formulae follow Savage and Heyer (1997).

We compare the newly collected specimens from this study with the following congeners from the Indo-China region: *Raorchestes annandalii* (Boulenger, 1906); *Raorchestes yadongensis* Zhang, Shu, Liu, Dong & Guo, 2022; *Raorchestes shillongensis* (Pillai & Chanda, 1973); *Raorchestes rezakhani* Al-Razi, Maria & Muzaffar, 2020; *Raorchestes cangyuanensis* Wu, Suwannapoom, Xu, Murphy & Che, 2019; *Raorchestes longchuanensis* (Yang & Li, 1978); *Raorchestes dulongensis* Wu, Liu, Gao, Wang, Li, Zhou, Yuan & Che, 2021; *Raorchestes hillisi* Jiang Ren, Guo, Wang & Li, 2020; *Raorchestes parvulus* (Boulenger, 1893); *Raorchestes menglaensis* (Kou, 1990); *Raorchestes huanglianshan* Jiang, Wang, Ren & Li, 2020; *Raorchestes malipoensis* Huang, Liu, Du, Bernstein, Liu, Yang, Yu & Wu, 2023; *Raorchestes hekouensis* Du, Xu, Liu & Yu, 2024. Specimens of congeners were examined from museum collections when available and supplemented with additional morphological data from the following literature: Zhang et al. (2022); Boruah et al. (2018); Al-Razi et al. (2020); Wu et al. (2019); Jiang et al. 2020; Yu et al. 2019; Du et al. (2024).

Molecular methods

Total genomic DNA was extracted from three specimens of *Raorchestes* spp. (SACON VA 806, VA 809, VA 805) with a QIAGEN DNA extraction and purification kit, following the manufacturer's protocols. One mitochondrial 16s rRNA gene was amplified using the primers 16sAR-L (5'-CGCCTGTTTATCAAAAACAT-3') and 16sB R-H (5'-CCGGTCTGAACTCAGATCACGT-3'), respectively (Kocher et al. 1989). Amplifications were performed in an Applied Bio Systems Veriti 96-well thermal cycler: 20 µl reactions with 4 µl of 5X Phusion HF buffer, 0.4 µl of 10 mM dNTP, 0.2 µl of Phusion DNA Polymerase, 0.1 µl each of forward and reverse primers, 2.0 µl of DNA template, and 13.2 µl of nuclease-free water with the following procedure: initial denaturation of DNA at 95 °C for 5 min, 35 cycles of denaturation at 95 °C for 1 m, annealing at 55 °C for 1 min, extension at 72 °C for 1 m, and at last, final extension at 72 °C for 10 min. The amplicon was checked by running it through an agarose gel electrophoresis for a clear band of the desired region in the amplified PCR product. The amplified PCR product was purified and sequenced commercially. The forward and reverse sequences thus obtained were aligned along with seventeen other sequences of *Raorchestes* of the *R. bombayensis* and *R. parvulus* groups (*sensu* Vijayakumar et al. 2014), which were selected based on high similarity in the BLAST search, and two species each of the genera *Philautus* and *Pseudophilautus*, with *Nasutixalus jerdonii* as the outgroup taxon. The sequences were edited manually by deleting sites that corresponded to gaps, if any, and aligned with Muscle in MEGA 6.0 (Tamura et al. 2013). This alignment was exported in FASTA and MEGA formats and was then used to determine uncorrected pairwise genetic distances between the samples with MEGA 6. The alignment was then subjected to a maximum likelihood phylogenetic analysis with RAxML GUI 2.0 using the general time reversible model GTR GAMMA (as RAxML uses only the general time reversible (GTR) model of sequence evolution) with 500 bootstrap replicates. The tree file thus generated was then visualized using Fig Tree v. 1.4.0.

Results

Three distinct forms were identified based on morphological and molecular analysis. Our molecular as well as morphological analyses clearly revealed that the collected specimens belong to the genus *Raorchestes* (Fig. 1, Table 1), with one of the species (SACON VA 806) matching the morphological characteristics of *I. kempiae* while also being genetically close to *R. cangyuanensis* with high support (ML BS: 100) and low genetic divergence (0.46% on the 16S rRNA gene, which falls within the typical range of intraspecific divergence rates in members of this genus (Vijayakumar et al. 2014; Yu et al. 2019). The other population, SACON VA 809 from the high elevation zone of the Garo Hills (Sakalgre, ~1000 m asl.), was found to match the morphological characteristics of *I. garo*, and the molecular analysis showed that it was allied to *R. shillongensis* of the Khasi Hills, Meghalaya, albeit with a poor bootstrap support (51). The third identified entity (SACON VA 805), clustered together with two other samples of *Raorchestes* used in this study with a high bootstrap support (ML BS 100). The following section deals with the systematics of the identified entities.

Table 1. Uncorrected 16s pairwise genetic divergences between the Rhacophoridae species studied.

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**22**	**23**	**24**
1																											
2	9.19																										
3	6.00	7.31																									
4	0.69	9.47	6.25																								
5	0.69	9.47	6.25	0.00																							
6	5.80	8.15	5.01	6.06	6.06																						
7	6.04	5.78	4.28	6.30	6.30	5.05																					
8	9.19	0.23	7.56	9.47	9.47	8.15	5.78																				
9	9.19	0.23	7.56	9.47	9.47	8.15	5.78	0.00																			
10	7.06	5.79	6.75	7.32	7.32	6.81	4.51	5.79	5.79																		
11	6.53	5.77	5.02	6.79	6.79	6.55	4.50	5.77	5.77	5.21																	
12	6.05	5.28	5.52	6.31	6.31	5.06	3.06	5.28	5.28	3.76	4.24																
13	6.53	5.77	5.02	6.79	6.79	6.55	4.50	5.77	5.77	5.21	0.00	4.24															
14	6.51	6.23	6.23	6.77	6.77	6.51	4.23	6.23	6.23	4.74	4.71	3.75	4.71														
15	9.38	7.56	6.78	9.65	9.65	8.05	5.49	7.56	7.56	6.76	5.24	6.24	5.24	6.94													
16	7.06	6.05	6.00	7.33	7.33	6.05	4.02	6.05	6.05	5.50	5.00	4.03	5.00	3.99	6.73												
17	7.29	7.59	6.78	7.55	7.55	7.33	5.75	7.59	7.59	6.26	5.23	5.02	5.23	5.48	8.29	6.53											
18	9.07	6.50	6.26	9.34	9.34	7.78	4.74	6.50	6.50	7.22	5.48	4.97	5.48	5.67	4.00	5.70	6.50										
19	9.39	7.07	6.27	9.67	9.67	7.53	5.99	7.07	7.07	6.51	5.76	6.25	5.76	6.46	1.39	6.74	7.80	4.01									
20	10.8	7.86	8.34	11.0	11.0	10.2	7.53	7.86	7.86	7.25	6.71	7.75	6.71	7.25	10.4	7.51	9.09	9.29	9.87								
21	8.03	7.05	6.78	8.30	8.30	7.30	4.75	7.05	7.05	5.76	5.47	4.26	5.47	4.23	7.25	5.74	3.03	4.50	6.76	8.06							
22	9.45	0.46	7.81	9.73	9.73	8.41	6.03	0.23	0.23	6.04	6.02	5.52	6.02	6.47	7.81	6.29	7.84	6.75	7.32	8.12	7.30						
23	7.52	5.25	6.73	7.78	7.78	7.01	4.23	5.25	5.25	4.97	5.45	4.47	5.45	4.70	6.95	5.48	6.72	6.18	6.47	6.97	5.71	5.50					
24	13.2	14.7	12.7	13.2	13.2	12.1	11.5	14.7	14.7	13.1	11.7	11.0	11.7	11.5	11.6	11.5	11.0	11.5	12.8	14.5	10.9	15.0	13.3				
25	18.4	15.4	18.8	18.8	18.8	17.2	16.2	15.4	15.4	17.8	16.2	17.2	16.2	15.3	16.9	16.5	16.3	17.6	16.4	16.2	15.6	15.7	17.9	13.5			
26	13.2	13.2	12.3	13.5	13.5	14.0	12.5	13.2	13.2	13.1	12.0	12.6	12.0	11.4	14.5	12.5	12.0	13.3	14.2	14.7	13.6	12.9	11.9	14.8	20.4		
27	16.4	16.3	16.7	16.7	16.7	17.3	15.1	16.3	16.3	16.1	16.9	16.4	16.9	15.7	16.6	15.6	15.4	16.0	17.2	17.2	14.3	16.6	15.4	17.7	18.6	11.9	
28	18.9	17.9	16.7	19.3	19.3	18.6	15.7	17.9	17.9	16.3	17.5	16.2	17.5	19.0	18.2	16.8	18.4	18.4	18.8	16.2	17.1	18.2	17.1	18.3	20.0	16.1	16.7

1 = VA-805 *Raorchestes asakgrensis* sp. nov.; 2 = VA 806 *Raorchestes kempiae* comb nov.; 3 = VA 809 *Raorchestes garo* comb nov.; 4 = MN524577.1 *Raorchestes* sp.; 5 = JX092712.1 *Raorchestes* sp.; 6 = MG980283.1 *Raorchestes shillongensis*; 7 = MN475871.1 *Raorchestes longchuanensis*; 8 = MW98630.1 *Raorchestes longchuanensis*; 9 = MN193413.1 *Raorchestes longchuanensis*; 10 = MN072374.1 *Raorchestes rezakhani*; 11 = KF366385.1 *Raorchestes ghatei*; 12 = MN475869.1 *Raorchestes gryllus*; 13 = JX092687.1 *Raorchestes* sp.; 14 = MT488411.1 *Raorchestes hillisi*; 15 = MW019900.1 *Raorchestes menglaensis*; 16 = MW537816.1 *Raorchestes dulongensis*; 17 = KP137388.1 *Raorchestes tuberohumerus*; 18 = MW019901.1 *Raorchestes parvulus*; 19 = MT488414.1 *Raorchestes huanglianshan*; 20 = MG980285.1 *Raorchestes* sp.; 21 = MK188865.1 *Raorchestes sanctisilvaticus*; 22 = MN475866.1 *Raorchestes cangyuanensis*; 23 = MT983169.1 *Raorchestes annandalii*; 24 = MH789426.1 *Pseudophilautus schmarda*; 25 = KP939071.1 *Pseudophilautus kani*; 26 = MW356660.1 *Philautus nepenthophilus*; 27 = KT445970.1 *Philautus nephophilus*; 28 = KU170003.1 *Nasutixalus jerdonii*.

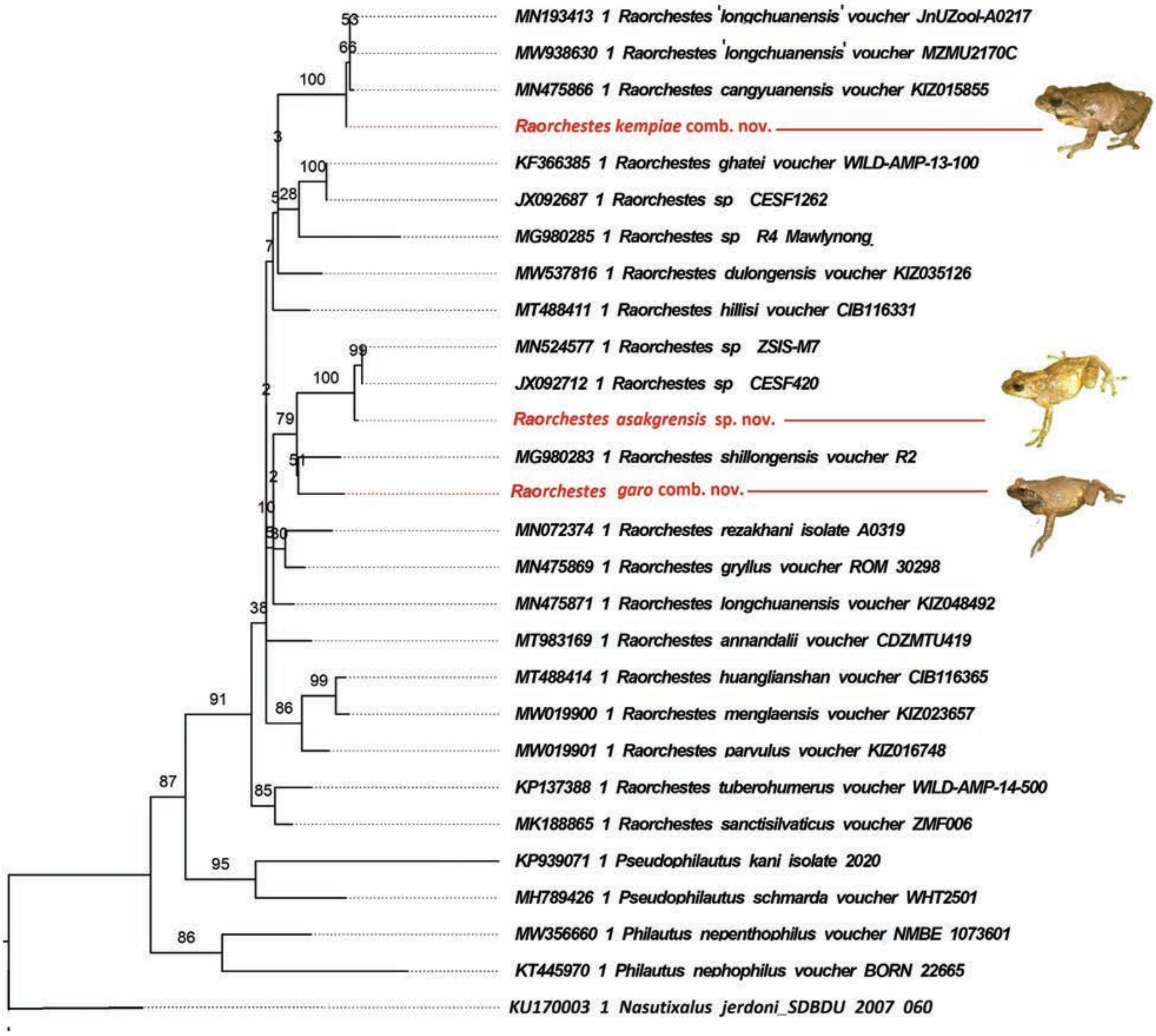

Figure 1. Maximum likelihood phylogenetic tree based on the 16S rRNA gene of select rhacophorid species, showing the phylogenetic positions of the *Raorchestes* spp. studied. Numbers on nodes represent bootstrap support values.

Systematics

Raorchestes garo (Boulenger, 1919), comb. nov.

Ixalus garo Boulenger 1919
Rhacophorus (*Philautus*) *garo* – Ahl 1931
Philautus garo – Bourret (1942)

Material studied. ***Holotype*:** ZSI 19187 (Fig. 2 above) sub adult, collected by S.W. Kemp, from "Above Tura".

Topotypes: SACON VA 809 (Fig. 2 below), 163, 305, 306, 308 adult males, collected by RSN on April 26th, 2022 from (25.51°N, 90.38°E, 895 m), and SACON VA 129, an adult female, on June 23rd, 2020 from Daribokgre Community Reserve (25.47297°N, 90.3148°E, 1200 m), East Garo Hills, Meghalaya, India.

Diagnosis. *Raorchestes garo* comb nov. is placed in the genus *Raorchestes* due to the combination of following characters: small body size, vomerine teeth absent, single translucent external subgular vocal sac present, and tips of all fingers and toes expanded into discs with circum-marginal grooves (see Biju et al. 2010). The species can be distinguished from congeners in the Indo-China region by the following combination of characters: A medium-sized (mean SVL 20.92 mm) arboreal species with a truncated snout scarcely projecting beyond the mandible; tympanum small, fairly distinct, ovoid, and supratympanic fold evident; tongue without papilla; ventral aspect of the hind limb dark orange in color; finger and toe discs well developed; tips of all fingers and toes brown to yellowish orange; toe webbing I1-1II1-1III3-3IV3-2.5V. Phylogenetically, *R. garo* clusters with *R. shillongensis* with a low bootstrap support (ML BS: 51) and shows the lowest genetic divergence from *R. longchuanensis* (4.28%).

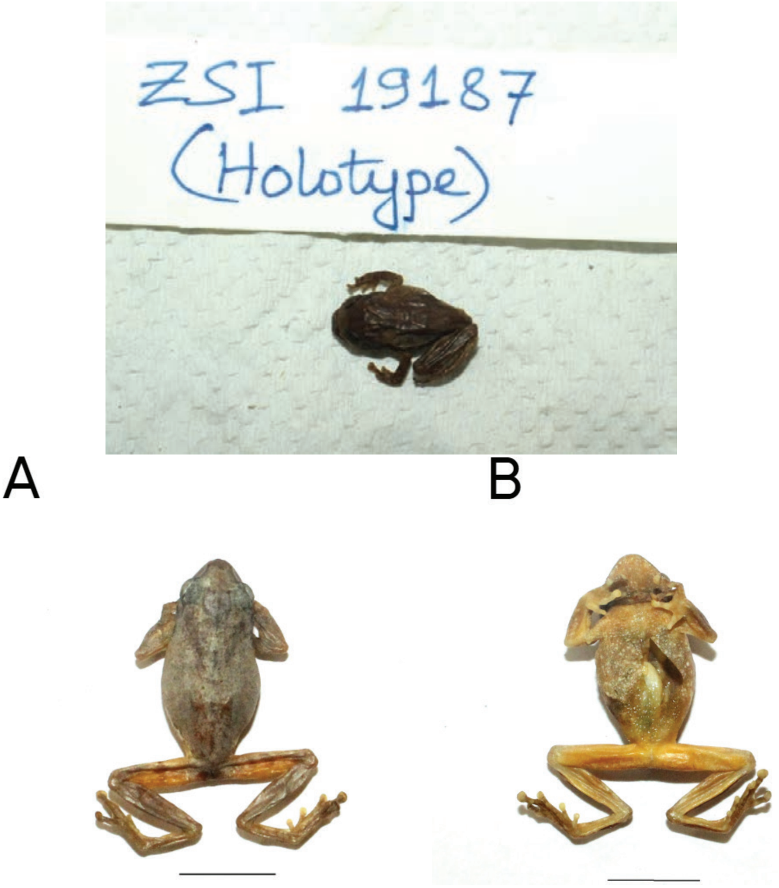

Figure 2. The holotype of *Ixalus garo* (ZSI 19187) (above) (not to scale); SACON VA 809 in preservation (below); **A.** Dorsal view; and **B.** Ventral view. Scale bars: 10 mm.

Description of a topotype. SACON VA 809 Medium-sized adult male (SVL 23.8 mm), dorsal skin mostly smooth with a few irregularly scattered spinules and lateral skin completely smooth, ventral skin slightly granulose, increasingly granulose towards the lower body. Head large (HL:SVL 0.30), wider than long (HL:HW 0.86). A short snout (ED:ES 0.95), sharply pointed in dorsal view. Canthus rostralis evident and rounded; loreal region slightly concave. Trunk short (AG:SVL 0.54) and stout (AG:BW 1.20); eyes large (ED:HL 0.43); nostrils, rounded, with no visible rim, situated almost midway between the snout and the eye (EN:ES 0.58), directed laterally; with a weakly developed rim around them. Tympanum distinct, ovoid (TYD 1.36). Inter-orbital space broader than inter-narial space (IO:IN 1.59). Upper arms short (UAL:SVL 0.20), shorter than lower arms (UAL:LAL 0.75); palm shorter than the upper arms (UAL:PAL 0.74); relative finger lengths III>IV>II>I. Subarticular tubercles distinct in both palmar and plantar surfaces, rounded; no webbing between fingers; fingers with well-developed ovoid-shaped discs. Thighs short, nearly half as long as the body (FEL:SVL 0.46), nearly as long as the tibia (FEL:TBL 0.97). Foot slightly shorter than the thigh (FEL:FOL 1.29), toes short, toe discs well developed and ovoid, smaller than finger discs, toes with partial webbing, webbing formula I1-1II1-1III3-3IV3- 2.5V. Dorsum uniformly brown with yellowish white spinules; loreal region dark brown; iris golden brown. Venter white, speckled with black and gray spots, and lower trunk grayish brown. Groin orange (bordered by golden yellow) turning red as it reaches the thighs (Fig. 3).

Variation. Morphometric measurements of the holotypes and other preserved topotypes examined are presented in Table 2. Variations noted during examination of preserved specimens and live individuals observed in the field. The single female specimen collected during this study is larger (SVL 26.1 mm) than all the males (range 15.58–23.8 mm; mean SVL 20.92 mm). Dorsal coloration variable from light to reddish or dark brown. Some individuals with an hourglass pattern, intensity of the dorsal hourglass pattern variable from feeble to bold and black (usually absent in males during the breeding season). Some individuals with a darker hourglass pattern and light background (in VA 305), and some with lighter hourglass pattern and darker background (in VA 308). Bands on the limbs feeble to bold. Ventral coloration uniform in all individuals.

Comparisons. *Raorchestes garo* is genetically divergent (4.28–8.34% on the 16S rRNA gene) from congeners; the least divergent species is *R. longchuanensis*, with a divergence of 4.28%. However, on the phylogenetic tree, it clusters with *R. shillongensis*,

Figure 3. *Raorchestes garo* (dorsolateral view of SACON VA 809, on top and dorsal view of SACON VA 809, bottom left and dorsal view of VA 305, adult male bottom right, showing hourglass pattern) from Garo Hills in life.

Table 2. Morphometric measurements (in mm) of *Raorchestes garo* (abbreviations explained in Material and methods).

Voucher no:	Sex	SVL	AG	BW	HL	HW	HD	ED	EN	ES	TYD	ET	UEW	IO	IN	UAL	LAL	PAL	FEL	TBL	TRL	FOL
Holotype (ZSI 19187)	NA	12.6	5.82	5.3	2.62	5.92	3.5	2	1.2	2.4	0.3	0.4	0.72	1.7	1.32	2.62	2.74	3.45	7.26	7.24	3.74	4.52
SACON VA-809	M	23.8	12.92	10.8	7.18	8.34	4.36	3.12	1.92	3.3	1.36	0.7	2.28	3.56	2.24	4.66	6.2	6.32	10.98	11.34	7.3	8.54
SACON VA 163	M	21.38	9.72	8.02	7.22	8.24	4.26	3.04	2.12	3.46	1.44	0.3	1.68	3.08	2.3	4.28	6.1	5.38	9.82	11.1	6.36	7.24
SACON VA-305	M	22.16	11.4	10.54	7.76	8.5	4.98	3.1	1.4	3.58	1	0.32	1.76	2.76	2.06	4.62	5.44	6.54	10.34	11.36	6.8	9.06
SACON VA-306	M	15.58	7.8	7.02	4.7	5.2	3.92	2.3	1.3	2.34	0.82	0	1.42	2.06	1.5	2.8	3.68	3.5	7.2	7.96	4.42	5.46
SACON VA-308	M	21.66	10.98	8.4	6.6	7.8	3.98	2.94	1.4	3.06	1.4	0	1.68	2.76	2.24	3.44	5.3	6.1	9.8	10	5.5	7.26
SACON VA 129	F	26.1	13.2	11.9	8.62	8.46	4.9	3.5	1.6	4.02	1.7	0.86	2.46	4.8	2.48	4.32	5.02	5.8	11.84	12.42	7.12	8.58

with a low node support (51). From congeners in the Indo-China region, it can be easily distinguished by: having an externally visible tympanum (absent in *R. kempiae*, *R. shillongensis*, *R. menglaensis* and *R. rezakhani*); ventral aspect of the hind limb dark orange in color (vs. uniform creamy white in *R. kempiae* and dark gray in *R. rezakhani*); toe webbing I1-1II1-1III3-3IV3-2.5V in *R. garo* (vs. I1-2II2-1III1-1IV1-2V in *R. kempiae*, I 2–2 II 2–2 III 2–3 IV 3–2 V in *R. malipoensis*, II 1–2 III 1–2.5 IV 2.5–1 V in *R. menglaensis*, II 1–2 III 1–2.5 IV 2.5–1 V in *R. hillisi*, II 1–2 III 1–2 – IV 2–1 V in *R. huanglianshan* and I2–2 II 1.75–2 III 1.5–3 IV 2.75–2 V in *R. rezakhani*); snout short and truncate in *R. garo* (vs. rounded in *R. kempiae*, *R. menglaensis*, *R. hillisi*, *R. hekouensis*, *R. malipoensis*, *R. longchuanensis* and *R. huanglianshan* and sharply projecting in *R. annandalii* and *R. yadongensis*).

Natural history and distribution. These frogs can be heard calling at dusk. Males were seen to be actively vocalizing from April to September. Most individuals recorded during this study were observed to be calling from shrubs (usually higher > 2.5 m above the ground). The species was infrequently encountered and was only recorded from areas above 800 m asl during this study. This species was recorded from three different localities in and around the West Garo Hills region: Sakalgre (25.51°N, 90.38°E; 895 m); Daribokgre (25.47°N, 90.31°E; 1200 m); and Mandalgre (25.48°N, 90.41°E; 1081 m). Based on the locations from the study, we calculated the extent of occurrence of this species with the current data from this study, and it was estimated to be 11.46 km^2. However, further surveys are required to robustly estimate the distribution range of this species.

Remarks. Boulenger (1919) described this species from the Garo Hills along with *Bufoides kempi* and *R. kempiae*. Our topotypical specimens are in agreement with the holotype and with Boulenger's description in most characters. Kharkongor et al. (2016) reported a population of *Raorchestes* from Upper Shillong with detailed morphological description as *Philautus garo*. Although their descriptions of these specimens closely match the description of *R. garo*, since these are not topotypical populations, i.e., the collection locations occur almost 200 km (aerial distance) from the type locality and from a different elevational range, further studies based on molecular analysis are required to see if this population is conspecific with *Raorchestes garo*.

Raorchestes kempiae (Boulenger, 1919), comb. nov.

Ixalus kempiae Boulenger, 1919
Rhacophorus (*Philautus*) *kempiae* – Ahl, 1931
Philautus kempiae – Bourret (1942)
Raorchestes cangyuanensis – Wu, Suwannapoom, Xu, Murphy & Che, 2019, syn. nov.

Material studied. ***Holotype*:** ZSI 18859 (Fig. 4 above); sub adult, collected by Agnes Kemp, from "Above Tura".

***Topotypes*:** Five adult males, SACON VA 806 (Fig. 4 below), 301, 302, 297, 299 from Mikadogre Community Reserve (25.433660°N, 90.398981°E, 174 m), South Garo Hills, Meghalaya, India, collected by RSN on January 23rd 2021.

Diagnosis. *Raorchestes kempiae* comb nov. is placed in the genus *Raorchestes* due to the combination of following characters: small body size, vomerine teeth absent, single translucent external subgular vocal sac present, and tips of all fingers and toes expanded into discs with circum-marginal grooves (see Biju et al. 2010). The species can be distinguished from congeners in the Indo-China region by the following combination of characters: A small-sized (mean SVL 24.1 mm in adult males) arboreal species with a rounded snout; tympanum indistinct and supratympanic fold prominent; vomerine teeth absent; single semi-transparent gular vocal sac; tongue without papilla; ventral aspect of the hind limb and groin yellow with black or brown spots; finger and toe discs well developed; toe webbing I1-2II2-1III1-1IV1-2V.

Description (based on an adult male SACON VA 806). Small-sized frog (SVL 23.8 mm), with robust body. Dorsal skin mostly smooth with a few scattered spinules, ventral skin slightly granulose. Head broader than long (HL:HW 0.83); large (HL:SVL 0.33); with a short-rounded snout (ED:ES 0.72). Canthus rostralis evident and rounded; loreal region concave. Trunk short (AG:SVL 0.41) and stout (AG:BW 1.02); eyes large (ED:HL 0.38); nostrils, ovoid, with no visible rim, situated closer to the snout tip than to the eyes (EN:ES 0.61), directed laterally. Inter-orbital space broader than inter-narial space (IO:IN 0.69). Tympanum indistinct. Upper arms short (UAL:SVL 0.28), shorter than lower arms (UAL:LAL 0.57); palm slightly shorter than the upper arms (UAL:PAL 0.54); relative finger lengths III>IV>II>I; Subarticular tubercles distinct in both palmar and plantar surfaces, rounded; Supernumerary tubercles present in the palm; no webbing between fingers; fingers with well-developed rounded discs. Thighs short, nearly half as long as the body (FEL:SVL 0.32), slightly shorter than the tibia (FEL:TBL 0.83). Foot shorter than the thigh (FEL:FOL 2.83), toes short with well-developed rounded discs, partial webbing, webbing formula I1-2II2-1III1-1IV1-2V. Dorsum uniformly brown with a faint hourglass pattern. Loreals pale brown, iris dark golden brown. Venter granular, pale gray, spotted with gray and brown in lower trunk and thighs. Lateral aspect of the hind limb and groin orangish yellow with black and brown blotches (Fig. 5).

Variation. Morphometric measurements of the holotypes and other preserved topotypes examined are presented in Table 3. Variations noted during examination of preserved specimens and live individuals observed in the field. Dorsal coloration ranging from light brown to brownish black with or without an ")(" pattern (present in VA 301 and VA 302). Brown or black stripes over the thighs and tibia. Some individuals have a bright yellow to white band across the interorbital region (Fig. 5).

Natural history and distribution. Males were usually observed to start calling at dusk from bamboo bushes. The species was recorded from Mikadogre Community Reserve (25.434°N, 90.399°E, 174 m) and Bollonggre village (25.489°N, 89.995°E, 150 m) from South Garo Hills and Nengmandalgre Fish Sanctuary (25.494°N, 90.548°E, 410 m) from East Garo Hills. Based on these locations and the locality provided by Wu et al. (2019), we estimated the extent of occurrence of this species to be 242,491 km². We observed it to be relatively abundant across an elevational gradient of 100 m asl to 1200 m asl. Therefore, we recommend that this species be downgraded from Critically Endangered to the least concern category.

Remarks. Wu et al. (2019) described *Raorchestes cangyuanensis* from Yunnan Province, China. However, they did not compare this species with the senior taxon *Ixalus kempiae*, likely because *I. kempiae* had remained poorly known since its description and there had been no literature available on this species based on new sightings since it was first described. It is also unusual for frogs of the genus *Raorchestes* to have such wide distribution ranges (see Vijayakumar et al. 2014). We conclude that *Raorchestes cangyuanensis* is a junior synonym of *Raorchestes kempiae* based on molecular and morphological evidence (see Tables 1, 3). In addition to this, Gouda et al. (2023) regarded *Raorchestes cangyuanensis* as a subjective junior synonym of *R. manipurensis* (Mathew & Sen, 2009). Our analysis also revealed that specimens referable to both these names actually show characters that are diagnostic of *Raorchestes kempiae* (Boulenger 1919), a species described much earlier but overlooked until now due to improper and deficient comparisons. However, since we did not have molecular data or access to type specimens of

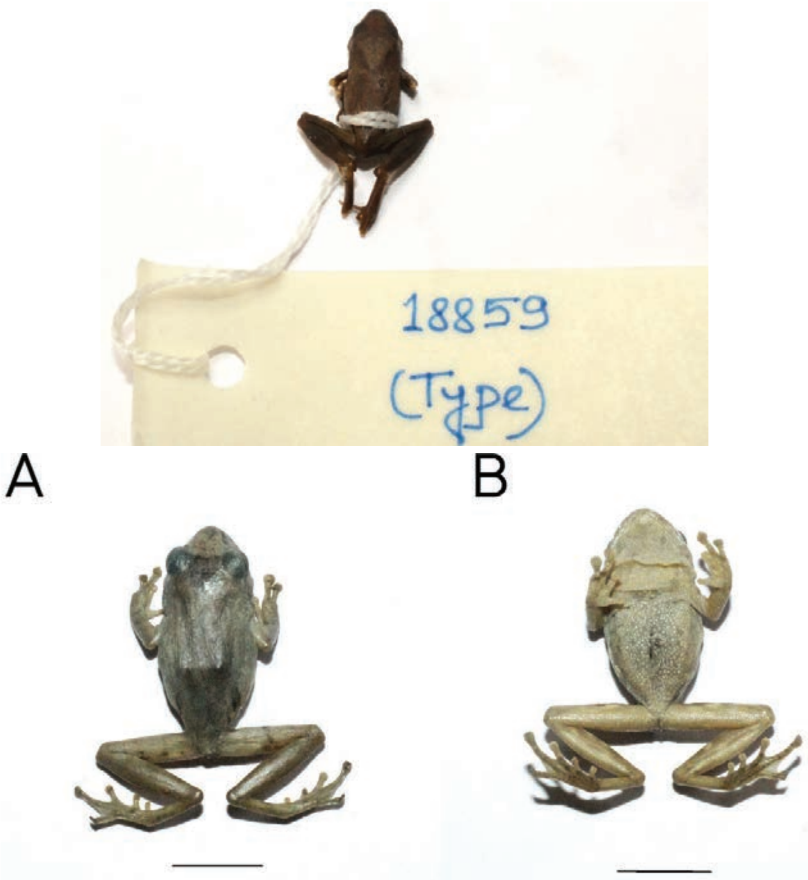

Figure 4. The holotype of *Ixalus kempiae* (ZSI 18859) - not to scale (above); SACON VA 806 in preservation (below). **A.** Dorsal view; and **B.** Ventral view. Scale bars: 10 mm.

Figure 5. *Raorchestes kempiae* (dorso-lateral view of SACON VA 806 on top and dorso-lateral view of an uncollected male, bottom) from Garo Hills in life.

Table 3. Morphometric measurements (in mm) of *R. kempiae*.

Voucher no:	Sex	SVL	AG	BW	HL	HW	HD	ED	EN	ES	TYD	ET	UEW	IO	IN	UAL	LAL	PAL	FEL	TBL	TRL	FOL
Holotype (ZSI 18859)	NA	16.3	6.02	6.14	5.22	5.76	3.14	2.44	1.7	2.04	0	0	1.4	1.7	1.4	2.42	2.82	2.46	6.7	7.92	5	U/N
SACON VA-806	Male	23.8	9.7	9.52	7.76	9.36	5.34	2.96	2.5	4.1	0	0	2.12	4.5	6.5	6.58	11.42	12.16	7.7	9.26	2.68	2.72
SACON VA-301	Male	21.9	7.94	9.82	6.42	8.58	4.3	3.24	1.88	3.52	0	0	1.88	3.8	4.9	5.78	9.9	10.86	6.5	9.2	2.3	2.92
SACON VA-302	Male	25	11.92	11.58	7.78	9.6	4.96	3.72	1.66	3.6	0	0	2.9	4.8	5.44	6.2	12.54	12.7	7.18	9.44	2.14	3.32
SACON VA-297	Male	25.2	13	11.66	8.14	9.56	5.98	4.16	1.92	3.86	0	0	2.6	3.94	5.26	6.12	12.2	13.12	7.4	10.26	2.24	3.32
SACON VA-299	Male	24.6	11.52	10.6	7.52	10.68	4.76	3.62	1.2	3.34	0	0	2.2	4.6	6.02	7.6	12.48	12.72	7.1	9.82	2.2	3.00

R. manipurensis, we do not address this in the current work and recommend that resolving this taxonomic uncertainty should be a priority for further study.

Comparisons. *Raorchestes kempiae* is genetically divergent (5.25–9.47% on the 16S rRNA gene) from congeners, with the least divergent species being Western Ghats endemic *R. ghatei*, with a divergence of 5.77%. From congeners in this region, it can be easily distinguished by: absence of an externally visible tympanum (vs. present in *R. garo*, *R. hekouensis*, *R. malipoensis*, *R. parvulus*, *R. dulongensis*, *R. hillisi*, *R. huanglianshan*); ventral aspect of the hind limb uniform creamy white (vs. dark gray in *R. rezakhani*); ventral surface mostly smooth with slight granulation (vs. granulated and well developed whitish tubercles in *R. hekouensis*); toe webbing I1-2II2-1III1-1IV1-2V in *R. kempiae* (vs. I1-1II1-1III3-3IV3-2.5V in *R. garo*, I 2–2 II 2–2 III 2–3 IV 3–2 V in *R. malipoensis*, II 1–2 III 1–2.5 IV 2.5–1 V in *R. menglaensis*, II 1–2 III 1–2.5 IV 2.5–1 V in *R. hillisi*, II 1–2 III 1–2–IV 2–1 V in *R. huanglianshan* and I2–2 II 1.75–2 III 1.5–3 IV 2.75–2 V in *R. rezakhani*); snout rounded in *R. kempiae* (vs. pointed in *R. annandalii* and short and truncate in *R. garo*).

Raorchestes asakgrensis Naveen, Chandramouli & Babu, sp. nov.

https://zoobank.org/3AC56A6A-DBAE-4F49-9B8C-D425E38E0B79

Material studied. ***Holotype*:** An adult female, SACON VA 805 (Fig. 6) from Eman Asakgre Community Reserve (25.36788°N, 90.54344°E, 174 m), Meghalaya, India, collected by RSN on August 15th, 2023.

***Paratypes*:** Three adult males, SACON VA 339, 340, and VA 343, from Eman Asakgre Community Reserve (25.36788°N, 90.54344°E, 174 m), Meghalaya, India, collected by RSN on August 15th, 2023.

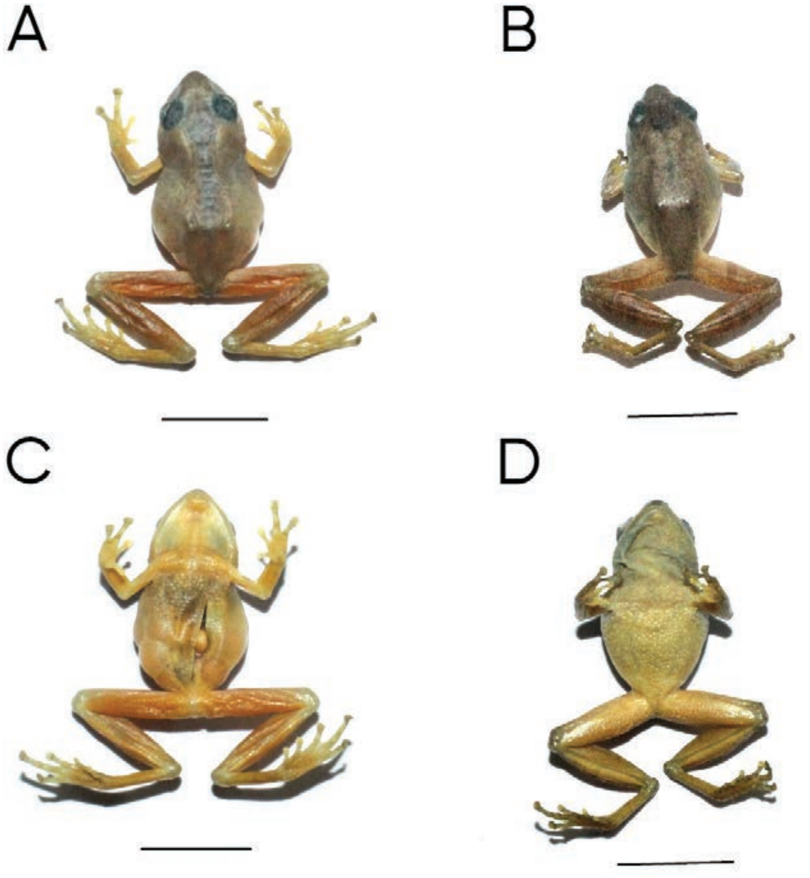

Figure 6. Adult female holotype (SACON VA 805) and adult male paratype (SACON VA 343) of *Raorchestes asakgrensis* sp. nov. in preservative, **A.** dorsal; **C.** ventral of holotype, and **B.** dorsal; **D.** ventral of paratype. Scale bars: 10 mm.

Diagnosis. *Raorchestes asakgrensis* sp. nov. is placed in the genus *Raorchestes* due to the combination of the following characters: small body size, vomerine teeth absent, single translucent external subgular vocal sac present, and tips of all fingers and toes expanded into discs with circum-marginal grooves (see Biju et al. 2010). The species can be distinguished from congeners in the Indo-China region by the following combination of characters: A small-sized (mean SVL 20.49 mm) arboreal species with an obtusely pointed snout; tympanum fairly visible with a visible supra-tympanic, relatively weakly developed; vomerine teeth absent; single semi-transparent gular vocal sac; tongue without papilla; ventral aspect of the hind limb uniform white as the venter; finger and toe discs well developed; toe webbing I1-2II2-2III2-1IV1-2V. Phylogenetically, *R. asakgrensis* forms a well-supported clade (BS: 100) consisting of two more Genbank sequences from 'India' (JX092712.1) and Nongkhyllem, Meghalaya (MN524578.1).

Description of the holotype. A small-sized *Raorchestes* (SVL 22.8 mm), dorsal and lateral skin smooth, venter granulated. Head wider than long (HL:HW 0.82); large (HL:SVL 0.28). A short snout (ED:ES 0.9), obtusely pointed in dorsal view. Canthus rostralis evident and rounded; loreal region concave. Trunk short (AG:SVL 0.43) and less gracile (AG:BW 0.95); eyes large (ED:HL 0.42); nostrils rounded, situated closer to the snout tip than to the eyes (EN:ES 0.53), directed laterally with no visible rim. Tympanum fairly visible, rounded in shape (TYD 1.92 mm); Supra-tympanic fold relatively weakly developed; Inter-orbital space broader than inter-narial space (IO:IN 1.14). Upper arms short (UAL:SVL 0.18), shorter than lower arms (UAL:LAL 0.79); palm shorter than the upper arms (UAL:PAL 0.75); relative finger lengths III>IV>II>I. Subarticular tubercles distinct in both palmar and plantar surfaces, rounded; Supernumerary tubercles present in the palm; no webbing between fingers; fingers with well-developed rounded discs. Thighs short, nearly half as long as the body (FEL:SVL 0.48), slightly shorter than the tibia (FEL:TBL 0.89). Foot slightly shorter than the thigh (FEL:FOL 1.12), toes, short with well-developed rounded discs and partial webbing, webbing formula I1-2II2-2III2-1IV1-2V. In life dorsum uniformly brown with a faint ")-(" mark; loreal yellowish brown; iris golden brown. Venter uniformly white, pale white eggs visible through ventral surface. Forearm and hindlimbs with dark brown bands (Fig. 7).

Variation. Measurement of the paratypes presented in Table 4. Variations noted during examination of preserved specimens and live individuals observed in the field. The single known female (Holotype VA 805) is larger (SVL 22.8 mm) than all the male specimens examined during this study (range 18.68–21.5 mm; mean SVL 19.72 mm). One of the male paratypes (VA 340) with a thin yellow line passing dorsally from the tip of the snout to the cloaca and extending through the thighs and shank (Fig. 7). Dorsal coloration pale to dark brown with or without a faint or dark hourglass pattern, ")(".

Comparisons. *Raorchestes asakgrensis* sp. nov. is genetically divergent (5.8–10.8%) from congeners, with the closest species being *R. shillongensis* with a divergence of 5.8%. On the phylogenetic tree, it clusters with a clade comprising *R. garo* and *R. shillongensis*, with a moderate node support (79). From species occurring in the Indo-China region, it can be distinguished by: having an obtusely pointed snout (vs. truncate and scarcely projecting in *R. garo*, rounded in *R. kempiae*, *R. menglaensis*, *R. hillisi*, *R. hekouensis*, *R. malipoensis*, *R. longchuanensis*, *R. dulongensis*, and *R. huanglianshan* and sharply projecting in *R. annandalii* and *R. yadongensis*), a fairly visible tympanum (vs. not externally visible in *R. kempiae*, *R. shillongensis*, *R. rezakhani*, *R. menglaensis*, and *R. parvulus*); and the presence of supernumerary tubercles on the palmar surface (vs. absent in *R. dulongensis*, *R. yadongensis*, and *R. hekouensis*). Ventral aspect of the hind limb uniformly creamy white in color (vs. dark orange in *R. garo* and *R. annandalii*, yellow with brown to black blotches in *R. kempiae*, and pale brown with white flecks in *R. parvulus*); toe webbing formula I 1-2 II 2-2 III 2-1 IV 1-2 V (vs. I 1-1 II 1-1 III 3-3 IV 3-2.5 V in *R. garo*, I1-2II2-1III1-1IV1-2V in *R. kempiae*, I 2–2 II 2–2 III 2–3 IV 3–2 V in *R. malipoensis*, II 1–2 III 1–2.5 IV 2.5–1 V in *R. menglaensis*, II 1–2 III 1–2.5 IV 2.5–1 V in *R. hillisi*, II 1–2 III 1–2 – IV 2–1 V in *R. huanglianshan*, I2–2II1.5–3.25III2–3.5IV3.25–2V in *R. parvulus* and I2–2 II 1.75–2 III 1.5–3 IV 2.75–2 V in *R. rezakhani*).

Remarks. The new species is clearly differentiated by a distinct set of morphological characters, particularly the presence of an externally visible tympanum and snout shape (see Fig. 7). Boruah et al. (2018) reported a *Raorchestes* sp. from regions north of the Khasi Hills, and it is likely that this could be *R. asakgrenesis*, as we also recorded this species from near Nongkhyllem Wildlife Sanctuary.

Natural history and distribution. The frogs were seen calling at dusk from bushes in and around the Eman Asakgre Community Reserve. A single amplecting pair was recorded at around 19:30 h perched on a bush 1.5 m above the ground during the pre-monsoon season following the first shower in May. The sequence generated during the current study also matches closely with a sequence from Nongkhyllem (MN524577.1), foothills of Khasi hills (about 150 km away from the type locality) in Meghalaya. In addition to this, during this study this species was also recorded from various locations across Garo hills (Sasatgre, Oragitok, and Dangkipara) and low-elevation regions of Khasi hills (Umling and Nongpoh). We calculated the extent of occurrence of this species with the current data from this study, and it was estimated to be 1,625 km^2 across an elevational range of 150 m asl to 950 m asl; however, further surveys are required to robustly estimate the distribution range of this species.

Etymology. The species is named after the type locality, Eman Asakgre Community Reserve, to honor the residents who greatly supported the surveys during which the type specimens were collected.

Figure 7. *Raorchestes asakgrensis* sp. nov. holotype, SACON VA 805, an adult female dorso-lateral view in life (above); paratype, SACON VA 340, an adult male dorso-lateral view in life (below).

Table 4. Morphometric measurements (in mm) of *R. asakgrensis* sp. nov.

Voucher no:	Sex	SVL	AG	BW	HL	HW	HD	ED	EN	ES	TYD	ET	UEW	IO	IN	UAL	LAL	PAL	FEL	TBL	TRL	FOL
SACON VA 805 Holotype	F	22.8	10.02	10.5	6.4	7.72	4.5	2.7	1.6	3	1.92	0.44	1.6	3.2	2.8	4.12	5.18	5.5	11.02	12.42	7.3	9.82
SACON VA 343	M	21.5	10.74	8.14	5.82	7.6	4.68	2.6	1.42	3.18	1.18	0.52	1.84	2.98	1.98	3.82	5.08	5.2	10.64	11.64	6.98	8.3
SACON VA 340	M	18.68	8.24	7.18	5.04	7.14	4.54	2.72	1.42	2.54	0.8	0.42	1.42	2.42	1.8	3.68	4.12	5	10	10.68	6.44	7.24
SACON VA 339	M	18.98	7.92	8.68	6.34	7.02	5.14	3.12	1.44	3.02	0.92	0.52	1.44	2.34	2.4	4.94	4.12	4.22	9.3	10.54	6.6	7.24

Discussion

To date, three species of bush frogs have been discovered and described from the Garo and Khasi hills: *Ixalus garo* and *I. kempiae* by Boulenger (1919), and *Philautus shillongensis* by Pillai and Chanda (1973). Boruah et al. (2018) redescribed *Raorchestes shillongensis* and clarified that its distribution is restricted to the higher elevations of the Shillong Plateau in the Khasi Hills. However, *Ixalus garo* and *I. kempiae*, originally described over a century ago, based on only a single subadult type specimen each, have remained poorly known since then, to the extent that even their generic placement has remained unclear (see Frost 2024). Our study, based on the mitochondrial 16S rRNA gene, shows that they belong to the genus *Raorchestes*. Boulenger (1919) described *Ixalus garo* based on a single specimen from above Tura in the Garo Hills that was characterized by a distinct tympanum that was 1/3rd the diameter of the eye, fingers, and toes with discs as large as the tympanum, toes webbed at the base, grayish with an hourglass pattern on the back, and a grayish belly dotted with white. The specimens allocated to *R. garo* described above agree with the description and exhibit no morphological differences from the holotype of the species; hence, we identified them as *Raorchestes garo*. Similarly, for *Ixalus kempiae*, Boulenger (1919) characterized it based on a rounded snout, hidden tympanum, large discs on fingers and toes, toes webbed at the base, grayish above with small black spots, a black cross-band between the eyes, a ")(" shaped marking on the back, and a whitish belly spotted and marbled with brown. Our newly collected material from the near-type locality not only matches the description of *Ixalus kempiae* by Boulenger (1919) but also matches the description of *Raorchestes cangyuanensis* by Wu et al. (2021). Therefore, we place the name *Raorchestes cangyuanensis* under the subjective junior synonymy of *Raorchestes kempiae* owing to its genetic congruence and morphological similarity with our new sample (see Tables 1, 3). In addition to this, the discovery of *Raorchestes asakgrensis* sp. nov. from a low elevation site near the foothills of the Garo Hills of Meghalaya, which is differentiated from all other sympatric species by a unique set of morphological characters as well as molecular differences, highlights the importance of integrative taxonomy, which combines multiple lines of evidence to accurately classify and recognize species. This study now confirms the presence of four species of bush frogs in the genus *Raorchestes* from the Garo and Khasi Hills of Meghalaya. In addition to these, Mathew and Sen (2010) reported the presence of *Raorchestes annandalii* (Boulenger, 1906) from Meghalaya. However, this poorly known species, originally described from Kurseong, Darjeeling, is separated from Meghalaya by the Brahmaputra River (see. Fig. 8), which serves as a barrier for many species in this region (Chaitanya et al. 2024). Thus, further studies are needed to confirm the presence of this species in Meghalaya.

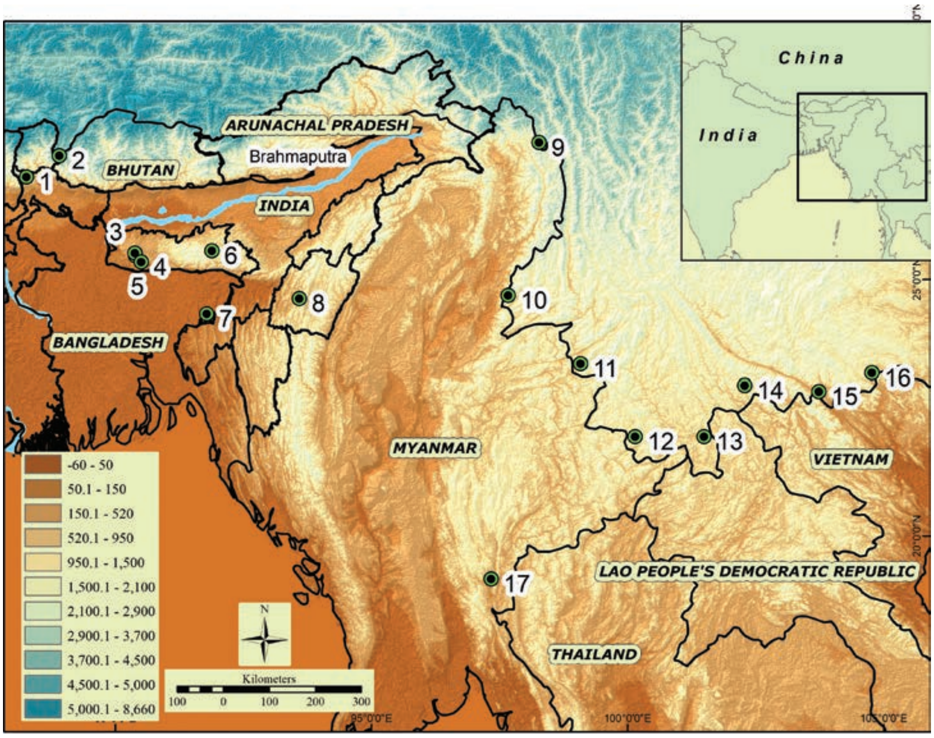

Figure 8. Map showing the type localities of *Raorchestes* species known from the Indo-China region, including 1. *R. annandalii*, 2. *R. yadongensis*, 3. *R. garo*, 4. *R. asakgrensis* sp. nov., 5. *R. kempiae*, 6. *R. shillongensis*, 7. *R. rezakhani*, 8. *R. manipurensis*, 9. *R. dulongensis*, 10. *R. longchuanensis*, 11. *R. "cangyuanensis"*, 12. *R. hillisi*, 13. *R. menglaensis*, 14. *R. huanglianshan*, 15. *R. hekouensis*, 16. *R. malipoensis*, and 17. *R. parvulus*.

Acknowledgements

This publication is partly supported by the project entitled "Characterization of Community Reserves and Assessment of their Conservation Values in Meghalaya," funded by the National Mission on Himalayan Studies (GBPNI/NMHS-2017-18/MG 32, dated: 28.03.2018) and the Stiftung Artenschutz amphibian Conservation Fund awarded to RSN. We sincerely thank the Additional Principal Chief Conservator of Forests (Wildlife) and Chief Wildlife Warden (CWLW), Meghalaya, and the officers of the Department of Forest and Environment, Government of Meghalaya, for facilitating permission from the Community Reserve Management Committees to carry out the field study (No. FWC/Research/15/603-04, dated 31 May 2019). We would like to thank Dr. Kaushik Deuti for facilitating the examination of type specimens at ZSI, Kolkata. RSN would like to express his gratitude to his field assistants, Mr. Lising Momin and Mr. Nelbin Sangma, for their invaluable support during the fieldwork. We would also like to thank the anonymous reviewers for their valuable comments that greatly improved the manuscript. We are indebted to the management committees of each community reserve for granting us permission to conduct this work.

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Appendix 1

Table A1. Genbank accession numbers of sequences used in this study.

Accession number and species	Reference
PQ512828 *Raorchestes asakgrensis* sp. nov.	This study
PQ512827 *Raorchestes kempiae* comb. nov.	This study
PQ585812 *Raorchestes garo* comb. nov.	This study
MN524577.1 *Raorchestes* sp.	Laskar et al. 2019; unpublished
JX092712.1 *Raorchestes* sp.	Vijayakumar et al. 2014
MG980283.1 *Raorchestes shillongensis*	Boruah et al. 2018; unpublished
MN475871.1 *Raorchestes longchuanensis*	Wu et al. 2019
MW938630.1 *Raorchestes longchuanensis*	Lalremsanga et al. 2021; unpublished
MN193413.1 *Raorchestes longchuanensis*	Al-Razi et al. 2020
MN072374.1 *Raorchestes rezakhani*	Al-Razi et al. 2020
KF366385.1 *Raorchestes ghatei*	Padhye et al. 2013
MN475869.1 *Raorchestes gryllus*	Wu et al. 2019
JX092687.1 *Raorchestes* sp.	Vijayakumar et al. 2014
MT488411.1 *Raorchestes hillisi*	Jiang et al. 2020
MW019900.1 *Raorchestes menglaensis*	Xu et al. 2021
MW537816.1 *Raorchestes dulongensis*	Wu et al. 2021
KP137388.1 *Raorchestes tuberohumerus*	Padhye et al. 2014; unpublished
MW019901.1 *Raorchestes parvulus*	Wu et al. 2020
MT488414.1 *Raorchestes huanglianshan*	Jiang et al. 2020
MG980285.1 *Raorchestes* sp.	Boruah et al. 2018; unpublished
MK188865.1 *Raorchestes sanctisilvaticus*	Warekar et al. 2018; unpublished
MN475866.1 *Raorchestes cangyuanensis*	Wu et al. 2019
MT983169.1 *Raorchestes annandalii*	Khatiwada 2020
MH789426.1 *Pseudophilautus schmarda*	Meegaskumbura et al. 2018
KP939071.1 *Pseudophilautus kani*	Sureshkumar and George 2015; unpublished
MW356660.1 *Philautus nepenthophilus*	Etter et al. 2021
KT445970.1 *Philautus nephophilus*	Dehling et al. 2016
KU170003.1 *Nasutixalus jerdonii*	Biju et al. 2016

ÖGH Herpetozoa
Austrian Herpetological Society

Herpetozoa 37: 373–378 (2024)
DOI 10.3897/herpetozoa.37.e136688

The advertisement call of *Leptobrachella dushanensis* (Anura, Megophryidae)

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https://zoobank.org/6B258E72-EB2E-42AE-A7B2-637ACCE1300B

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Academic editor: Günter Gollmann ♦ **Received** 9 September 2024 ♦ **Accepted** 15 November 2024 ♦ **Published** 2 December 2024

Abstract

We describe the advertisement call of the Dushan Leaf Litter Toad, *Leptobrachella dushanensis*, based on recordings obtained from three individuals from its type locality. The advertisement call of *L. dushanensis* consists of a single note with a dominant frequency of 4.84–4.91 kHz. Furthermore, we compare the differences in advertisement calls between *L. dushanensis* and its sister species, *Leptobrachella dong*. The advertisement calls of *L. dushanensis* and *L. dong* can be clearly distinguished. Our findings provide bioacoustic evidence supporting the taxonomic status of *L. dushanensis* and provide basic data for future behavioral and ecological studies on *Leptobrachella*.

Key Words

acoustic differences, bioacoustics, frogs, southern China

Introduction

The Asian leaf litter toads of the genus *Leptobrachella* Smith, 1925, are the largest clade of the family Megophryidae Bonaparte, 1950, currently comprising 105 species (Frost 2024). Species of the genus *Leptobrachella* are widely distributed, ranging from southern China, northeastern India, and Myanmar, through Thailand and Vietnam, and extending to the Malay Peninsula, Borneo, and Natuna Island (Frost 2024). During the breeding season, *Leptobrachella* species often inhabit rocks on the stream banks (Fei et al. 2005). Molecular phylogenetic frameworks proposed many cryptic species in the genus (Chen et al. 2018). In the past five years, more than 30% of new species in *Leptobrachella* have been described (Frost 2024). High levels of morphological similarity and rampant homoplasy appear to have misled estimates of diversity and evolutionary relationships (Shen et al. 2021). Addressing the aforementioned issues requires the application of integrative taxonomy, combining methods from morphology, genetics, and bioacoustics to reveal the species diversity within the genus *Leptobrachella*.

Vocalizations of anuran amphibians have received much attention in studies of behavioral ecology and species identification (Cui et al. 2012; Zhu et al. 2021; Shen et al. 2024). In Anura, nearly all species are capable of vocalization (Köhler et al. 2017). To further understand the relationship between frog behaviors and vocal communication, researchers have divided frog calls into four types: feeding calls, aggressive calls, reproductive calls, and defensive calls. Advertisement calls, courtship calls, amplectant calls, release calls, post-oviposition male release calls, and rain calls are examples of reproductive calls (Toledo et al. 2015; Köhler et al. 2017).

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Furthermore, advertisement calls exhibit substantial variation across species, making them a viable foundation for the systematic classification and identification of cryptic species (Sullivan et al. 1996).

Leptobrachella dushanensis (Anura, Megophryidae) is a newly described species in Southern China (Li et al. 2024). Information about their acoustic signals is not available in the literature. Here, we describe the advertisement call of *L. dushanensis* for the first time to understand its characteristics. Furthermore, we focus on comparing the advertisement call of *L. dushanensis* with that of its sister species, *Leptobrachella dong*. Our findings provide acoustic evidence that supports the taxonomic differentiation of *L. dushanensis* and contribute basic data for future behavioral and ecological studies on *Leptobrachella* species.

Materials and methods

Call recordings

A total of 197 calls were recorded from three individuals of *L. dushanensis,* collected from its type locality Dushan County, Guizhou Province, China (107.63°E, 25.94°N, ca. 1200 m elev., 17.0–19.0 °C air temperature, 98% ambient humidity) on April 1, 2024, between 19:00–23:00 h. Additionally, to compare the differences in advertisement calls between *L. dushanensis* and its sister species, *Leptobrachella dong,* we also collected advertisement calls from *L. dong*. A total of 117 calls were recorded from four individuals of *L. dong,* collected from Congjiang County, Guizhou Province, China (108.28°E, 25.57°N, ca. 1190 m elev., 12.0–13.0 °C air temperature, 90% ambient humidity) on March 4, 2022, between 19:00–23:00 h. Calls from each individual were obtained using a digital recorder, a SONY ICD-PX470 (sampling rate 44.1 kHz, 16-bit resolution). Each call was recorded within a 0.5 m distance from the calling individual. All recordings were obtained from isolated individuals, without overlapping calls or mixed choruses, and saved as WAV files. The recordings are publicly available in Figshare at https://doi.org/10.6084/m9.figshare.26969512.

Snout-vent lengths (SVLs) of all recorded males were measured in situ using a precision digital caliper to the nearest 0.1 mm. One *L. dushanensis* (specimen number: DS20240401001) and one *L. dong* (specimen number: CJ20220304001) were collected for species identification, and the others were released to their original habitat after measurement. After taking photos of the two specimens, we euthanized them using isoflurane and then fixed the specimens in 10% buffered formalin. Tissue samples were taken and preserved separately in 95% ethanol before fixation. Specimens were deposited in the Moutai Institute, China. Mitochondrial COI genes were extracted and amplified from muscle samples and sequenced, and the obtained sequence was verified and uploaded to GenBank (https://www.ncbi.nlm.nih.gov/). The sequencing results were compared to finally determine the species collected.

Acoustic analyses

The advertisement call characteristics were analyzed with the software Raven Pro 1.6 (K. Lisa Yang Center for Conservation Bioacoustics [at the Cornell Lab of Ornithology] 2023). Temporal properties were measured using Raven's waveform display. Spectral properties were measured by averaging the spectrum over the entire duration of a call (Hann window, DFT = 512 samples, overlap = 50%, hop size = 256 samples). Only calls that had a high signal-to-noise ratio and were free from overlapping calls of nearby males were used for the analysis. We used "call-centred" terminology as summarised by Köhler et al. (2017), in which the fundamental unit was defined as a "call"; each "call" contains a single "note," and each "note" contains several pulses. We measured all parameters and characteristics following the procedure described by Köhler et al. (2017) and Qian et al. (2023), including (1) call duration (ms), CD (2) inter-call interval, CI (3) call rate (calls/s), CR (4) note per call, NPC (5) no. of pulses per call, NP (6) pulse rate (pulses/s), PR (7) dominant frequency (kHz), DF. Oscillograms and spectrograms were generated using Seewave v.2.2.0 (Sueur et al. 2008) and TuneR 1.4.2 (Ligges et al. 2013) packages in R programme 4.2.2 (R Core Team 2021) with a "Hanning" window size of 256 samples and an overlap of 50%. Descriptive statistics of call characteristics (mean, standard deviation (SD), and range) were computed using SPSS 23.0. Furthermore, principal component analysis (PCA) was conducted to compare the differences in advertisement calls between *L. dushanensis* and its sister species, *L. dong*.

Results

Call characteristics

Males were observed calling on rocks in streams surrounded by shrubs. After molecular identification, we determined that the species captured in Dushan County is *L. dushanensis* (Fig. 1A). The GenBank accession number for the COI sequence is PQ288957 (specimen number: DS20240401001). The SVL of *L. dushanensis* ranged from 31.9 mm to 32.6 mm (Table 1). We analyzed the spontaneous vocalizations (197 calls) from three *L. dushanensis* males. The advertisement calls of *L. dushanensis* are pulse-repetition calls (Fig. 2A, B). All calls of *L. dushanensis* exhibited a consistent structure, with the amplitude reaching its peak at the beginning of the call, then gradually decreasing. About one-third into the call, the amplitude increased again but did not exceed the initial peak before gradually decreasing until the end of the call (Fig. 2B). The advertisement call of *L. dushanensis* comprised a single note with a call duration ranging from 78.19–123.78 ms (Table 1). The inter-call interval ranged from 394.88–499.59 ms. The call rate ranged from 1.78–2.31 calls/s. The pulse number ranged from 14.00–21.00, and the pulse rate ranged from 164.26–174.06 pulses/second. The dominant frequency

Table 1. Descriptive statistics for call parameters of advertisement calls of *Leptobrachella dushanensis*.

Individuals	Calls analyzed	Air temperature (°C)	SVL (mm)	Call duration (ms)	Inter-call interval (ms)	Call rate	Note per call	Note rate	No. of pulses per call	Pulse rate	Dominant frequency (kHz)
DS20220401001	75	17.0	31.9	123.78 ± 16.61 94.00–167.10	499.59 ± 49.57 410.80–594.31	1.78 ± 0.14 1.65–1.93	1	NA	21.00 ± 4.50 14.00–33.00	164.26 ± 21.72 134.30–223.40	4.91 ± 0.15 4.82–5.17
DS20240401002	77	19.0	32.3	86.76 ± 9.73 68.40–115.40	469.77 ± 77.65 303.27–669.00	2.09 ± 0.18 1.92–2.39	1	NA	15.00 ± 1.50 12.00–24.00	174.06 ± 12.40 152.01–205.72	4.87 ± 0.17 4.82–5.51
DS20240401003	45	19.0	32.6	78.19 ± 6.52 64.60–97.10	394.88 ± 68.53 288.00–588.02	2.31 ± 0.15 2.19–2.48	1	NA	14.00 ± 0.50 10.00–20.00	164.56 ± 12.13 139.32–195.67	4.84 ± 0.10 4.82–5.51
All Individuals	3/197	17.0–19.0	31.9–32.6	78.19–123.78	394.88–499.59	1.78–2.31	1	NA	14.00–21.00	164.26–174.06	4.84–4.91

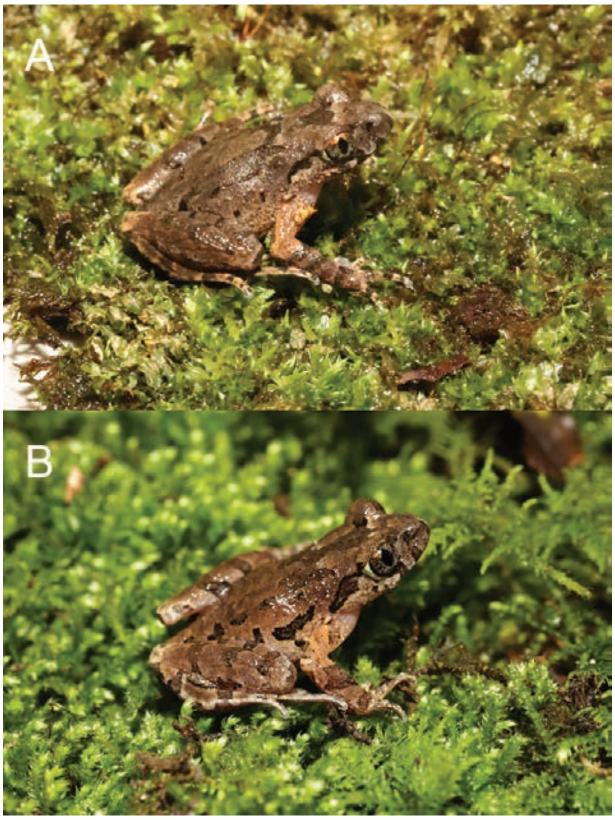

Figure 1. Photograph of the specimen. **A.** Adult male of *Leptobrachella dushanensis* (DS20240401001) from Dushan County, Guizhou Province, China; **B.** Adult male of *Leptobrachella dong* (CJ20220304001) from Congjiang County, Guizhou Province, China.

ranged from 4.84–4.91 kHz, and no obvious harmonics were found (Fig. 2B, Table 1).

After molecular identification, we confirmed that the species collected from Congjiang County is *L. dong* (Fig. 1B). The GenBank accession number for the COI sequence of *L. dong* is PQ438741 (specimen number: CJ20220304001). The SVL of *L. dong* ranged from 29.4 mm to 29.8 mm (Table 2). We analyzed the spontaneous vocalizations of 117 calls from four *L. dong* males. The advertisement calls of *L. dong* are also pulse-repetition calls (Fig. 2C, D). The advertisement call of *L. dong* reaches its peak amplitude at the beginning and then gradually decreases until reaching approximately halfway through the call. Subsequently, at around two-thirds of the call duration, the amplitude suddenly increases again but does not exceed the initial peak, and then gradually decreases until the end of the call (Fig. 2D). The advertisement call of *L. dong* comprised a single note with a call duration ranging from 63.54–90.82 ms (Table 2). The inter-call interval ranged from 299.20–380.01 ms. The call rate ranged from 2.23–2.72 calls/s. The pulse number ranged from 9.00–11.00, with a pulse rate of 102.13–133.77 pulses/second. The dominant frequency was 4.82 kHz, and no obvious harmonics were detected (Fig. 2D, Table 2).

Comparison of advertisement calls

The principal component analysis (PCA) of call parameters between *L. dushanensis* and *L. dong* resulted in two PCs, explaining a cumulated variance of 79.77% (Fig. 3). The PC1 axis accounted for 62.09% of the total variation and was primarily associated with temporal parameters, as it comprised the number of pulses, call duration, and pulse rate (Table 3). The PC2 axis explains 17.69% of the variation, primarily driven by the spectral parameter of dominant frequency (Table 3). The advertisement calls of the two species showed no overlap in PC space, indicating acoustic separation between the species.

Discussion

From the perspective of phylogenetic relationships, *L. dushanensis* was assigned to the *L. oshanensis* species group (Li et al. 2024). Previous studies have shown that species within the *L. oshanensis* species group typically produce more complex advertisement calls (Wang et al. 2019; Shi et al. 2021; Shen et al. 2023). However, unlike other species within the species group, we only recorded simple calls of *L. dushanensis* and did not notice any complex calls. Compared to the published calls of the other species in the *L. oshanensis* species group, the advertisement call of *L. dushanensis* differs considerably, reinforcing the specific identity of this species. For example, the species of *Leptobrachella yeae*, *Leptobrachella jinyunensis*, *Leptobrachella chishuiensis*, *Leptobrachella bijie*, *Leptobrachella jinshaensis*, *Leptobrachella suiyangensis*, *Leptobrachella purpuraventra*, *Leptobrachella wulingensis*, and *Leptobrachella oshanensis* exhibit multiple types of advertisement calls (Wang et al. 2019; Li et al. 2020; Cheng et al. 2021; Shi et al. 2021; Shen et al. 2023;

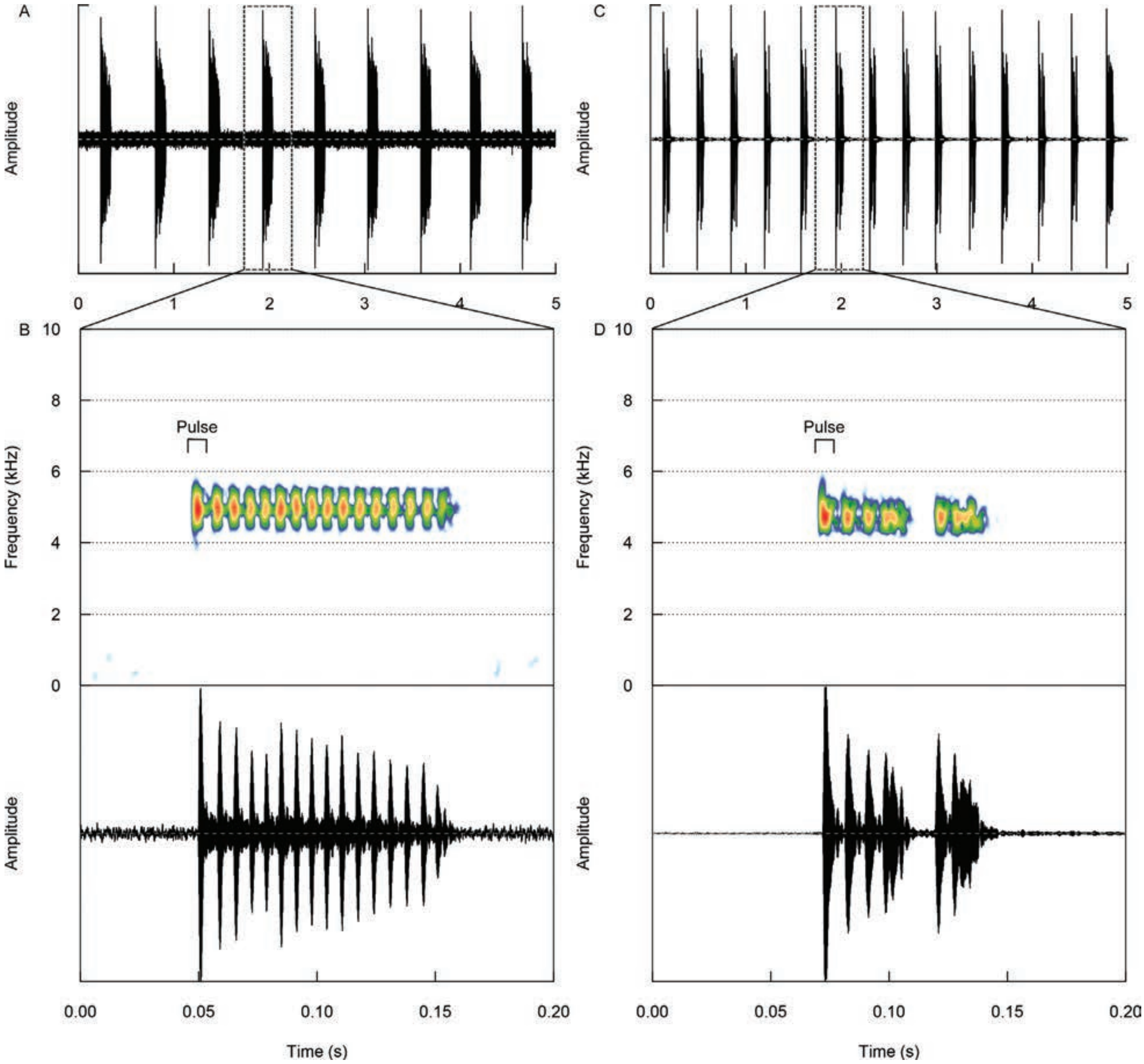

Figure 2. Advertisement calls of the two *Leptobrachella* species. **A.** 5 s oscillograms showing nine calls of *Leptobrachella dushanensis*; **B.** 0.2 s oscillograms and corresponding spectrograms showing one call of *L. dushanensis*; **C.** 5 s oscillograms showing fourteen calls of *Leptobrachella dong*; **D.** 0.2 s oscillograms and corresponding spectrograms showing one call of *L. dong*.

Table 2. Descriptive statistics for call parameters of advertisement calls of *Leptobrachella dong*.

Individuals	Calls analyzed	Air temperature (°C)	SVL (mm)	Call duration (ms)	Inter-call interval (ms)	Call rate	Note per call	Note rate	No. of pulses per call	Pulse rate	Dominant frequency (kHz)
CJ20220304001	27	13.0	29.6	82.55 ± 5.08 65.60–94.00	380.01 ± 42.06 312.95–478.13	2.23	1	NA	11.00 ± 0.00 8.00–13.00	121.87 ± 5.43 106.71–133.63	4.82
CJ20220304002	30	13.0	29.8	63.54 ± 4.84 48.40–72.80	336.30 ± 47.67 274.14–441.65	2.57	1	NA	9.00 ± 1.13 5.00–13.00	133.77 ± 21.62 70.80–165.41	4.82
CJ20220304003	30	12.0	29.5	90.82 ± 8.50 63.10–105.60	311.65 ± 37.97 270.60–435.54	2.55	1	NA	10.50 ± 0.50 6.00–12.00	102.13 ± 7.24 79.24–119.18	4.82
CJ20220304004	30	12.5	29.4	66.56 ± 6.15 54.40–81.60	299.20 ± 27.75 242.72–360.42	2.72	1	NA	9.00 ± 0.50 5.00–10.00	112.59 ± 16.92 66.89–147.06	4.82
All Individuals	4/117	12.0–13.0	29.4–29.8	63.54–90.82	299.20–380.01	2.23–2.72	1	NA	9.00–11.00	102.13–133.77	4.82

Shi et al. 2023; Qian et al. 2023). The dominant frequency of *L. dushanensis* (4.88 kHz) is lower than that of *Leptobrachella alpian* (6.70 kHz; Xu et al. 2005). As the sister species of *L. dushanensis*, *L. dong* exhibits a similar advertisement call structure to that of *L. dushanensis*. Their advertisement calls can be distinguished by call parameters such as the number of pulses, call duration, pulse rate, and dominant frequency (Table 3). In taxonomic studies, an increasing number of researchers are utilizing acoustic diagnostic traits to identify cryptic species (Cui et al. 2023;

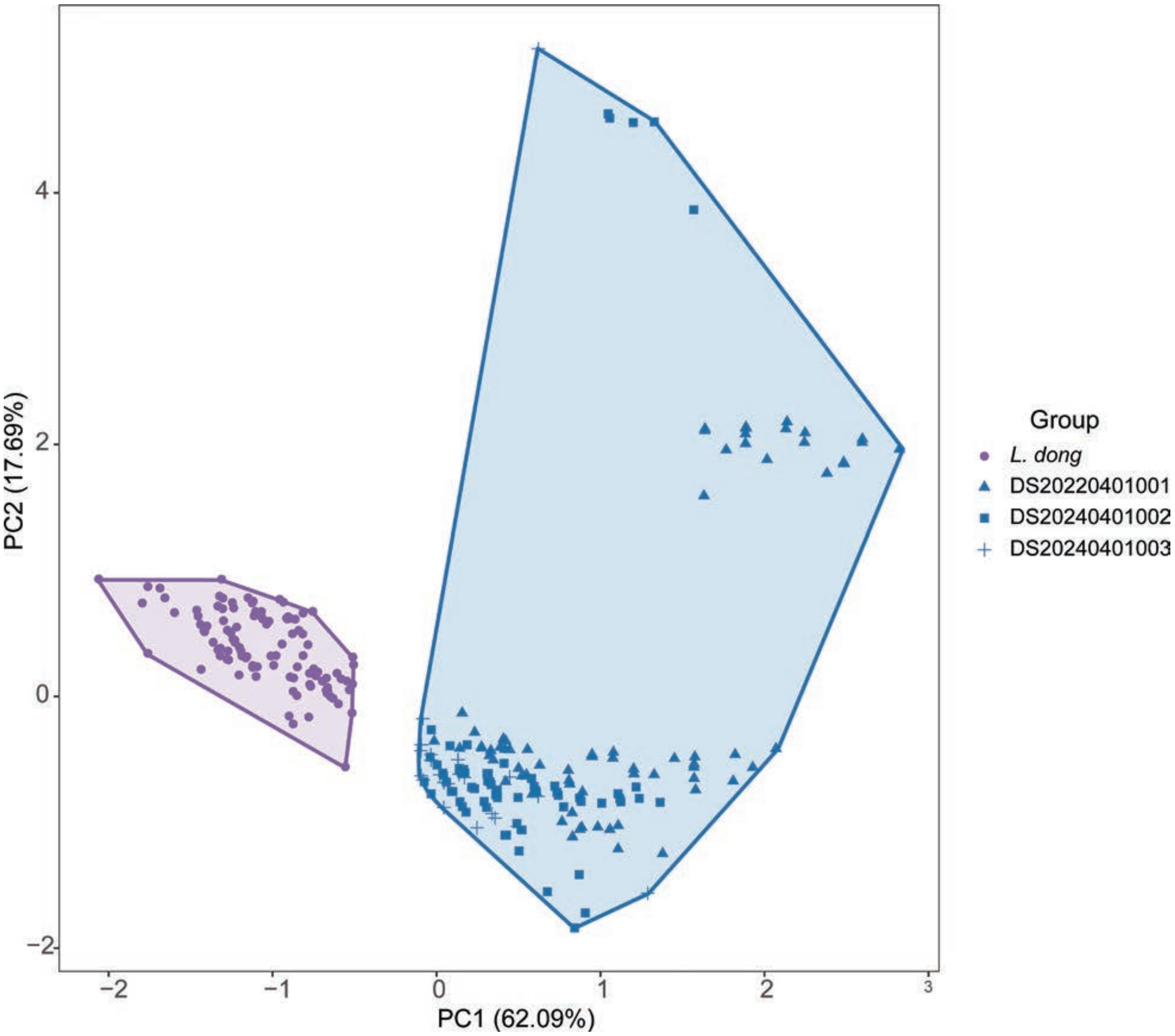

Figure 3. Plots of the first principal component (PC1) versus the second (PC2) for advertisement calls of *Leptobrachella dushanensis* and *Leptobrachella dong* from a principal component analysis.

Table 3. Factor loadings of the principal component analysis (PCA) on the advertisement call parameters of the *Leptobrachella dushanensis* and *Leptobrachella dong*.

Call parameters	Principal components				
	1	2	3	4	5
Call duration	0.830	0.074	-0.551	0.030	0.043
Inter-call interval	0.765	-0.395	0.210	0.463	-0.003
Number of pulses	0.967	-0.004	-0.178	-0.175	-0.060
Pulse rate	0.814	-0.192	0.428	-0.341	0.031
Dominant frequency	0.483	0.829	0.247	0.138	0.001
Eigenvalue	3.104	0.884	0.624	0.381	0.006
Variance (%)	62.085	17.686	12.473	7.628	0.128
Cumulative variance (%)	62.085	79.771	92.244	99.872	100.000

Chen et al. 2024). Species within the genus *Leptobrachella* are highly morphologically conserved, but *L. dushanensis* exhibits distinct advertisement call characteristics in its calls compared to other congeneric species, supporting the species-specific nature of its acoustic signals. The differentiation of advertisement calls in *Leptobrachella* can serve as evidence for their differentiation in taxonomic status. Our study will support further studies on the behavior, physiology, and ecology of the species.

Acknowledgements

This work was supported by the projects from the National Natural Science Foundation of China (Nos. 32270498, 31960099, 32260136, and 32070426), Guizhou Provincial Science and Technology Projects (Nos. ZK[2022]540 and [2023]099), and high-level personnel research start-up funding projects of Moutai Institute (Nos. mygccrc[2022]055, mygccrc[2022]067, and mygccrc[2022]083).

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ÖGH Herpetozoa
Austrian Herpetological Society

Herpetozoa 37: 379–390 (2024)
DOI 10.3897/herpetozoa.37.e137809

A new endemic insular species of the genus *Colubroelaps* (Squamata, Serpentes, Colubroidea) from Khanh Hoa Province, Vietnam

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Academic editor: Silke Schweiger ♦ **Received** 25 September 2024 ♦ **Accepted** 22 November 2024 ♦ **Published** 4 December 2024

Abstract

A new species of the poorly known genus *Colubroelaps* Orlov, Kharin, Ananjeva, Nguyen & Nguyen, 2009, is described based on a single female specimen collected from Hon Tre Island, Khanh Hoa Province, Vietnam. The new species, *Colubroelaps adleri* **sp. nov.**, can be distinguished from its sister species *Colubroelaps nguyenvansangi* Orlov, Kharin, Ananjeva, Nguyen & Nguyen, 2009 by the following combination of morphological characters: small body size (TL 402 mm); tail relatively short (ratio TaL/TL 0.10); dorsal scales in 14–14–14 rows; supralabials six, third and fourth entering orbit; infralabials seven; loreal present; ventral scales 234; subcaudals 30, all divided; cloacal plate divided; dorsal coloration pale brown, with narrow and interrupted dark stripe along spine; body flanks dark-gray lacking bluish iridescence; ventrally uniform off-white; head black with rostral, nasals, prefrontals, preoculars, loreal, and the two anterior supralabials, as well as anterior parts of supraoculars and frontal dirty yellowish-brown with dark-brown spots. This discovery represents the second known species of the genus *Colubroelaps* and the first record of the genus in the coastal areas of southern-central Vietnam. The new species is likely micro-endemic to the small island of Hon Tre within Nha Trang Bay in Khanh Hoa Province, Vietnam, at an elevation of 30 m a.s.l., and was recorded only from the heavily disturbed secondary forest area of the island. The new species is under threat due to the intensifying development of tourist infrastructure on Hon Tre Island. We suggest that the new species be considered Endangered (EN) following the IUCN's Red List categories.

Key Words

Colubroelaps adleri sp. nov., conservation, Hon Tre Island, morphology, Nha Trang Bay, taxonomy

Introduction

The monotypic genus *Colubroelaps* Orlov, Kharin, Ananjeva, Nguyen & Nguyen, 2009, is one of the most mysterious groups of snakes. This genus, colloquially known as lace snakes (Poyarkov et al. 2023), remained unnoticed for over a century of herpetological exploration of Vietnam and was only recently erected by Orlov et al. (2009). In its original description, the genus *Colubroelaps* was morphologically characterized as a small, very thin colubrid snake "with elapsoid morphotype but no fangs and venomous glands" (Orlov et al. 2009); body vermiform, round in cross section, total length up to 500 mm; small head roundish and short, not distinguished from the body, slightly dorso-ventrally depressed and covered by large regular shields; eyes very small with a round pupil; dorsal scales uniform, rhomboid, tile-shaped, smooth; the number of scale

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rows around the body is almost equal along all the body length; cloacal plate divided; maxillae slightly arched, longer than palatinum and hardly shorter than pterygoideum; palatinum with external process, joined with internal process of maxillae; pterygoideum with triangular hollow for palatinum; accordingly, palatinum has triangular projection for pterygoideum in its lower part; hypapophyses on the posterior trunk vertebrae, a diastema, and sulcate teeth on the posterior end of the maxillae are absent (cited from Orlov et al. 2009 with minor edits). In the original description, the genus was provisionally included in the family Colubridae Oppel, 1811, with the remark that the morphometric and scalation characters indicate similarities to both Colubridae and Elapidae Boie, 1827 snakes (Orlov et al. 2009). However, Zaher et al.'s (2019) subsequent examination of the skull morphology using micro-CT X-ray scanning demonstrated that *Colubroelaps* has hinged teeth more similar to those of the family Sibynophiidae Dunn, 1928 than to colubrids or elapids. The assumption by Zaher et al. (2019) that *Colubroelaps* was incorrectly assigned to the family Colubridae by Orlov et al. (2009) and should be regarded as a member of Sybinophiidae is now widely accepted (Uetz et al. 2024). However, any phylogenetic data on *Colubroelaps* are still unavailable; therefore, herein we follow Poyarkov et al. (2023) and take a cautious approach and refer to the taxon as a member of Colubroidea incertae sedis pending further phylogenetic assessment.

Currently, the genus *Colubroelaps* is only known for a single species named *C. nguyenvansangi* Orlov, Kharin, Ananjeva, Nguyen & Nguyen, 2009. The Nguyen Van Sang's lace snake was described based on a single adult female collected in 2003 from the Loc Bac Forest Enterprise, Bao Lam District, Lam Dong Province, southern Vietnam. Because of its fossorial lifestyle, this species is quite elusive and difficult to detect in the field. Until today, *C. nguyenvansangi* has only been reported from six localities in southern Vietnam, namely, Loc Bac and Di Linh districts in Lam Dong Province, Bu Gia Map National Park and Bu Dang District in Binh Phuoc Province, Tuy Duc District in Dak Nong Province, Buon Ma Thuot City and Krong Buk District in Dak Lak Province, and Cat Tien National Park in Dong Nai Province (see Nguyen et al. 2024, Fig. 1). Moreover, the occurrence of this species in eastern Cambodia is anticipated (Orlov et al. 2009; Poyarkov et al. 2023; Nguyen et al. 2024). The recent analysis of distribution and climatic niche modeling by Nguyen et al. (2024) demonstrated that *C. nguyenvansangi* is associated with seasonally dry, semi-deciduous, lowland, and mid-elevation tropical forests of southern Vietnam (see Vassilieva et al. 2016), and its distribution is restricted to the western foothills of the Langbian Plateau (see Fig. 1).

Khanh Hoa Province is located in the southern part of the coastal region of Vietnam and currently harbors 176,382 ha of evergreen forest (Anonymous, 2023). However, the biodiversity of this province is still poorly studied, in particular the herpetofauna. Ten new species were recently described with their type localities from Khanh Hoa Province within the last fifteen years, namely: *Kalophrynus honbaensis* Vassilieva, Galoyan, Gogoleva & Poyarkov, 2014; *Kurixalus viridescens* Nguyen, Matsui & Hoang, 2014; *Acanthosaura murphyi* Nguyen, Do, Hoang, Nguyen, McCormack, Nguyen, Orlov, Nguyen & Nguyen, 2018; *Cyrtodactylus cucdongensis* Schneider, Phung, Le, Nguyen & Ziegler, 2014; *C. raglai* Nguyen, Duong, Grismer & Poyarkov, 2021; *C. yangbayensis* Ngo & Chan, 2010; *Gekko truongi* Phung & Ziegler, 2011; *Sphenomorphus yersini* Nguyen, Nguyen, Nguyen, Orlov & Murphy, 2018; *Lycodon anakradaya* Nguyen, Duong, Wood & Grismer, 2022; and *L. truongi* Nguyen, Duong, Wood & Grismer, 2022 (see Poyarkov et al. 2021, 2023; Uetz et al. 2024). These discoveries, along with the complex terrain of the Khanh Hoa Province covering several isolated mountain systems and a number of offshore islands, suggest that this area may still harbor unrecognized herpetofaunal diversity.

During our recent field surveys on Hon Tre Island in Nha Trang Bay, Khanh Hoa Province, we encountered a specimen of *Colubroelaps* sp. that was superficially similar to *C. nguyenvansangi* in its overall morphological habitus and body coloration. Closer morphological examination of scalation, pattern, color, and morphometric characteristics of the Hon Tre specimen demonstrated clear morphological differences from *C. nguyenvansangi* in a number of taxonomically important traits. Therefore, in the present paper, we describe the *Colubroelaps* population from Hon Tre Island as a new species.

Materials and methods

Sampling

Fieldwork was carried out in Hon Tre Island within Nha Trang Bay, Khanh Hoa Province, Vietnam, by N. A. Poyarkov and A. M. Bragin from 15 to 20 of June 2023 (Fig. 1, locality 1). We obtained geographic coordinates and altitude using a Garmin GPSMAP 60CSx GPS receiver (USA) and recorded them in datum WGS 84. The specimen was collected by hand, photographed in life, and euthanized using MS-222 solution within 24 h after capture. The specimen was fixed in 4% buffered formalin for 24 h and later stored in 70% ethanol. The specimen was subsequently deposited in the herpetological collection of the Zoological Museum of Moscow State University (ZMMU), Moscow, Russia. Specimen collection and animal use protocols were approved by the Institutional Ethical Committee of the Joint Vietnam-Russia Tropical Science and Technology Research Center (VRTC). Fieldwork in Hon Tre Island, including collection of animals in the field, was authorized by the People's Committee of Khanh Hoa Province, Vietnam (#5565/UBND-KT of 08.06.2023) granted to VRTC. Abbreviations. **Mt.**: Mountain; **NP.**: National Park; **Isl.**: Island; **asl.**: above sea level.

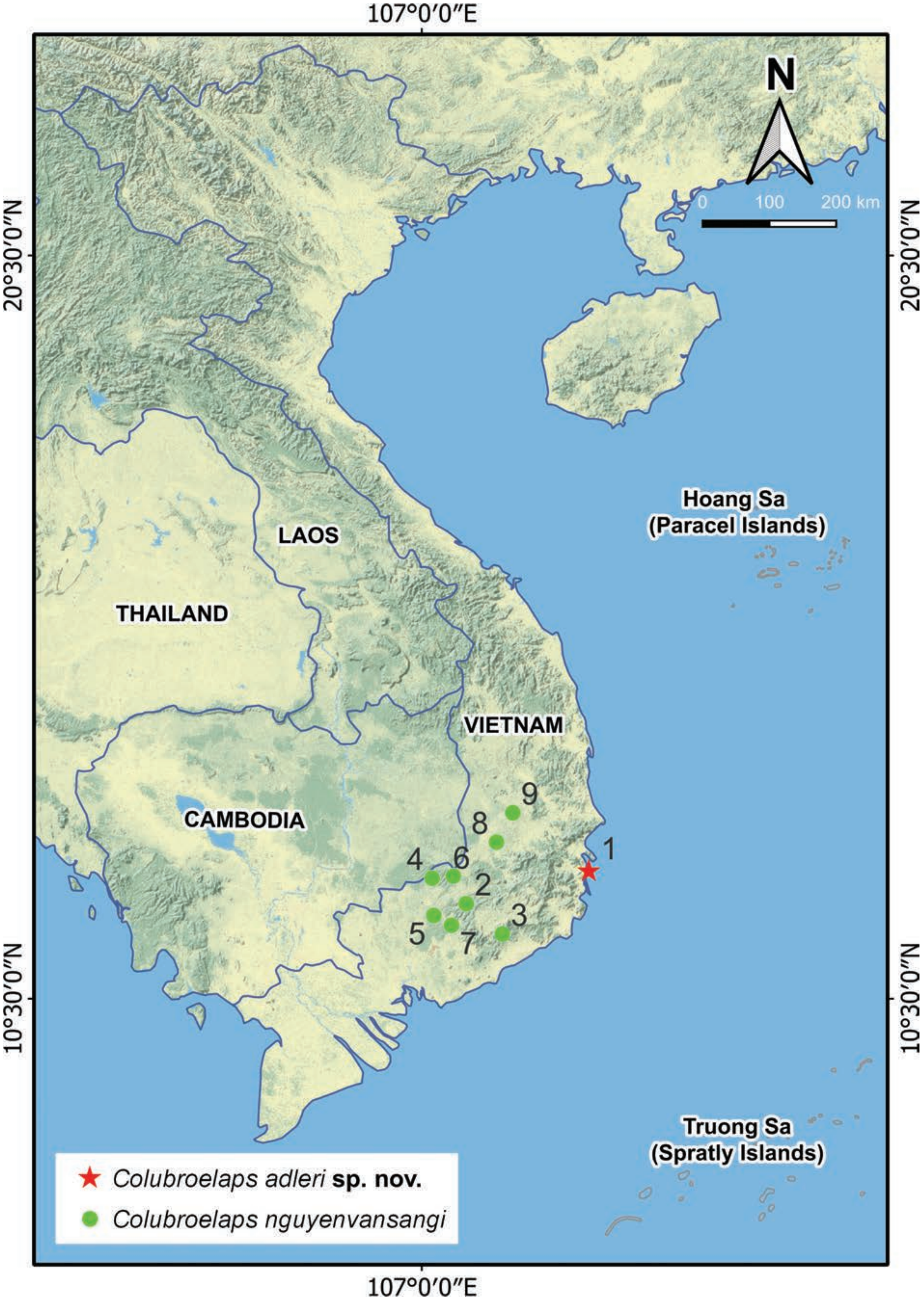

Figure 1. Distribution of the genus *Colubroelaps* in Vietnam. Localities**:** *Colubroelaps adleri* sp. nov. (red): (1) Hon Tre Island within Nha Trang Bay, Khanh Hoa Province (type locality); *C. nguyenvansangi* (green): (2) Loc Bac, Lam Dong Province (type locality); (3) Di Linh, Lam Dong Province; (4) Bu Gia Map NP, Binh Phuoc Province; (5) Bu Dang, Binh Phuoc Province; (6) Tuy Duc, Dak Nong Province; (7) Cat Tien NP, Dong Nai Province; (8) Buon Ma Thuot, Dak Lak Province; (9) Krong Buk, Dak Lak Province.

Morphological differentiation

Measurements and meristic counts followed Orlov et al. (2009) and Poyarkov et al. (2019, 2022). We presented the paired meristic characters in left/right order. We used a Mitutoyo digital caliper to take the following measurements to the nearest 0.1 mm: **ED**, horizontal eye diameter; **HD**, maximum head depth; **HL**, head length (from the tip of rostral to the posterior end of the jaw); **HW**, maximum head width; **SnL**, snout length (from the tip of rostral to the anterior eye margin); **SVL**, snout-vent length; **TaL**, tail length; **TL**, total length. The following meristic characters were examined: **ASR**, anterior number of dorsal scale rows (at one HL behind the head); **IL**, number of infralabial scales; **MSR**, number of dorsal scale rows at midbody (counted at the level of the middle of the distance between the snout tip and the cloaca); **PO**, number of postoculars; **PrO**, number of preoculars; **Lor**, number of loreals; **AT**, number of anterior temporals; **PT**, number of posterior emporals; **PSR**, posterior number of dorsal scale rows (at one HL before the cloacal plate); **PreV**, number of preventrals (scales directly preceding the ventrals, unpaired, wider than long but not in contact on each side with the 1st dorsal scale row); **SC**, number of subcaudal scales, not including the terminal pointed scute; **SL**, number of supralabial scales; **VEN**, number of ventral scales (counted following Dowling 1951). We determined the sex by examining the cloacal area for the presence of hemipenis and performing minor dissections of the body.

We conducted a detailed comparison of the morphological and coloration characters of the examined specimen with three available specimens of *C. nguyenvansangi*, including the holotype ZISP 25682, reported by Orlov et al. (2009) and stored in the Zoological Institute, Russian Academy of Sciences, St. Petersburg, Russia (ZISP), and two additional specimens from the herpetological collection of ZMMU. The morphological data for all examined specimens is presented in Table 1.

Results

Our morphological analysis, which recovered a number of important diagnostic characters summarized below, allows unambiguously distinguishing the population of *Colubroelaps* sp. from Khanh Hoa Province from *C. nguyenvansangi*. These results support our hypothesis that this recently discovered lineage of *Colubroelaps* sp. from Khanh Hoa Province represents a previously unknown species, which we formally describe below.

Colubroelaps adleri sp. nov.

https://zoobank.org/F64EE8E0-B851-4923-8299-75793283310E

Table 1, Figs 2, 3A–C, 4A

Holotype. • ZMMU Re-18000 (field tag NAP-15227), adult female, collected by Nikolay A. Poyarkov and Andrey M. Bragin on June 17, 2023, from Dam Bay Research Station, Hon Tre Island, within Nha Trang Bay in Vinh Nguyen Ward, Nha Trang City, Khanh Hoa Province, Vietnam (12.198°N, 109.289°E; at the elevation of 30 m asl.).

Diagnosis. *Colubroelaps adleri* sp. nov. can be distinguished from *C. nguyenvansangi* by the following combination of morphological characters: body size small (TL 402 mm); tail relatively short (ratio TaL/TL 0.10); dorsal scales in 14–14–14 rows; supralabials six, third and fourth entering orbit; infralabials seven; loreal present; ventral scales 234; subcaudals 30, all divided; cloacal plate divided; dorsal coloration pale brown with narrow and interrupted dark stripe along spine; body flanks dark gray lacking bluish iridescence; ventrally uniform off-white; head black with rostral, nasals, prefrontals, preoculars, loreal, and the two anterior supralabials, as well as the anterior parts of supraoculars and frontal shields dirty yellowish-brown with dark brown spots.

Description of the holotype. Adult female specimen in a good state of preservation (Fig. 2). Body strongly elongated, very thin, vermiform, round in cross-section. Tail obtusely rounded, comparatively short, the tip of tail blunt (SVL 362 mm; TaL 40.2 mm; TL 402.2 mm; ratio TaL/TL 0.10) (Fig. 2E–G). Head very small, rounded, slightly dorso-ventrally depressed, covered by large, regular, symmetric shields (Figs 2A–D, 3A–C), not distinct from the neck (HL 7.99 mm; HW 5.16 mm; ratio HW/HL 0.65). Snout wide, short, bluntly rounded in dorsal view (Fig. 2A, B), slightly tapering in lateral view (Fig. 2C, D). Rostral barely visible in dorsal aspect, triangular (Figs 2A, 3A). Eyes very small with round pupils (ED 0.51 mm, EN 1.46 mm, SnL 2.78 mm) (Fig. 2C, D). Single nasal on each side of head (1/1 nasals); nostril oval-shaped, with horizontal orienatation, completely enclosed in nasal scale, located closer to the posterior edge of nasal (Figs 2C, D, 3B); single (1/1) loreal, small, almost triangular in shape, shorter than the eye diameter, in contact with nasal, 1st and 2nd supralabial, preocular, prefrontal and internasal; single (1/1) preocular, large, elongated pentagonal in shape, subequal to the eye diameter, in contact with 2nd and 3rd supralabial, the orbit, supraocular, prefrontal and loreal; single (1/1) postocular, trapezoidal in shape, smaller than the eye diameter, in contact with 4th supralabial, parietal, supraocular and the orbit; single (1/1) supraocular, large and wide, ca. twice longer than the eye diameter, in contact with the orbit, postocular, parietal, frontal, prefrontal and preocular (Figs 2C, D, 3B). Six supralabials on each side of the head (6/6), fourth one the largest, third and fourth supralabials in contact with eye, posterior edge of fourth supralabial in contact with anterior temporal, separating fifth supralabial from parietal; fourth supralabial in contact with parietal, postocular, and anterior temporal (Figs 2C, D, 3A); seven (7/7) infralabials, the anterior-most pair in contact with each other behind the mental (Figs 2B, 3C); the first four pairs of infralabials in contact with the anterior pair of chin shields (Figs 2B, 3C); fourth and fifth infralabials in contact with the poste-

Table 1. Morphological data on the type series of *Colubroelaps adleri* sp. nov. and *C. nguyenvansangi*; diagnostic differences from the new species are marked in bold. **Notes**: for abbreviations, see the Materials and methods section; "*": data on the holotype of *C. nguyenvansangi* as reported by Orlov et al. (2009), when different from our observations.

Species	***Colubroelaps adleri* sp. nov**	***C. nguyenvansangi***		
Collection number	**ZMMU Re-18000**	**ZISP 25682**	**ZMMU Re-15484**	**ZMMU Re-15485**
Type status	Holotype	Holotype	—	—
Locality	Dam Bay, Hon Tre Isl., Khanh Hoa Prov., Vietnam	Loc Bac, Bao Lam Dist., Lam Dong Prov., Vietnam	Bu Gia Map NP., Binh Phuoc Prov., Vietnam	Cat Tien NP., Tan Phu Dist., Dong Nai Prov., Vietnam
Sex	Female	Female	Male	Male
SVL (mm)	362	393	399	368
TaL (mm)	40.2	**107**	**121**	**98**
TL (mm)	402	**500**	**520**	**466**
TaL/TL	0.10	**0.21**	**0.23**	**0.21**
HL (mm)	8.0	5.9	5.9	5.5
HW (mm)	5.2	4.3	4.3	4.0
SnL (mm)	2.8	2.0	2.4	2.1
ED (mm)	0.5	0.8	1.0	0.8
EN (mm)	1.5	1.2	1.2	1.1
IO (mm)	3.0	2.7	2.8	2.5
SL	6/6	6/6	6/6	6/6
IL	7/7	7/7 (6/6)*	7/7	7/7
PrO	1/1	1/1	1/1	1/1
PO	1/1	1/1	1/1	1/1
Lor	1/1	1/1 (0/0)*	1/1	1/1
AT	1/1	1/1	1/1	1/1
PT	2/2	2/2	2/2	2/2
ASR	14	**16**	**16**	**16**
MSR	14	**16**	**16**	**16**
PSR	14	**16**	**16**	**16**
VEN	234	**267**	**282**	**292**
SC	30	**81**	**87**	**86**
Cloacal plate	divided	divided	divided	divided
Dorsal color and pattern	light brown with interrupted dark stripe along spine	**reddish-brown with continuous stripe along spine**	**reddish-brown with continuous stripe along spine**	**reddish-brown with continuous dark stripe along spine**
Pattern in the anterior part of head	dirty yellow-brown, narrow	**uniform light yellow or white, wide**	**uniform light yellow or white, wide**	**uniform light yellow or white, wide**

rior pair of chin shields (Figs 2B, 3C). Three gular scales aligned between the chin shields and the first preventral (Figs 2B, 3C). One pair of enlarged internasals, in contact with each other; one pair of enlarged prefrontals, in contact with each other; one wide, pentagonal frontal shield; one pair of wide, triangular parietals, in contact with each other, anteriorly separated by the protruding posterior edge of frontal (Figs 2A, 3B), posteriorly barely extending beyond posterior edges of upper posterior temporals (Fig. 3B). Single (1/1) anterior temporal, quadrangular in shape; two (2/2) posterior temporals, lower one the largest, two and a half times larger than upper posterior temporal (Fig. 3A, B), upper one rectangular-shaped, narrow, notably protruding beyond the upper edge of lower posterior temporal (Figs 2C, D, 3B), in dorsal aspect notably protruding beyond the posterior edge of parietal (Fig. 3B). Dorsal scales in 14–14–14 rows. Dorsal scales rhomboid, tile-shaped, all smooth, and of the same size (Fig. 2G). Ventrals 234; cloacal plate divided (Fig. 2E); 30 subcaudals, all divided (Fig. 2E, F).

Coloration in life. Body glossy but lacking the metal/bluish iridescence (Figs 2A–G, 4A). Dorsal background coloration pale brown, with narrow and interrupted dark stripe along spine (Figs 2G, 4A), formed by small, ca. 1 scale in size, dark brown to black, diamond-shaped blotches (Fig. 2A); body flanks dark gray, nearly black, with indistinct light gray mottling along the scale edges; ventral surface of body and tail off-white with light greenish tint (Fig. 2E, F). Head black to dark brown with rostral, nasals, prefrontals, preoculars, loreal, the first two supralabials, as well as the anterior part of supraoculars and frontal shield dirty yellowish-brown with dark brown irregular spots (Fig. 2A–D). Ventral surfaces of the head white with chocolate-brown blotches (Fig. 2B). After one year in preservative, the background dorsal color faded to beige-gray, and yellowish parts faded to off-white; overall, the pattern of dark markings remained unchanged.

Comparisons. The main differences between the new species and *C. nguyenvansangi* are summarized in Table 1. *Colubroelaps adleri* sp. nov. can be easily distinguished from *C. nguyenvansangi* by having notably shorter tail (TaL/TL 0.10 in a single female vs. 0.21 in females and 0.21–0.23 in males; however, this character should be taken cautiously: the tail of *Colubroelaps adleri* sp. nov. holotype may be incomplete; cases of caudal autotomy were earlier reported for the members of Sibynophiidae and likely may also occur in *Colubroelaps*; see Mendelson, 1991), lower number of body scale rows (DSR 14–14–14 vs. 16–16–16); lower number of ventrals (VEN 234 in a single female vs. 267 in female, 282–292 in males); much lower number of subcaudals (SC 30 in a single female vs. 81 in female, 86–87 in males).

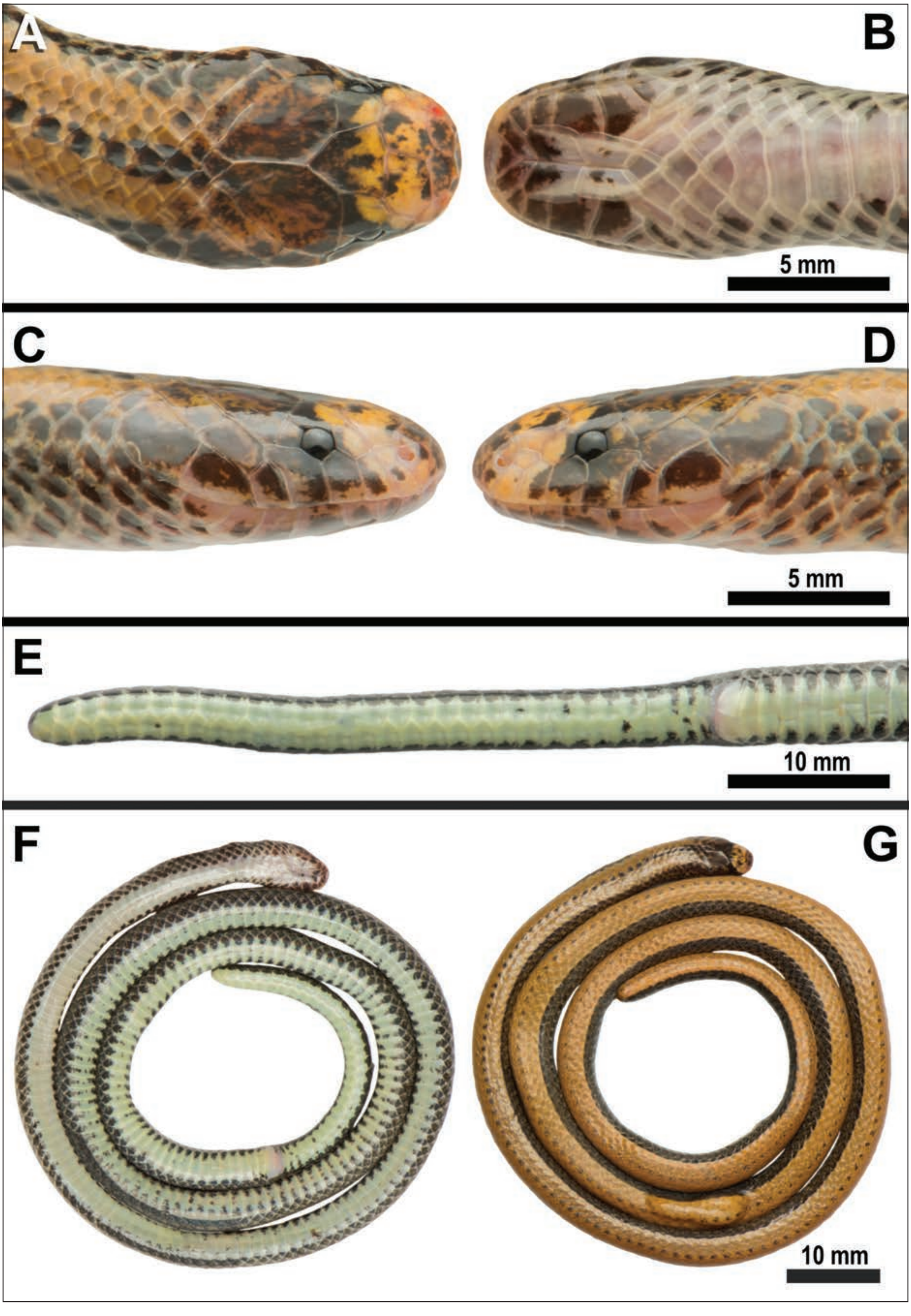

Figure 2. The holotype of *Colubroelaps adleri* sp. nov. in life (ZMMU Re-18000, adult female) from Hon Tre Island, Khanh Hoa Province, Vietnam. **A.** Dorsal view of the head; **B.** Ventral view of the head; **C.** Lateral view of the right side of the head; **D.** Lateral view of the left side of the head; **E.** Ventral view of the tail; **F.** Ventral view of the body; **G.** Dorsal view of the body. Scale bars: 5 mm (**A–D**); 10 mm (**E–G**). Photographs by A. M. Bragin.

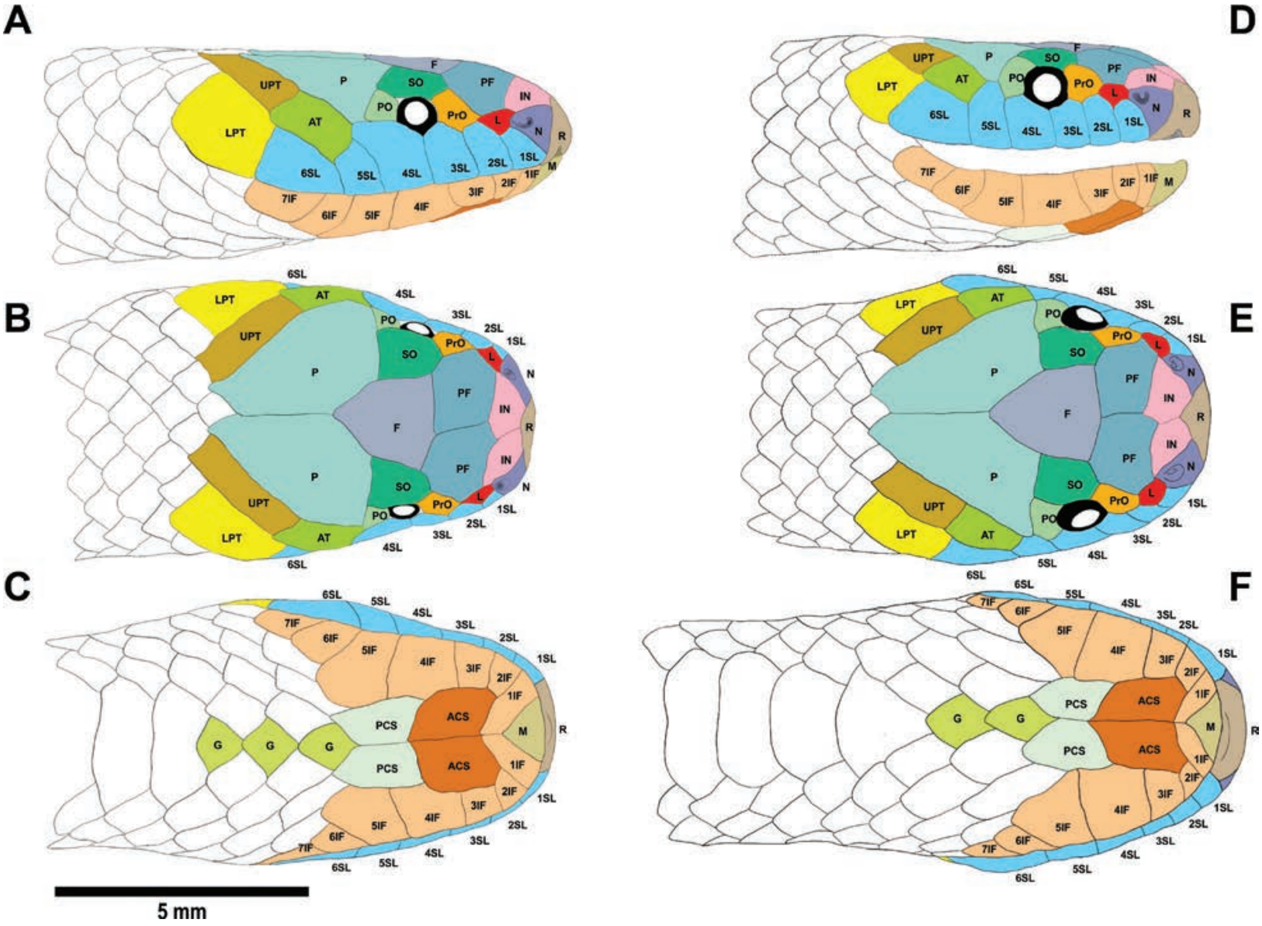

Figure 3. Line drawings of head scalation of the *Colubroelaps adleri* sp. nov. holotype (ZMMU Re-18000, adult female; **A–C**) and of the *C. nguyenvansangi* holotype (ZISP 25682, adult female; **D–F**). Line drawings show the head scales in lateral (**A, D**), dorsal (**B, E**), and ventral (**C, F**) aspects. Abbreviations: ACS = anterior chin shield; AT = anterior temporal; F = frontal; G = gular scale; IL = infralabial; IN = internasal; L = loreal; LPT = lower posterior temporal; M = mental; N = nasal; P = parietal; PCS = posterior chin shield; PF = prefrontal; PO = postocular; PrO = preocular; R = rostral; SL = supralabial; SO = supraocular; UPT = upper posterior temporal. Scale bar equals 5 mm. Line drawings by A. M. Bragin.

Furthermore, the new species can be easily diagnosed from *C. nguyenvansangi* by having three gular scales between the chin shields and the first preventral (vs. two) (Fig. 3C, F); by fourth infralabial in contact with parietal and anterior temporal, separating fifth infralabial from parietal and postocular (vs. fifth infralabial in contact with parietal and postocular) (Fig. 3A, D); by upper posterior temporal being elongated and much narrower than lower one, noticeably protruding beyond the level of the posterior edge of parietals (vs. upper posterior temporal small, as long as lower posterior temporal, not protruding beyond the level of the posterior edge of parietals) (Fig. 3A–E). *Colubroelaps adleri* sp. nov. can be further diagnosed from *C. nguyenvansangi* by having pale brown dorsum with narrow and interrupted dark stripe along spine (vs. reddish-brown dorsum with narrow and continuous dark stripe along spine) (Fig. 4A, B); by lacking the metallic iridescence on dorsum and body flanks (vs. body with bluish metallic iridescence) (Fig. 4A, B); and by having dirty yellowish-brown anterior part of head with irregular dark brown blotches (vs. uniform light yellow to white blotch on anterior part of head lacking dark markings) (Fig. 4A, B).

Etymology. The species epithet '*adleri*' is a patronymic adjective in genitive singular. We name the new species in honor of Dr. Kraig Adler, Professor Emeritus at Cornell University (New York, USA), in recognition of his outstanding support to the international herpetological community as well as his remarkable scientific contribution to Asian herpetology. We suggest the following common names for the new species: "*Adler's lace snake*" (in English), "*Shnurkovaya zmeya Adlera*" (Шнурковая змея Адлера, in Russian), and "*Rắn hổ nước Át-Lơ*" (in Vietnamese).

Distribution and natural history notes. Currently, *Colubroelaps adleri* sp. nov. is known only from a single locality in secondary dry maritime evergreen forest on Hon Tre Island, Khanh Hoa Province, South Central Coastal Region of Vietnam (Figs 1, 5). The new species is also expected to inhabit other islands of the Nha Trang Bay, though they are much smaller than Hon Tre, and on some of them, forest vegetation has been greatly destroyed. The only known specimen of *Colubroelaps adleri* sp. nov.

Figure 4. Two species of the genus *Colubroelaps* in life. **A.** *Colubroelaps adleri* sp. nov., holotype ZMMU Re-18000, adult female from Dam Bay Research Station, Hon Tre Island, Khanh Hoa Province, Vietnam; **B.** *Colubroelaps nguyenvansangi*, ZMMU Re-15485, adult male from Cat Tien NP., Tan Phu District, Dong Nai Province, Vietnam. Photographs by: A. M. Bragin (**A**); E. A. Galoyan (**B**).

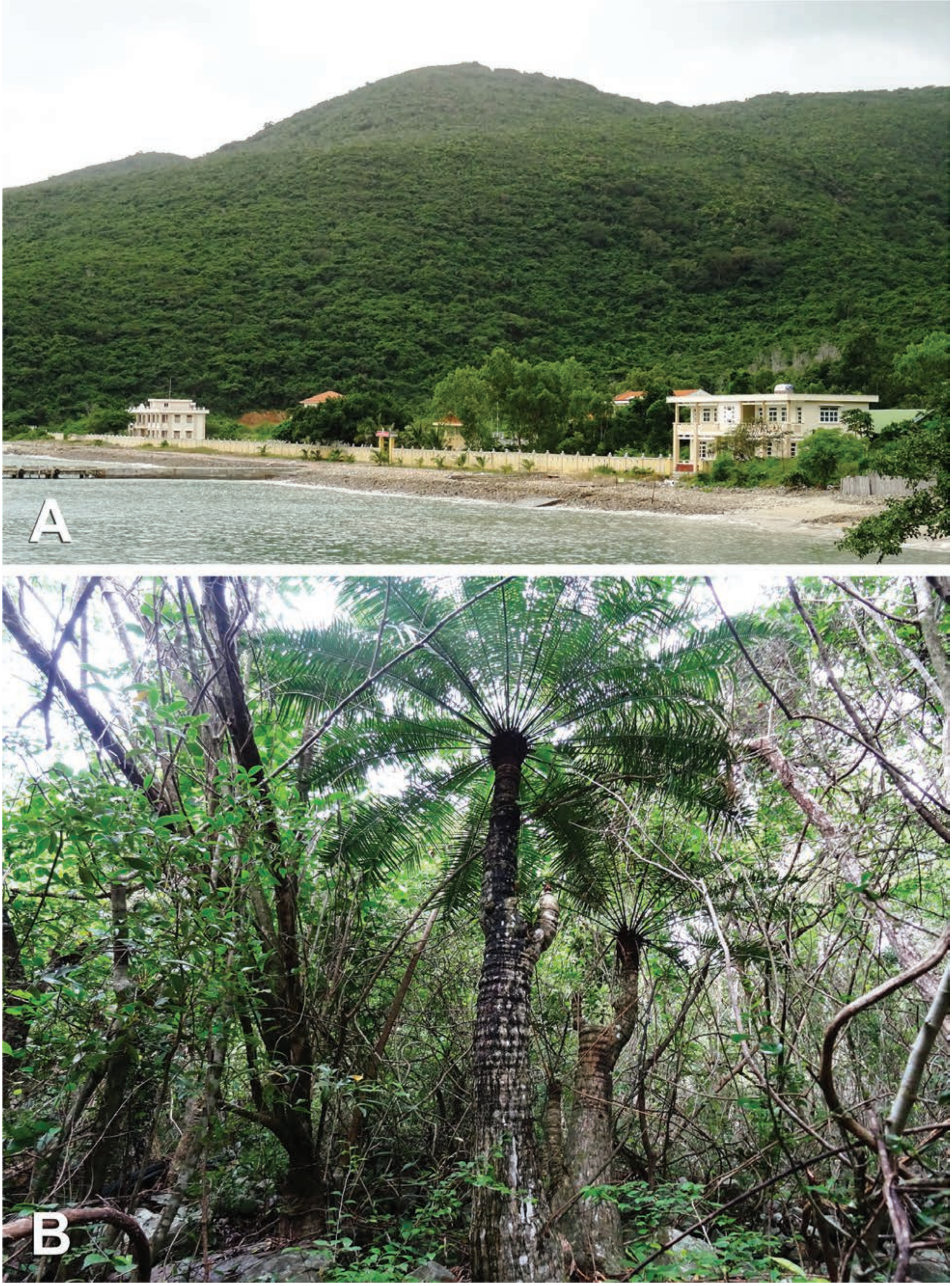

Figure 5. Habitat of *Colubroelaps adleri* sp. nov. **A.** Macrohabitat; **B.** Microhabitat of the new species in Dam Bay Research Station, Hon Tre Island, Nha Trang, Khanh Hoa Province, Vietnam. Photographs by A. V. Alexandrova.

was collected during the daytime (14h00) while crossing the road. The individual was collected near a garbage dump at the Dam Bay Research Station at 30 m a.s.l. elevation (Fig. 5A), ca. 10 m from a dry maritime mixed low evergreen forest. The forest near the type locality is dominated by *Buchanania reticulata* Hance, *Choerospondias axillaris* (Roxb.) Burtt. & Hill, *Pentaspadon annamense* (Evrard & Tardieu) Phạmh., *Spondias pinnata* (L.f.) Kurz, and *Ormosia* sp., including occasional trees of *Sindora siamensis* Teijsm. ex Miq., *Streblus ilicifolius* (S. Vidal) Corner, and *Eurya turfosa* Gagnep, and with an undergrowth formed primarily by *Dracaena* sp. and with occasional specimens of *Cycas rumphii* Miq. (Fig. 5B) (plant identification—A. N. Kuznetsov, pers. comm.). Other species of snakes recorded in sympatry with the new species at the type locality included *Lycodon davisonii* (Blanford, 1878), *L. capucinus* (Boie, 1827), *Ptyas korros* (Schlegel, 1837), *Ophiophagus hannah* (Cantor, 1836), and *Trimeresurus albolabris* (Gray, 1842).

A parasitic invasion of the Acanthocephala (Kölr.) worm was found in the subcutaneous cavity between the skin and the body muscles in the posterior third of the specimen length on its dorsal side (Figs 2C, 4A). The presence of this parasite may indicate that *Colubroelaps adleri* sp. nov. likely feeds on insects, as these animals are the intermediate hosts for acanthocephalan parasitic worms. All other aspects of the ecology of *Colubroelaps adleri* sp. nov., including information on diet, preferred microhabitats, reproduction, and predators, remain unknown.

Discussion

Orlov et al. (2009) described the new genus and species, *C. nguyenvansangi*, based on a single female specimen (ZISP 25682) collected from Lam Dong Province, Vietnam. To date, this species' rarity and elusive habits have largely prevented the publication of morphological data on additional specimens. In this study, based on re-examination of the female holotype as well as an examination of two additional male specimens, we, for the first time, provide data on morphological variation within *C. nguyenvansangi*. The additional materials examined include an adult male ZMMU Re-15484 from Bu Gia Map NP, Binh Phuoc Province, and an adult male ZMMU Re-15485 from Cat Tien NP., Dong Nai Province; the morphological data on the three specimens of *C. nguyenvansangi* is summarized in Table 1.

During the re-examination of the *C. nguyenvansangi* holotype (ZISP 25682), we found several inconsistencies contradicting its original description by Orlov et al. (2009). In particular, the holotype of *C. nguyenvansangi* clearly showed the presence of a loreal, same as in the other two specimens of *C. nguyenvansangi* examined by us (see Table 1, Fig. 3D, E). However, Orlov et al. (2009: 235) indicated that loreal is absent in ZISP 25682 but reported the presence of a posterior nasal in this specimen ("nostril large, situated between two nasals, more close to posterior edge of anterior nasal on the border with posterior nasal; no loreal," p. 235). We do not agree with this interpretation, as in all three *C. nguyenvansangi* specimens examined by us, the nostril was clearly completely enclosed within the undivided nasal scale (Fig. 3D, E). Therefore, we re-interpret the 'posterior nasal' scale of Orlov et al. (2009) as a loreal. Another inconsistency is that Orlov et al. (2009: 236) added a somehow contradictory statement regarding the number of supralabials in ZISP 25682: "supralabials 6–6, the 1st is the smallest and the 7th is the largest" (p. 236). After re-examination of the holotype specimen, we can confirm that ZISP 25682 indeed has 6/6 supralabials, the same as all other *C. nguyenvansangi* specimens examined by us (Table 1); therefore, this inconsistency is likely due to a typographic mistake. Furthermore, Orlov et al. (2009: 236) stated that *C. nguyenvansangi* has six infralabials ("there are 6–6 infralabial shields; the fourth and fifth are the largest," p. 236). Our re-examination of the holotype ZISP 25682 clearly indicates that it has 7/7 infralabials (Fig. 3D, F); this character is shared by all other *C. nguyenvansangi* specimens examined by us (Table 1). Finally, all three specimens of *C. nguyenvansangi* available for our examination had 16–16–16 dorsal scale rows, including the holotype, while Orlov et al. (2009: 235) stated that this specimen has 15–15–15 dorsal scale rows.

Therefore, based on re-examination of the holotype and examination of two additional specimens of *C. nguyenvansangi*, both males, we provide below a revised diagnosis for this species as follows: body size comparatively large (TL 466–520 mm in males, 500 mm in a single female), tail relatively long (TaL/TL ratio 0.21–0.23 in males, 0.21 in a single female); dorsal scales in 16–16–16 rows; supralabials six, third and fourth entering orbit; infralabials seven; loreal present; 1/1 nasal, nostril completely enclosed in nasal scale; ventral scales 282–292 in males, 267 in a single female; subcaudals 86–87 in males, 81 in a single female, all divided; cloacal plate divided; dorsally reddish-brown with narrow and continuous dark stripe along spine; flanks dark gray, nearly black with bluish iridescence; ventral surfaces bluish-white; head black with rostral, nasals, prefrontals, preoculars, loreal, and the two anterior supralabials and the anterior parts of supraocular and frontal shields uniform light yellow or white lacking dark markings.

Based solely on morphological characters, we have identified the Hon Tre specimen of *Colubroelaps* as a new species. Further molecular analyses are required to clarify the degree of genetic divergence between the two species of *Colubroelaps*, as well as to shed light on the phylogenetic position of this enigmatic genus within Colubroidea. *Colubroelaps adleri* sp. nov. occurs at low elevations of ca. 30 m asl. and appears to be restricted to the unique maritime dry evergreen forests of southern Vietnamese coasts, in contrast to its sister species *C. nguyenvansangi*, which occurs at elevations of 100–930 m asl. in lowland and mid-elevation seasonally dry monsoon semideciduous forests of southern Vietnam as defined by Poyarkov et al. (2021, 2023). To date, the new species, *Colubroelaps adleri* sp. nov., is known only from a single specimen that was discovered during a herpetological survey in a very narrow area on a small offshore island within the Nha Trang Bay. This is a highly secretive snake that appears to have a fossorial lifestyle. Our intensive surveys in similar habitats over several other forested areas of the coastal region in central and southern Vietnam (Ba Ria-Vung Tau, Binh Thuan, Ninh Thuan, Khanh Hoa, and Phu Yen provinces) failed to discover any additional populations of *Colubroelaps*. Furthermore, the new species is likely affected by the growing anthropogenic pressure and forest destruction in Hon Tre Island due to the expanding construction of roads and tourism infrastructure. In particular, the recent development of the VinPearl Resort and the construction of the VinPearl Land amusement park, as well as the world's largest oversea cable car route, have led to an enormous growth in the number of tourists visiting the small island of Hon Tre. In 2023, VinPearl welcomed nearly 10,000 visitors a day (https://vietnamlife.asia/vinpearl-land-nha-trang/). The immense financial returns of tourism development largely outweigh concerns for biodiversity conservation; therefore, urgent measures have to be developed at the local and central government levels to protect the remaining patches of the maritime forests of Hon Tre Island. Moreover, due to its uniqueness and rarity, *Colubroelaps adleri* sp. nov. is also likely to become a target species for illegal pet trade. Considering the available information, we recommend that *Colubroelaps adleri* sp. nov. be classified as Endangered (EN) according to the IUCN's Red List categories (IUCN Standards and Petitions Subcommittee 2019). Given Hon Tre Island's small size and rapidly growing tourism infrastructure, the new species may actually be Vietnam's most endangered snake. Further research is urgently required to clarify the extent of *Colubroelaps adleri* sp. nov. distribution, natural history, and population trends, thereby facilitating the development of adequate conservation actions.

Our discovery of *Colubroelaps adleri* sp. nov. significantly expands the knowledge on the geographic distribution of the genus *Colubroelaps*, which is now for the first time recorded from the coastal areas of southern and central Vietnam (Fig. 1). The discovery of a new previously unnoticed species of the enigmatic genus *Colubroelaps* in coastal areas of southern and central Vietnam highlights the overlooked biodiversity and further underlines the importance of this area as a local center of herpetofaunal diversity and endemism (Poyarkov et al. 2021, 2023). Recent studies have reported on numerous new species of reptiles (e.g., Pauwels et al. 2018; Ngo et al. 2020; Ostrowski et al. 2020, 2021; Do et al. 2023; Kliukin et al. 2023; 2024; Ngo et al. 2023; Nguyen et al. 2023; Idiiatullina et al. 2024) and amphibians (e.g., Duong et al. 2018; Nguyen et al. 2018; Pham et al. 2020; Hoang et al. 2021; Gorin et al. 2023) from this region. Also, we would like to note that the remaining forest communities in coastal areas of Vietnam's southern and central coastal regions are under great threat of deforestation (Meijer 1973; De Koninck 1999; Laurance 2007; Meyfroidt and Lambin 2008). The discovery of *Colubroelaps adleri* sp. nov. further underlines the key importance of these unique coastal tropical forests for the conservation of herpetofaunal diversity in Southeast Asia. It is therefore critical to urgently conduct further comprehensive investigations and taxonomic studies on herpetofauna in this region to gain a deeper understanding of its biodiversity, especially the offshore islands, as they may be more vulnerable to antropogenic threats.

Acknowledgments

The fieldwork in Vietnam was completed within the frameworks and with partial financial support from the research project E-1.2 of the Joint Vietnam-Russia Tropical Science and Technology Research Center for 2024 (Task No. 3). Permission to conduct fieldwork in Khanh Hoa Province was granted by the Bureau of Forestry, Ministry of Agriculture and Rural Development of Vietnam, as well as by the local administration, the Forest Protection Department of the Peoples' Committee of Khanh Hoa Province (permit number #5565/UBND-KT of 08.06.2023). We are deeply grateful to Eduard A. Galoyan (ZMMU, Russia), Alina V. Alexandrova (MSU, Russia), and Thuc Van Phan (SIFASV, Vietnam) for providing photos and information. We also warmly thank Duc Trong Nguyen (SIFASV, Vietnam) for help in the preparation of the map. We thank Valentina F. Orlova (ZMMU) and Natalia B. Ananjeva (ZISP) for permission to examine specimens under their care. We thank Sabira S. Idiiatullina (MSU) for assistance in the laboratory. NAP and AMB are grateful to Andrei N. Kuznetsov, Hoi Dang Nguyen, Svetlana P. Kuznetsova, and Leonid P. Korzoun for their support and organization of fieldwork. We are thankful to Andrei N. Kuznetsov for help with plant identification. We are grateful to two anonymous reviewers for their comments on the earlier version of the manuscript. This work was supported by the Russian Science Foundation to N. A. Poyarkov (Grant No. 22-14-00037, specimen collection and preservation and morphological analyses, data analysis) and in part by the Rufford Foundation to T. V. Nguyen (Grant No. 45888-2; data analysis).

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OGH Herpetozoa
Austrian Herpetological Society

Herpetozoa 37: 391–420 (2024)
DOI 10.3897/herpetozoa.37.e137394

Phylogeographic pattern and taxonomic revision of the *Kaloula baleata* species complex (Amphibia, Anura, Microhylidae) with description of two new species from Indochina

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https://zoobank.org/E97A128A-13B5-4781-8FEC-ADC5CB37AB45

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Academic editor: Peter Mikulíček ♦ **Received** 18 September 2024 ♦ **Accepted** 22 November 2024 ♦ **Published** 11 December 2024

Abstract

Despite increased attention by molecular taxonomists, the herpetofauna of Southeast Asia still hides many undescribed species among far-ranging taxa. In this study, we re-examine the mitochondrial diversity of painted frogs of the microhylid genus *Kaloula*, based on ~2,455 bp of published and new 12S and 16S rRNA sequences, and describe two new species from the tropical forests of southern Vietnam and central Laos based on integrative evidence. These species, which belong to the *K. baleata* complex, feature species-level mitochondrial divergence (> 4.4% at 16S rRNA) and are both morphologically well-differentiated from each other and from the recently described *K. indochinensis*, to which they were previously confounded. Comparative examinations also indicate distinct male advertisement calls and unique coloration features. Based on genetic barcoding, we preliminarily revise the species distribution ranges in the *K. baleata* complex, which support a general pattern of biogeographic partitioning that has been widely retrieved among the Indochinese amphibians studied so far. Molecular diversity within *K. baleata* further suggests genetic structure across Sundaland, namely three shallow mitochondrial haplogroups worthy of fine-scale phylogeographic and taxonomic investigations. Furthermore, our study highlights the propensity of amphibian species "hidden in plain sight," even among recently studied taxa, thus calling for caution when specifying type specimens—the type series of *K. indochinensis*, described in 2013, includes specimens of the one of the new species. Our study emphasizes the continued need for thorough herpetological surveys even in supposedly well-known parts of Indochina and sets the ground for future research in *Kaloula* painted frogs, notably to test evolutionary and taxonomic hypotheses with genomic loci.

Key Words

16S rRNA, bioacoustics, DNA barcoding, Laos, morphology, Southeast Asia, systematics, taxonomy, Vietnam

PENSOFT.

Introduction

Many widely distributed amphibian genera in Asia have undergone a considerable increase in their numbers of recognized species since the turn of the 21st century. For example, among microhylids of the genus *Microhyla* Tschudi, 1838, the number of recognized species jumped from 22 to 51 within two decades (Poyarkov et al. 2020a; Zhang et al. 2022; Trofimets et al. 2024). The number of species within the genus *Micryletta* Dubois, 1987, rose from three in the 20th century to 13 species nowadays (Suwannapoom et al. 2020; Poyarkov et al. 2021a; Sankar et al. 2022). The genus *Kalophrynus* Tschudi, 1838, included from 13 to currently 27 recognized species (Fukuyama et al. 2021). Two new microhylid genera were discovered in Southeast Asia in the past several years, namely, *Siamophryne* Suwannapoom, Sumontha, Tunprasert, Ruangsuwan, Pawangkhanant, Korost & Poyarkov, 2018 (one single known species) and *Vietnamophryne* Poyarkov, Suwannapoom, Pawangkhanant, Aksornneam, Duong, Korost & Che, 2018 (six species described so far; see Suwannapoom et al. 2018; Poyarkov et al. 2018a, 2021b; Gorin et al. 2021; Frost 2024).

Quite a few genera of Asian microhylids, however, have so far evaded the focus of taxonomic studies, and their species numbers thus remained relatively stable. One notable example is the genus *Kaloula* Gray, 1831, also known as Asian painted frogs (Frost 2024). *Kaloula* is a widely distributed genus of microhylids, with 18 species recognized up to date. Painted frogs inhabit East Asia, from Korea and northeastern China to Southeast Asia, including Indochina to the Sunda Islands and the Philippines to the south, and Bangladesh and northeastern India to the west (Othman et al. 2022; Frost 2024). The genus *Kaloula* is characterized by the following morphological attributes: pupil round; tongue oval, entire, and free behind; two transverse ridges across the palate in front of the pharynx; a strong bony ridge behind each choanae; the first finger shorter than the second and the outer toe shorter than the third; medium body size (SVL 35–60 mm); smooth or slightly rough dorsum with irregular dark markings; tips of the fingers dilated into disks and truncated; fingers free of the web; nearly full webbing on toes in males and reduced webbing in females (e.g., Gray 1831; Sengupta et al. 2009; Gorin et al. 2021). Recent contributions to the diversity of this genus include the review by Blackburn et al. (2013), which focused on *Kaloula* species inhabiting Southeast Asian islands, mainly the Philippine Archipelago. In this study, the authors conducted phylogenetic analysis on 140 *Kaloula* individuals from all over Southeast Asia and indicated the presence of at least six undescribed lineages of putative species status. Following the study of Blackburn et al. (2013), two new species of the *K. baleata* complex were described from Indochina, namely *K. indochinensis* Chan, Blackburn, Murphy, Stuart, Emmett, Ho & Brown, 2013 (Chan et al. 2013), and from Peninsular Malaysia, namely *K. latidisca* Chan, Grismer & Brown, 2014 (Chan et al. 2014). In both cases, authors provided only limited morphological data to substantiate species divergence, did not provide any bioacoustic data, and based their phylogenetic estimations entirely on the previously published mtDNA data of Blackburn et al. (2013).

In parallel, a new species, *K. nonggangensis* Mo, Zhang, Zhou, Chen, Tang, Meng & Chen, 2013, was described from the Guangxi Province of China (Mo et al. 2013) on the basis of morphological and genetic data. More recently, the subspecies *K. baleata ghoshi* Cherchi, 1954, endemic to the Andaman Islands, was elevated to full species status based on morphological differentiation, accompanied by new data on natural history, bioacoustics, and distribution (Chandramouli and Prasad 2018). Lastly, the phylogeography of the genus *Kaloula* in East Asia was re-summarized by Ohtman et al. (2022), based mostly on previously published data, and without emphasizing undescribed diversity.

In this work, we present an updated mitochondrial DNA (hereafter mtDNA)-based genealogy for the genus *Kaloula*, combining sequences available in GenBank with new sequences obtained from an extensive sampling in Indochina, as well as integrative taxonomic revisions in the *K. baleata* species complex, specifically for two populations from central Laos and southern Vietnam previously assigned to *K. indochinensis* (Chan et al. 2013). These populations are deeply divergent in the mitochondrial phylogeny and feature morphological and bioacoustic differentiation from related taxa, hence substantiating their description as new species.

Materials and methods

Sample collection

Fieldwork in Vietnam was conducted by NAP, AMB, and VAG in 2009–2023; fieldwork in Laos was conducted by NLO and KDM in 2009. We obtained geographic coordinates and elevation data using a Garmin GPSMAP 60CSx (USA) and recorded them in the WGS84 datum. The specimens collected were initially fixed in 4% formalin for 24 h and later transferred to 75% ethanol for storage; muscle or liver tissues were taken prior to the fixation in formalin and preserved in 95% ethanol for genetic analysis. Specimens were subsequently deposited in the herpetological collections of the Zoological Museum of Lomonosov Moscow State University (ZMMU, Moscow, Russia) and of the Zoological Institute, Russian Academy of Sciences (ZISP, St. Petersburg, Russia).

Laboratory methods

For the molecular phylogenetic analyses, total genomic DNA was isolated using the standard phenol-chloroform-proteinase K extraction procedures with consequent isopropanol precipitation for a final concentration of about 1 mg/ml (protocols followed the works of Hillis et al. 1996; Sambrook and Russell 2001). We visualized the isolated total genomic DNA using agarose electrophoresis in the presence of ethidium bromide. We measured the concentration of total DNA in 1 µL using NanoDrop 2000 (Thermo Scientific) and consequently adjusted it to ca. 100 ng DNA/µL.

We amplified mtDNA fragments covering the 16S rRNA gene and the adjacent tRNA genes to obtain a 1,003 bp-long continuous fragment. The 16S rRNA gene is widely used for biodiversity surveys in amphibians (Vences et al. 2005; Vieites et al. 2009) and has been analyzed in the most recent phylogenetic studies on Microhylinae (Matsui et al. 2011; Garg et al. 2019; Gorin et al. 2020, 2021). We performed DNA amplification in 20 µL reactions using ca. 50 ng genomic DNA, 10 nmol of each primer, 15 nmol of each dNTP, 50 nmol additional $MgCl_2$, Taq PCR buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.1 mM $MgCl_2$, and 0.01% gelatine), and 1 unit of Taq DNA polymerase. Primers used in PCR and sequencing include 16sL-2188 (CTGACCGTGCAAAGGTAGCGTAATCACT) and 16H-1 (CTCCGGTCTGAACTCAGATCACGTAGG) (Hedges et al. 1994; Matsui et al. 2005). The PCR conditions involved an initial denaturation step of 5 min at 94 °C, followed by 43 cycles of denaturation for 1 min at 94 °C, primer annealing for 1 min using the TouchDown program, reducing 1 °C every cycle, extension for 1 min at 72 °C, and a final extension step for 5 min at 72 °C (Gorin et al. 2020, 2021).

PCR products were loaded onto 1.0% agarose gels in the presence of ethidium bromide and visualized in agarose electrophoresis. When distinct bands were obtained, we purified PCR products using 2 µL of a 1:4 dilution of ExoSapIt (Amersham) per 5 µL of PCR product prior to cycle sequencing. A 10 µL sequencing reaction included 2 µL of template, 2.5 µL of sequencing buffer, 0.8 µL of 10 pmol primer, 0.4 µL of BigDye Terminator version 3.1 Sequencing Standard (Applied Biosystems), and 4.2 µL of water. The cycle sequencing used 35 cycles of 10 sec at 96 °C, 10 s at 50 °C, and 4 min at 60 °C. We purified the cycle sequencing products by ethanol precipitation. We carried out sequence data collection and visualization on an ABI 3730xl Automated Sequencer (Applied Biosystems). We deposited the obtained sequences in GenBank under the accession numbers PQ268497–PQ268520.

Phylogenetic analyses

To reconstruct the matrilineal genealogy, we used newly obtained 16S rRNA sequences of *Kaloula* spp. from Laos and Vietnam and the sequences of 12S rRNA and 16S rRNA mtDNA fragments of the *K. baleata* species complex members from Thailand, Malaysia, and Indonesia, as well as other *Kaloula* species obtained from GenBank. Table 1 summarizes the information on GenBank Accession Numbers, museum vouchers, and the locality of origin for the sequences used in this study. We also added sequences of *Uperodon taprobanicus* (Parker, 1934) as a sister group; a sequence of *Kalophrynus interlineatus* (Blyth, 1855) was used to root the tree; the final alignment included 2,455 bp. In total, we obtained data for 16S rRNA for 233 specimens, which included 15 out of 18 currently recognized *Kaloula* species, including the topotype specimens of *K. baleata* (Müller, 1836) (type locality: Java, Indonesia) (see Table 1); geographic distribution of the sampled populations of the *K. baleata* species complex is shown in Fig. 1.

We initially aligned nucleotide sequences using ClustalX 1.81 (Thompson et al. 1994) with default parameters and then optimized them manually in BioEdit 7.0.5.2 (Hall 1999) and MEGA 11.0 (Tamura et al. 2013). We utilized ModelFinder (Kalyaanamoorthy et al. 2017) to determine the most suitable evolutionary models for our data set analysis. According to the Akaike Information Criterion (AIC), the best-fitting models of DNA evolution for 12S rRNA, tRNA-Val, and 16S rRNA genes of mtDNA are GTR+I+G, K2P+G, and GTR+I+G, respectively. We determined mean uncorrected genetic distances (p-distances) between sequences with MEGA 11.0.

We inferred the matrilineal genealogy using Bayesian Inference (BI) and Maximum Likelihood (ML) approaches. We conducted BI using MrBayes 3.1.2 (Ronquist and Huelsenbeck 2003). Metropolis-coupled Markov chain Monte Carlo (MCMCMC) analyses were run with one cold chain and three heated chains for one million generations and sampled every 1,000 generations. We performed two independent MCMCMC runs, and the initial 100 trees were discarded as burn-in. We assessed confidence in tree topology based on the frequency of nodal resolution (posterior probability; BI PP) (Huelsenbeck and Ronquist 2001). We used IQ-TREE (Nguyen et al. 2015) to reconstruct ML-trees. A total of 10,000 ultrafast bootstrap replications for ML analysis (UFB) (Minh et al. 2013) assessed the confidence in tree topology for ML analysis. In both datasets, we regarded tree nodes with BI PP and UFB values over 0.95 to be sufficiently resolved a priori. We considered BI PP and UFB values between 0.95 and 0.90 as tendencies. Lower values were considered to indicate unresolved nodes (Huelsenbeck and Hillis 1993; Minh et al. 2013).

Morphological analysis

Measurements were taken using a digital caliper under a light dissecting microscope to the nearest 0.01 mm, subsequently rounded to 0.1 mm. The adult morphometrics and character terminology follow Poyarkov et al. (2018b): (1) snout-vent length (SVL; measured from the tip of the snout to cloaca); (2) head length (HL; measured from the tip of snout to hind border of jaw angle); (3) snout length (SL; measured from the anterior corner of eye to the tip of snout); (4) eye length (EL; measured as the distance between anterior and posterior corners of the eye); (5) nostril-eye length (N-EL; measured as the distance between the anterior corner of the eye and the nostril center); (6) head width (HW; measured as the maximum width of head on the level of mouth angles in ventral view); (7) internarial

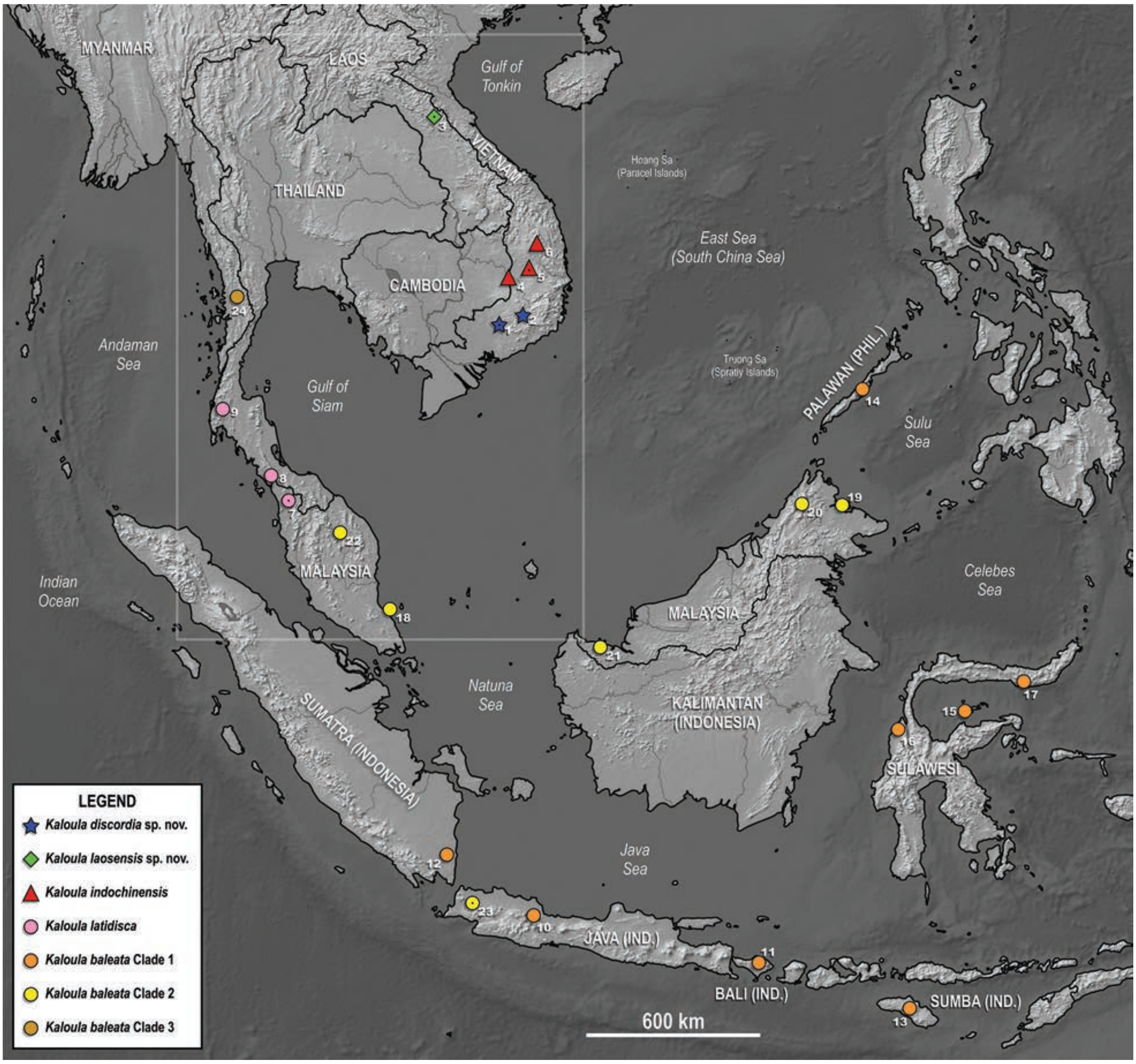

Figure 1. Geographic origins of the specimens of the *Kaloula baleata* species complex included in phylogenetic analyses. Dots in the symbols denote type localities. Symbol colors correspond to those used in Fig. 2. Numbers near symbols correspond to the locality numbers given in Table 1. The white rectangle represents the area of inset map detailing the distribution of the *K. baleata* species complex in Indochina in Fig. 5.

distance (IND; measured as the distance between the central points of nostrils); (8) interorbital distance (IOD; measured as the shortest distance between the medial edges of eyeballs in dorsal view); (9) upper eyelid width (UEW; measured as the maximum distance between the medial edge of eyeball and the lateral edge of upper eyelid); (10) tympanum length, measured as the horizontal tympanum diameter (TMP); (11) forelimb length (FLL; measured as the length of straightened forelimb to the tip of third finger); (12) lower arm and hand length (LAL; measured as the distance between elbow and the tip of third finger); (13) hand length (HAL; measured as the distance between the proximal end of outer palmar (metacarpal) tubercle and the tip of third finger); (14) first finger length (1FL, measured as the distance between the tip and the distal end of inner palmar tubercle); (15) inner palmar tubercle length (IPTL; measured as the maximum distance between proximal and distal ends of inner palmar tubercle); (16) outer palmar tubercle length (OPTL; measured as the maximum diameter of outer palmar tubercle); (17) third finger disk diameter (3FDD); (18) hindlimb length (HLL; measured as the length of straightened hindlimb from groin to the tip of fourth toe); (19) tibia length (TL; measured as the distance between the knee and tibiotarsal articulation); (20) foot length (FL; measured as the distance between the distal end of tibia and the tip of fourth toe); (21) first toe length (1TOEL), measured as the distance between the distal end of inner metatarsal tubercle and the tip of first toe; (22) fourth toe disk diameter (4TDD); (23) outer metatarsal tubercle length (OMTL). Webbing and subarticular tubercle formulas follow those of Savage (1975). We took all measurements on the right side of the examined specimen. We determined the sex of specimens by examining the presence of eggs in the abdomen of a dissected specimen.

Table 1. Museum voucher information, geographic localities, and GenBank accession numbers of specimens and sequences used in this study. Exact locality information is unknown for specimens obtained via the pet trade or those published in some earlier works. Locality numbers given in brackets in bold after the locality name correspond to the numbers shown in Fig. 1.

No.	Species	Locality	Museum / Sample ID	Accession numbers		Reference
	Ingroup			12S rRNA	16S rRNA	
***Kaloula baleata* species complex**						
1	*Kaloula discordia* sp. nov.	Vietnam, Dong Nai Prov., Cat Tien NP (**1**)	ZMMU-A-4642		PQ268510	this work
2	*Kaloula discordia* sp. nov.	Vietnam, Dong Nai Prov., Cat Tien NP (**1**)	ZMMU-A-4739		PQ268511	this work
3	*Kaloula discordia* sp. nov.	Vietnam, Dong Nai Prov., Cat Tien NP (**1**)	ZISP 15285		PQ268512	this work
4	*Kaloula discordia* sp. nov.	Vietnam, Dong Nai Prov., Cat Tien NP (**1**)	ZMMU-A-8134		PQ268513	this work
5	*Kaloula discordia* sp. nov.	Vietnam, Dong Nai Prov., Cat Tien NP (**1**)	ZMMU-A-8135		PQ268514	this work
6	*Kaloula discordia* sp. nov.	Vietnam, Lam Dong Prov., Loc Bac (**2**)	ZMMU NAP-02826		PQ268515	this work
7	*Kaloula discordia* sp. nov.	Vietnam, Lam Dong Prov., Loc Bac (**2**)	ZMMU NAP-03384		PQ268516	this work
8	*Kaloula laosensis* sp. nov.	Laos, Khammouane Prov., Nakai-Nam Theun NP (**3**)	ZISP 15284		PQ268517	this work
9	*Kaloula laosensis* sp. nov.	Laos, Khammouane Prov., Nakai-Nam Theun NP (**3**)	ZMMU-A-8144		PQ268518	this work
10	*Kaloula indochinensis*	Vietnam, Dak Lak Prov., Yok Don NP (**4**)	ZMMU-A-8147		PQ268497	this work
11	*Kaloula indochinensis*	Vietnam, Dak Lak Prov., Yok Don NP (**4**)	ZMMU-A-8148		PQ268498	this work
12	*Kaloula indochinensis*	Vietnam, Gia Lai Prov., Krong Pa (**5**)	ROM32925		KC180032	de Sa et al. 2012
13	*Kaloula indochinensis*	Vietnam, Gia Lai Prov., Krong Pa (**5**)	ZISP-Gia Lai 1		PQ268499	this work
14	*Kaloula indochinensis*	Vietnam, Gia Lai Prov., Krong Pa (**5**)	ZISP-Gia Lai 2		PQ268500	this work
15	*Kaloula indochinensis*	Vietnam, Gia Lai Prov., Krong Pa (**5**)	ROM32925	KC822572		Blackburn et al. 2013
16	*Kaloula indochinensis*	Vietnam, Gia Lai Prov., Krong Pa (**5**)	ROM32932	KC822573		Blackburn et al. 2013
17	*Kaloula indochinensis*	Vietnam, Gia Lai Prov., Krong Pa (**5**)	ROM32943	KC822574		Blackburn et al. 2013
18	*Kaloula indochinensis*	Vietnam, Gia Lai Prov., Kon Ka Kinh NP (**6**)	ZMMU-A-6315		PQ268501	this work
19	*Kaloula indochinensis*	Vietnam, Gia Lai Prov., Kon Ka Kinh NP (**6**)	ZMMU-A-6316		PQ268502	this work
55	*Kaloula latidisca*	Malaysia, Kedah, Gubir (**7**)	LSUHC5074	KC822576		Blackburn et al. 2013
56	*Kaloula latidisca*	Thailand, Satun Prov., Baan Suan Tondin (**8**)	ZMMU-A-8145		PQ268503	this work
57	*Kaloula latidisca*	Thailand, Suratthani Prov., Khao Sok (**9**)	ZMMU-A-8146		PQ268504	this work
20	*Kaloula baleata* clade 1	Indonesia, Java isl., Jawa Barat (**10**)	TNHC67086	KC822569		Blackburn et al. 2013
21	*Kaloula baleata* clade 1	Indonesia, Bali Prov., Bali isl. (**11**)	JAM3232	KC822570		Blackburn et al. 2013
22	*Kaloula baleata* clade 1	Indonesia, Sumatra, Way Jepara (**12**)	ZMMU A-04412		PQ268507	this work
23	*Kaloula baleata* clade 1	Indonesia, Sumba (**13**)	KUHE32313	AB634629	AB634687	Matsui et al. 2011
24	*Kaloula baleata* clade 1	Philippines, Palawan isl. (**14**)	ACD1303	KC822582		Blackburn et al. 2013
25	*Kaloula baleata* clade 1	Philippines, Palawan isl. (**14**)	ACD1307	KC822583		Blackburn et al. 2013
26	*Kaloula baleata* clade 1	Indonesia, Sulawesi (–)	JAM3573		KY132185	Alexander et al. 2017
27	*Kaloula baleata* clade 1	Indonesia, Sulawesi Tengah Prov., Pulau Batudaka (**15**)	JAM-3853		KC822568	Blackburn et al. 2013
28	*Kaloula baleata* clade 1	Indonesia, Sulawesi Tengah Prov., Donggala (**16**)	LSUMZ-83998		KC822566	Blackburn et al. 2013
29	*Kaloula baleata* clade 1	Indonesia, Sulawesi Utara Prov., Bogani Nani Wartabone NP (**17**)	LSUMZ-83999		KC822567	Blackburn et al. 2013
30	*Kaloula baleata* clade 2	Malaysia (–)	FRIM1066	KC822579		Blackburn et al. 2013
31	*Kaloula baleata* clade 2	Malaysia (–)	DWNP975	KC822580		Blackburn et al. 2013
32	*Kaloula baleata* clade 2	Malaysia, Johor, Pulau Aceh (**18**)	LSUHC5712	KC822577		Blackburn et al. 2013
33	*Kaloula baleata* clade 2	Malaysia, Sabah, Sepilok (**19**)	LSUHC6156	KC822578		Blackburn et al. 2013
35	*Kaloula baleata* clade 2	Malaysia, Sabah, Kinabalu (**20**)	ZMHA10028		GU154880	Das and Haas 2010
34	*Kaloula baleata* clade 2	Malaysia, Sarawak, Kuching (**21**)	ID8317	KC822581		Blackburn et al. 2013
36	*Kaloula baleata* clade 2	Malaysia, Pahang, Taman Negara (**22**)	ZMMU-A-6152		PQ268508	this work
37	*Kaloula baleata* clade 2	Malaysia, Pahang, Taman Negara (**22**)	ZMMU-A-6153		PQ268509	this work
38	*Kaloula baleata* clade 2	Indonesia, Java, Bogor (**23**)	released (Java)		PQ268505	this work
39	*Kaloula baleata* clade 2	Indonesia, Java, Bogor (**23**)	released (Java)		PQ268506	this work
40	*Kaloula baleata* clade 3	Myanmar, Tanintharyi Div., Yeybu (**24**)	USNM-587865		MT608909	Mulcahy et al. unpublished
41	*Kaloula baleata* clade 3	Myanmar, Tanintharyi Div., Yeybu (**24**)	USNM-587869		MT608910	Mulcahy et al. unpublished
42	*Kaloula baleata* clade 3	Myanmar, Tanintharyi Div., Yeybu (**24**)	USNM-587870		MT608911	Mulcahy et al. unpublished
43	*Kaloula baleata* clade 3	Myanmar, Tanintharyi Div., Yeybu (**24**)	USNM-587871		MT608912	Mulcahy et al. unpublished
44	*Kaloula baleata* clade 3	Myanmar, Tanintharyi Div., Yeybu (**24**)	USNM-587602		MT608913	Mulcahy et al. unpublished
45	*Kaloula baleata* clade 3	Myanmar, Tanintharyi Div., Yeybu (**24**)	USNM-587603		MT608914	Mulcahy et al. unpublished
46	*Kaloula baleata* clade 3	Myanmar, Tanintharyi Div., Yeybu (**24**)	USNM-587863		MT608915	Mulcahy et al. unpublished
47	*Kaloula baleata* clade 3	Myanmar, Tanintharyi Div., Yeybu (**24**)	USNM-587864		MT608916	Mulcahy et al. unpublished
48	*Kaloula baleata* clade 3	Myanmar, Tanintharyi Div., Yeybu (**24**)	USNM-587866		MT608917	Mulcahy et al. unpublished

No.	**Species**	**Locality**	**Museum / Sample ID**	**Accession numbers**		**Reference**
	Ingroup			**12S rRNA**	**16S rRNA**	
49	*Kaloula baleata* clade 3	Myanmar, Tanintharyi Div., Yeybu (**24**)	USNM-587867		MT608918	Mulcahy et al. unpublished
50	*Kaloula baleata* clade 3	Myanmar, Tanintharyi Div., Yeybu (**24**)	USNM-587868		MT608919	Mulcahy et al. unpublished
51	*Kaloula baleata* clade 3	Myanmar, Tanintharyi Div., Yeybu (**24**)	USNM-587604		MT608920	Mulcahy et al. unpublished
52	*Kaloula baleata* clade 3	Myanmar, Tanintharyi Div., Yeybu (**24**)	USNM-587861		MT608921	Mulcahy et al. unpublished
53	*Kaloula baleata* clade 3	Myanmar, Tanintharyi Div., Yeybu (**24**)	USNM-587862		MT608922	Mulcahy et al. unpublished
54	*Kaloula baleata* clade 3	Myanmar, Tanintharyi Div., Yeybu (**24**)	USNM-586944		MG935845	Mulcahy et al. 2018
Other *Kaloula* species						
58	*Kaloula borealis*	South Korea, Jeju isl.	KUHE-33139		AB634688	Matsui et al. 2011
59	*Kaloula borealis*	South Korea, Jeju isl.	NIBRAM-000123		JQ815297	Jeong et al. 2013
60	*Kaloula borealis*	South Korea, Jeju isl.	NIBRAM-100348		JQ815298	Jeong et al. 2013
61	*Kaloula borealis*	–	-	JQ692869		Hwang and Lee, 2012
62	*Kaloula borealis*	China, Beijing, Xiangshan	KIZHERP-0173		JX678908	Li et al. 2012
63	*Kaloula borealis*	China, Beijing, Xiangshan	KIZHERP-0174		JX678909	Li et al. 2012
64	*Kaloula conjuncta*	Philippines, Negros isl.	RMB 2252, PNM / CMNH	AY326064		Darst and Canatella, 2003
65	*Kaloula conjuncta*	Philippines, Luzon isl., Palola	ACD 769	KC822537		Blackburn et al. 2013
66	*Kaloula conjuncta*	Philippines, Mindanao isl., Bukidnon Prov.	ACD 996	KC822536		Blackburn et al. 2013
67	*Kaloula conjuncta*	Philippines, Semirara isl., Antique Prov.	KU301849	KC822532		Blackburn et al. 2013
68	*Kaloula conjuncta*	Philippines, Semirara isl., Antique Prov.	KU301854	KC822533		Blackburn et al. 2013
69	*Kaloula conjuncta*	Philippines, Polillo isl., Quezon Prov.	KU303279	KC822527		Blackburn et al. 2013
70	*Kaloula conjuncta*	Philippines, Luzon isl., Laguna Prov.	KU320031	KC822538		Blackburn et al. 2013
71	*Kaloula conjuncta*	Philippines, Mindoro isl., Mindoro Occidental Prov.	KU323280	KC822535		Blackburn et al. 2013
72	*Kaloula conjuncta*	Philippines, Mindoro isl., Mindoro Oriental Prov.	RMB509	KC822534		Blackburn et al. 2013
73	*Kaloula conjuncta*	Philippines, Luzon isl., Quezon Prov.	TNHC59628	KC822539		Blackburn et al. 2013
74	*Kaloula conjuncta*	Philippines, Luzon isl., Albay Prov.	TNHC62972	KC822528		Blackburn et al. 2013
75	*Kaloula conjuncta*	Philippines, Luzon isl., Albay Prov.	TNHC62973	KC822526		Blackburn et al. 2013
76	*Kaloula conjuncta*	Philippines, Luzon isl., Albay Prov.	TNHC62975	KC822530		Blackburn et al. 2013
77	*Kaloula conjuncta*	Philippines, Luzon isl., Albay Prov.	TNHC62976	KC822529		Blackburn et al. 2013
78	*Kaloula conjuncta*	Philippines, Luzon isl., Albay Prov.	TNHC62986	KC822531		Blackburn et al. 2013
79	*Kaloula conjuncta*	Philippines, Camigun Sur isl., Camiguin Sur Prov.	KU309658	KC822588		Blackburn et al. 2013
80	*Kaloula conjuncta*	Philippines, Mindanao isl., Davao del Sur Prov.	TNHC59632	KC822521		Blackburn et al. 2013
81	*Kaloula conjuncta*	Philippines, Mindanao isl., Davao del Sur Prov.	TNHC59635	KC822520		Blackburn et al. 2013
82	*Kaloula conjuncta*	Philippines, Mindanao isl., Davao del Sur Prov.	TNHC59636	KC822522		Blackburn et al. 2013
83	*Kaloula conjuncta*	Philippines, Mindanao isl., Davao del Sur Prov.	TNHC59669	KC822524		Blackburn et al. 2013
84	*Kaloula conjuncta*	Philippines, Mindanao isl., Davao del Sur Prov.	TNHC59670	KC822523		Blackburn et al. 2013
85	*Kaloula conjuncta*	Philippines, Mindanao isl., Davao City Prov.	TNHC59870	KC822525		Blackburn et al. 2013
86	*Kaloula conjuncta*	Philippines, Negros isl., Negros Oriental Prov.	KU328639	KC822518		Blackburn et al. 2013
87	*Kaloula conjuncta*	Philippines, Negros isl., Negros Oriental Prov.	KU328640	KC822519		Blackburn et al. 2013
88	*Kaloula kalingensis*	Philippines, Palaui isl., Cagayan Prov.	KU328643	KC822603		Blackburn et al. 2013
89	*Kaloula kalingensis*	Philippines, Palaui isl., Cagayan Prov.	KU328644	KC822604		Blackburn et al. 2013
90	*Kaloula kalingensis*	Philippines, Luzon isl., Kalinga Prov.	TNHC60118	KC822605		Blackburn et al. 2013
91	*Kaloula kalingensis*	Philippines, Luzon isl., Kalinga Prov.	RMB2210	KC822608		Blackburn et al. 2013
92	*Kaloula kalingensis*	Philippines, Luzon isl., Kalinga Prov.	RMB3137	KC822609		Blackburn et al. 2013
93	*Kaloula kalingensis*	Philippines, Luzon isl., Kalinga Prov.	TNHC59647	KC822610		Blackburn et al. 2013
94	*Kaloula kokacii*	Philippines, Luzon isl., Camarines del Norte Prov.	KU313824	KC822596		Blackburn et al. 2013
95	*Kaloula kokacii*	Philippines, Luzon isl., Camarines Sur Prov.	KU328629	KC822595		Blackburn et al. 2013
96	*Kaloula kokacii*	Philippines, Luzon isl., Albay Prov.	KU328630	KC822590		Blackburn et al. 2013
97	*Kaloula kokacii*	Philippines, Luzon isl., Quezon Prov.	KU328634	KC822593		Blackburn et al. 2013
98	*Kaloula kokacii*	Philippines, Luzon isl., Camarines Sur Prov.	TNHC62684	KC822594		Blackburn et al. 2013
99	*Kaloula kokacii*	Philippines, Luzon isl., Albay Prov.	TNHC62685	KC822589		Blackburn et al. 2013
100	*Kaloula kokacii*	Philippines, Luzon isl., Albay Prov.	TNHC62687	KC822591		Blackburn et al. 2013
101	*Kaloula kokacii*	Philippines, Luzon isl., Sorsogon Prov.	TNHC62688	KC822592		Blackburn et al. 2013
102	*Kaloula mediolineata*	Thailand, Tak Prov.	KUHE35178	AB634631	AB634689	Matsui et al. 2011
103	*Kaloula mediolineata*	Vietnam, Gia Lai Prov.	ROM32838	KC822571		Blackburn et al. 2013
104	*Kaloula mediolineata*	Thailand, Nakhorn Ratchasima Prov.	KU328285	KC822508		Blackburn et al. 2013
105	*Kaloula mediolineata*	Thailand, Nakhorn Ratchasima Prov.	KU328280	KC822509		Blackburn et al. 2013

No.	Species	Locality	Museum / Sample ID	Accession numbers		Reference
	Ingroup			**12S rRNA**	**16S rRNA**	
106	*Kaloula mediolineata*	Thailand, Ubon Ratchatani	FMNH265819	KC822510		Blackburn et al. 2013
107	*Kaloula nonggangensis*	China, Guanxi Prov., Nonggang	NHMG-1106040		KC567231	Chen et al. 2013
108	*Kaloula nonggangensis*	China, Guanxi Prov., Nonggang	NHMG-1108035		KC567232	Chen et al. 2013
109	*Kaloula nonggangensis*	China, Guanxi Prov., Nonggang	NHMG-1108036		KC567233	Chen et al. 2013
110	*Kaloula nonggangensis*	China, Guanxi Prov., Nonggang	NHMG-CHN-T20120901		KC567234	Chen et al. 2013
111	*Kaloula picta*	–	KUHE UN	AB634686		Matsui et al. 2011
112	*Kaloula picta*	–	USFS56931		KC180019	de Sa et al. 2012
113	*Kaloula picta*	Philippines, Luzon isl., Cavite Prov.	DLSUD021	KP298039		Brown et al. 2015
114	*Kaloula picta*	Philippines, Palawan isl., Palawan Prov.	ACD1206	KC822541		Blackburn et al. 2013
115	*Kaloula picta*	Philippines, Leyte isl., Leyte Prov.	ACD1242	KC822563		Blackburn et al. 2013
116	*Kaloula picta*	Philippines, Palawan isl., Palawan Prov.	ACD1261	KC822542		Blackburn et al. 2013
117	*Kaloula picta*	Philippines, Palawan isl., Palawan Prov.	ACD1304	KC822540		Blackburn et al. 2013
118	*Kaloula picta*	Philippines, Luzon isl., Pampanga Prov.	ACD1390	KC822546		Blackburn et al. 2013
119	*Kaloula picta*	Philippines, Leyte isl., Leyte Prov.	ACD1501	KC822562		Blackburn et al. 2013
120	*Kaloula picta*	Philippines, Luzon isl., Cavite Prov.	DLSUD020	KC822552		Blackburn et al. 2013
121	*Kaloula picta*	Philippines, Camiguin Sur Isl., Camiguin Sur Prov.	KU301873	KC822565		Blackburn et al. 2013
122	*Kaloula picta*	Philippines, Luzon isl., Laguna Prov.	KU326261	KC822554		Blackburn et al. 2013
123	*Kaloula picta*	Philippines, Luzon isl., Laguna Prov.	KU326262	KC822555		Blackburn et al. 2013
124	*Kaloula picta*	Philippines, Luzon isl., Quezon Prov.	KU326265	KC822548		Blackburn et al. 2013
125	*Kaloula picta*	Philippines, Luzon isl., Cagayan Prov.	RMB4223	KC822551		Blackburn et al. 2013
126	*Kaloula picta*	Philippines, Luzon isl., Cagayan Prov.	RMB4224	KC822550		Blackburn et al. 2013
127	*Kaloula picta*	Philippines, Leyte isl., Leyte Prov.	RMB4294	KC822564		Blackburn et al. 2013
128	*Kaloula picta*	Philippines, Leyte isl., Leyte Prov.	RMB4344	KC822561		Blackburn et al. 2013
129	*Kaloula picta*	Philippines, Bohol isl., Bohol Prov.	TNHC56430	KC822559		Blackburn et al. 2013
130	*Kaloula picta*	Philippines, Bohol isl., Bohol Prov.	TNHC56431	KC822560		Blackburn et al. 2013
131	*Kaloula picta*	Philippines, Cebu isl., Cebu Prov.	TNHC56468	KC822543		Blackburn et al. 2013
132	*Kaloula picta*	Philippines, Mindanao isl., Davao del Sur Prov.	TNHC59653	KC822558		Blackburn et al. 2013
133	*Kaloula picta*	Philippines, Mindanao isl., Davao del Sur Prov.	TNHC59654	KC822557		Blackburn et al. 2013
134	*Kaloula picta*	Philippines, Mindanao isl., Davao del Sur Prov.	TNHC59871	KC822556		Blackburn et al. 2013
135	*Kaloula picta*	Philippines, Luzon isl., Albay Prov.	TNHC62470	KC822549		Blackburn et al. 2013
136	*Kaloula picta*	Philippines, Luzon isl., Zambales Prov.	TNHC62471	KC822547		Blackburn et al. 2013
137	*Kaloula picta*	Philippines, Polillo isl., Quezon Prov.	USNM-FS56932	KC822545		Blackburn et al. 2013
138	*Kaloula picta*	Philippines, Polillo isl., Quezon Prov.	USNM-FS-56936	KC822544		Blackburn et al. 2013
139	*Kaloula pulchra*	Thailand, Nong Khai	KUHE 22206	AB634632	AB634690	Matsui et al. 2011
140	*Kaloula pulchra*	Thailand, Kanchanaburi, Thong Pha Phum	KUHE 35171	AB201183	AB201194	Matsui et al. 2005
141	*Kaloula pulchra*	Myanmar, Sagaing	USFS34083		KC180025	de Sa et al. 2012
142	*Kaloula pulchra*	Vietnam, Cat Ba	ZMMU-A-4761-1		PQ268519	this work
143	*Kaloula pulchra*	Vietnam, Cat Ba	ZMMU-A-4761-2		PQ268520	this work
144	*Kaloula pulchra*	Bangladesh, Sylhet	IABHU-3781 / Kpul-Bd1		AB530543	Hasan et al. 2012
145	*Kaloula pulchra*	Bangladesh, Bandarban	IABHU 3783 / Kpul-Bd2		AB530544	Hasan et al. 2012
146	*Kaloula pulchra*	Myanmar	KIZHERP0439		JX678910	Li et al. 2012
147	*Kaloula pulchra*	Thailand, Ranong	-		AB530633	Hasan et al. 2014
148	*Kaloula pulchra*	Indonesia, Sulawesi, Makassar	-		AB530639	Hasan et al. 2014
149	*Kaloula pulchra*	Malaysia	VUB0677		EF017955	Van Bocxlaer et al. 2006
150	*Kaloula pulchra*	–	-	NC006405		Zhang et al. 2005
151	*Kaloula pulchra*	Myanmar	KIZHERP3003		JX678902	Li et al. 2012
152	*Kaloula pulchra*	China, Yunnan, Jinghong	KIZHERP0121		JX678903	Li et al. 2012
153	*Kaloula pulchra*	China, Yunnan, Mengla	KIZHERP0129		JX678904	Li et al. 2012
154	*Kaloula pulchra*	China, Guangxi, Qinzhou	KIZHERP0171		JX678907	Li et al. 2012
155	*Kaloula pulchra*	Thailand, Phang Nga	P994		KR827832	Grosjean et al. 2015
156	*Kaloula pulchra*	China, Hainan	GRE119/G027		KR827833	Grosjean et al. 2015
157	*Kaloula pulchra*	Myanmar, Tanintharyi	USNM:Herp:586945		MG935846	Mulcahy et al. 2018
158	*Kaloula pulchra*	Myanmar, Tanintharyi	USNM:Herp:586946		MG935847	Mulcahy et al. 2018
159	*Kaloula pulchra*	Myanmar, Sagaing	USNM:Herp:520322		MG935848	Mulcahy et al. 2018
160	*Kaloula pulchra*	Myanmar, Sagaing	USNM:Herp:520326		MG935849	Mulcahy et al. 2018
161	*Kaloula pulchra*	Myanmar, Bago	MBM-USNMFS35512		MG935850	Mulcahy et al. 2018

No.	Species	Locality	Museum / Sample ID	Accession numbers		Reference
	Ingroup			12S rRNA	16S rRNA	
162	*Kaloula pulchra*	Myanmar, Yangon	MBM-JBS19849		MG935851	Mulcahy et al. 2018
163	*Kaloula pulchra*	Myanmar, Sagaing	USNM:Herp:523967		MG935852	Mulcahy et al. 2018
164	*Kaloula pulchra*	Myanmar, Mandalay	MBM-USNMFS36482		MG935853	Mulcahy et al. 2018
165	*Kaloula pulchra*	China, Guangxi, Nonggang	NHMG<CHN>:1106008		KC567235	Mo et al. 2013
166	*Kaloula pulchra*	China, Guangxi, Nonggang	NHMG<CHN>:1106009		KC567236	Mo et al. 2013
167	*Kaloula pulchra*	China	-		AF315162	Jiang and Zhou., 2001
168	*Kaloula pulchra*	Southeast Asia	ZCYK-Kpul1		LC640532	Kambayashi et al. 2022
169	*Kaloula pulchra*	Bangladesh	-		MN477194	Rony et al. unpublished
170	*Kaloula pulchra*	Vietnam, Ha Tinh Prov., Huong Son	AMCC 106697	DQ283397		Frost et al. 2005
171	*Kaloula pulchra*	pet trade	RdS 02	DQ283398		Frost et al. 2005
172	*Kaloula pulchra*	–	SIH-09		AY330893	Hoegg et al. 2004
173	*Kaloula pulchra*	Thailand	ACD1538	KC822621		Blackburn et al. 2013
174	*Kaloula pulchra*	Laos, Khammouane Prov., Boudalapha	FMNH225128	KC822620		Blackburn et al. 2013
175	*Kaloula pulchra*	Laos, Bolikhamxay Prov., Thaphabat	FMNH225129	KC822619		Blackburn et al. 2013
176	*Kaloula pulchra*	Malaysia, Selangor Prov.	JAM1753	KC822616		Blackburn et al. 2013
177	*Kaloula pulchra*	Malaysia, Pahang Prov., Tioman isl.	JAM1857	KC822615		Blackburn et al. 2013
178	*Kaloula pulchra*	Malaysia, Pahang, Pulau Tioman	LSUHC3869	KC822575		Blackburn et al. 2013
179	*Kaloula pulchra*	Malaysia (continental)	LSUHC3870	KC852906		Blackburn et al. 2013
180	*Kaloula pulchra*	Indonesia, Sumatra isl.	MF0766	KC822623		Blackburn et al. 2013
181	*Kaloula pulchra*	Indonesia, Sumatra isl.	MF0812	KC822624		Blackburn et al. 2013
182	*Kaloula pulchra*	China	NHMS3208	KC822614		Blackburn et al. 2013
183	*Kaloula pulchra*	Indonesia, Sulawesi isl., Propinsi Sulawesi Selatan	TNHC59422	KC822618		Blackburn et al. 2013
184	*Kaloula pulchra*	Indonesia, Sulawesi isl., Propinsi Sulawesi Selatan	TNHC59423	KC822617		Blackburn et al. 2013
185	*Kaloula pulchra*	Vietnam	TZ629	KC822622		Blackburn et al. 2013
186	*Kaloula pulchra*	China	-		AF315130	Jiang and Zhou., 2001
187	*Kaloula rigida*	Philippines, Luzon isl., Benguet Prov.	ACD1570	KC822636		Blackburn et al. 2013
188	*Kaloula rigida*	Philippines, Luzon isl., Isabela Prov., San Mariano	ACD1954	KC822631		Blackburn et al. 2013
189	*Kaloula rigida*	Philippines, Luzon isl., Isabela Prov., San Mariano	ACD2043	KC822632		Blackburn et al. 2013
190	*Kaloula rigida*	Philippines, Luzon isl., Isabela Prov., San Mariano	ACD2044	KC822634		Blackburn et al. 2013
191	*Kaloula rigida*	Philippines, Luzon isl., Kalinga Prov.	ACD2032	KC822633		Blackburn et al. 2013
192	*Kaloula rigida*	Philippines, Luzon isl., Sierra Madres	ACD660	KC822626		Blackburn et al. 2013
193	*Kaloula rigida*	Philippines, Luzon isl., Kalinga Prov., Balbalan	ACD756	KC822628		Blackburn et al. 2013
194	*Kaloula rigida*	Philippines, Luzon isl., Isabela Prov., San Mariano	KU326470	KC822635		Blackburn et al. 2013
195	*Kaloula rigida*	Philippines, Luzon isl., Cagayan Prov., Pagudpud	KU328628	KC822627		Blackburn et al. 2013
196	*Kaloula rigida*	Philippines, Luzon isl., Cagayan Prov., Gattaran	RMB4226	KC822625		Blackburn et al. 2013
197	*Kaloula rigida*	Philippines, Luzon isl., Kalinga Prov., Balbalan	TNHC59644	KC822629		Blackburn et al. 2013
198	*Kaloula rigida*	Philippines, Luzon isl., Kalinga Prov., Balbalan	TNHC60119	KC822630		Blackburn et al. 2013
199	*Kaloula rugifera*	China, Sichuan, Mianyang	KIZHERP071601		JX678911	Li et al. 2012
200	*Kaloula rugifera*	China, Sichuan, Mianyang	KIZHERP071602		JX678912	Li et al. 2012
201	*Kaloula rugifera*	China, Sichuan, Mianyang	CIB20110802028		KC567237	Mo et al. 2013
202	*Kaloula verrucosa*	China, Sichuan, Huidong	CIB20090349		KC567238	Mo et al. 2013
203	*Kaloula verrucosa*	China, Sichuan, Huidong	CIB20090351		KC567239	Mo et al. 2013
204	*Kaloula verrucosa*	China, Yunnan, Chuxiong	KIZHERP2015		JX678900	Li et al. 2012
205	*Kaloula verrucosa*	China, Yunnan, Wuliangshan	KIZHERP2020		JX678901	Li et al. 2012
206	*Kaloula verrucosa*	China	NMNS 3246	KC822507		Blackburn et al. 2013
207	*Kaloula walteri*	Philippines, Mindanao isl., Bukidnon Prov., Malagaylay	ACD994	KC822640		Blackburn et al. 2013
208	*Kaloula walteri*	Philippines, Polillo isl., Quezon Prov., Polillo	KU303285	KC822642		Blackburn et al. 2013
209	*Kaloula walteri*	Philippines, Luzon isl., Quezon Prov., Lucban	KU327338	KC822639		Blackburn et al. 2013
210	*Kaloula walteri*	Philippines, Luzon isl., Quezon Prov., Tayabas	RMB3701	KC822641		Blackburn et al. 2013
211	*Kaloula walteri*	Philippines, Luzon isl., Quezon Prov., Lucban	TNHC59667	KC822637		Blackburn et al. 2013
212	*Kaloula walteri*	Philippines, Luzon isl., Quezon Prov., Lucban	TNHC60116	KC822638		Blackburn et al. 2013
213	*Kaloula* sp.	Philippines, Luzon isl., Laguna Prov.	ACD1692	KC822602		Blackburn et al. 2013
214	*Kaloula* sp.	Philippines, Luzon isl., Sierra Madres	ACD2479	KC822606		Blackburn et al. 2013
215	*Kaloula* sp.	Philippines, Luzon isl., Isabela Prov., Palanan	ACD650	KC822607		Blackburn et al. 2013
216	*Kaloula* sp.	Philippines, Luzon isl., Laguna Prov., Los Banos	ACD943	KC822597		Blackburn et al. 2013
217	*Kaloula* sp.	Philippines, Luzon isl., Laguna Prov., Los Banos	ACD945	KC822598		Blackburn et al. 2013

No.	**Species**	**Locality**	**Museum / Sample ID**	**Accession numbers**		**Reference**
	Ingroup			**12S rRNA**	**16S rRNA**	
218	*Kaloula* sp.	Philippines, Luzon isl., Laguna Prov., Los Banos	FMNH267555	KC822599		Blackburn et al. 2013
219	*Kaloula* sp.	Philippines, Luzon isl., Aurora Prov., Aurora Memorial NP	RMB750	KC822601		Blackburn et al. 2013
220	*Kaloula* sp.	Philippines, Luzon isl., Aurora Prov., Aurora Memorial NP	RMB783	KC822600		Blackburn et al. 2013
221	*Kaloula* sp.	Philippines, Panay isl., Antique Prov., Sibalom	GVAG253	KC822515		Blackburn et al. 2013
222	*Kaloula* sp.	Philippines, Panay isl., Antique Prov., Sibalom	GVAG255	KC822514		Blackburn et al. 2013
223	*Kaloula* sp.	Philippines, Sibuyan isl., Rombolon Prov.	KU328607	KC822511		Blackburn et al. 2013
224	*Kaloula* sp.	Philippines, Sibuyan isl., Rombolon Prov.	KU328608	KC822512		Blackburn et al. 2013
225	*Kaloula* sp.	Philippines, Panay isl., Antique Prov.	MG012	KC822513		Blackburn et al. 2013
226	*Kaloula* sp.	Philippines, Panay isl., Antique Prov.	TNHC56341	KC822516		Blackburn et al. 2013
227	*Kaloula* sp.	Philippines, Panay isl., Antique Prov.	TNHC56343	KC822517		Blackburn et al. 2013
228	*Kaloula* sp.	Philippines, Samar isl., Eastern Samar Prov., Taft	KU310699	KC822587		Blackburn et al. 2013
229	*Kaloula* sp.	Philippines, Leyte isl., Leyte Prov., Danao	KU328632	KC822584		Blackburn et al. 2013
230	*Kaloula* sp.	Philippines, Leyte isl., Leyte Prov., Danao	KU328633	KC822585		Blackburn et al. 2013
231	*Kaloula* sp.	Philippines, Leyte isl., Leyte Prov., Baybay	KU328645	KC822586		Blackburn et al. 2013
232	*Kaloula* sp.	Philippines, Panay isl., Antique Prov., Sibalom	MG0000	KC822611		Blackburn et al. 2013
233	*Kaloula* sp.	Philippines, Panay isl., Antique Prov., Sibalom	MG0001	KC822612		Blackburn et al. 2013
234	*Kaloula* sp.	Philippines, Panay isl., Antique Prov., Sibalom	MG0002	KC822613		Blackburn et al. 2013
	Outgroups					
235	*Uperodon taprobanicus*	Bandgladesh, Mymensingh	Ktap-Bd		AB530545	Hasan et al. 2012
236	*Uperodon taprobanicus*	Sri Lanka	KUHE-37252	AB634633	AB634691	Matsui et al. 2011
237	*Uperodon taprobanicus*	–	-		AY948729	Roelants et al. 2007
238	*Kalophrynus interlineatus*	–	KIZ-HERP-0169		JX678906	Li et al. 2012

These characters were measured in specimens from Khammouane Province, Laos, and Dong Nai and Lam Dong provinces, Vietnam, as well as specimens of *K. indochinensis* from the type locality (Gia Lai Province, central Vietnam). We performed a Principal Component Analysis (PCA) in Statistica v10.0 (StatSoft, Inc. 2011) to examine overall morphological variation among the populations of *K. indochinensis* sensu stricto and the populations of *Kaloula* spp. from central Laos and southern Vietnam. We conducted PCA using SVL and size-corrected values for characters 2–23 (their ratios to SVL), following the methodology of Nishikawa et al. (2007). The first two principal components (i.e., those that explained the highest proportion of variance) were extracted for display with an ordination plot.

Furthermore, we compare external morphological characters based on information taken from the literature (e.g., Chan et al. 2013, 2014; Mo et al. 2013).

Bioacoustic analysis

Male advertisement calls of *Kaloula* sp. were recorded in Cat Tien National Park, near Nam Cat Tien Village, Tan Phu District, Dong Nai Province, Vietnam, on August 4, 2023 (11.410618°N, 107.419334°E; altitude 119 m a.s.l.); the record was taken at an air temperature of 24.0 °C. Male advertisement calls (described as calls hereafter) were recorded with an Apple iPhone X (iPhone 10); the calls were recorded at a distance of approximately 0.1–0.2 m from the calling males; an HTC-2 Digital LCD Thermometer Hygrometer with an outdoor sensor attached was used to take ambient temperatures and humidity at the calling site after the recording. The software Raven Pro 1.6 (http://www.birds.cornell.edu/raven) was used to analyze the recorded calls. Audio spectrograms were calculated with a fast Fourier transform (FFT) of 512 points, 90% overlap, and 135 Hz grid spacing using the Hanning window. The terminology of call analysis and description using a call-centered approach (defining uninterrupted units as calls whenever they are separated by long silent intervals) follows Koehler et al. (2017). We examined oscillograms (waveforms) and audio spectrograms of vocalizations and measured the call duration (in milliseconds—ms), intercall interval duration (ms), call repetition rate (calls/s and calls/min), number of notes per call (notes/call), harmonic frequency (kHz), and dominant frequency (kHz). We qualitatively compared the call of *Kaloula* sp. from Cat Tien National Park with the call parameters of *K. indochinensis* sensu stricto from Gia Lai Province, Vietnam, given by Nguyen et al. (2022).

Results

Genealogical mitochondrial relationships

The trees recovered by the BI and ML analyses featured essentially similar topologies, with the only differences being the relationships between the higher *K. baleata* clades. Our mtDNA-genealogy confirms the monophyly of the genus *Kaloula* (0.98/96; hereafter node support values are given for BIPP/UFB, respectively) with respect to its sister genus *Uperodon* Duméril & Bibron, 1841 (Fig. 2). Relationships between different groups

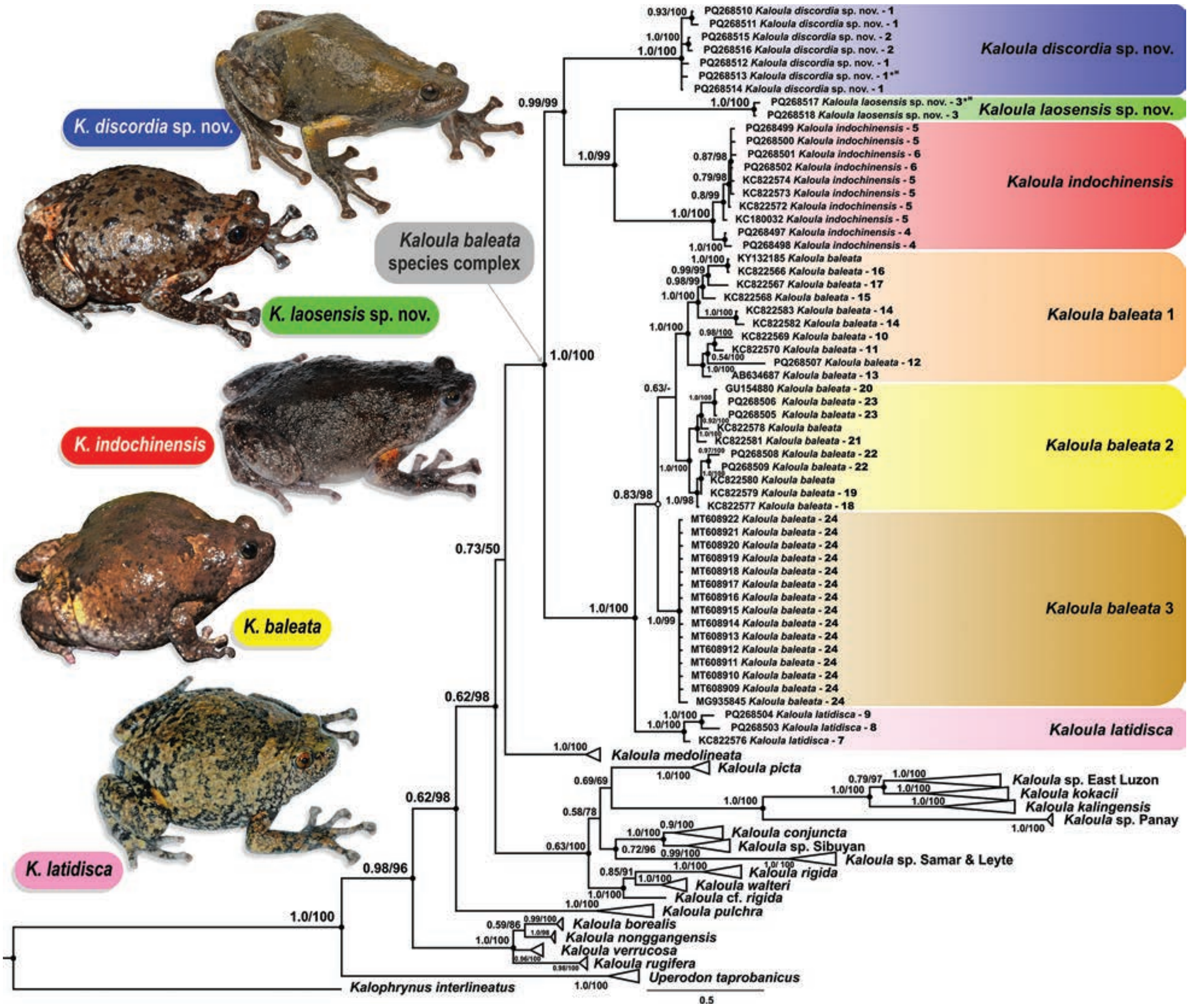

Figure 2. Genealogical relationships of the genus *Kaloula* based on the analysis of mtDNA fragments, including 12S rRNA, tRNAVal, and 16S rRNA gene sequences. Numbers at tree nodes correspond to PP/UFB support values, respectively. Black circles correspond to well-supported (PP ≥ 0.95 or UFB ≥ 90) nodes. Numbers in bold following sample names correspond to localities in Fig. 1 and Table 1. The information on GenBank Accession Numbers, museum vouchers, and localities of origin for sequences used in this study is summarized in Table 1. The holotypes of *Kaloula discordia* sp. nov. and *Kaloula laosensis* sp. nov. are marked with asterisks (*^{H}). Photographs by N.A. Poyarkov and N.L. Orlov.

within *Kaloula*, however, were not statistically supported. The East Asian diversification of *Kaloula*, including *K. borealis* (Barbour, 1908), *K. nonggangensis*, *K. verrucosa* Boulenger, 1904, and *K. rugifera* Stejneger, 1924 (1.0/100), is suggested as a sister group with respect to all other *Kaloula* species, but this topology got strong support only in the ML analysis (0.62/98). *Kaloula pulchra* Gray, 1831, is recovered as a sister species to all remaining species of *Kaloula*, again without significant nodal support by BI PP (0.62/98). All the remaining *Kaloula* species are grouped in three major clades, with their respective branching being unresolved (0.73/50): (1) the *K. baleata* species complex; (2) *K. mediolineata* Smith, 1917; (3) the *Kaloula* species found across the Southeast Asian Islands. In the latter, populations from the Philippine Archipelago and adjacent Southeast Asian islands, including *K. picta* (Duméril & Bibron, 1841), *K. kalingensis* Taylor, 1922, *K. kokacii* Ross & Gonzales, 1992, *K. conjuncta* (Peters, 1863), *K. rigida* Taylor, 1922, *K. walteri* Diesmos, Brown & Alcala, 2002, together with unnamed candidate species (noted sp.), are grouped in a clade, again with little support by BI PP (0.63/100). *Kaloula rigida* is paraphyletic with respect to *K. walteri* in the 12S-16S tree. The sister species of the *K. baleata* species complex is given as *K. mediolineata,* again with little support (0.73/50). The *K. baleata* species complex is monophyletic (1.0/100) and features two major clades (Fig. 2): (1) One clade is composed of *K. latidisca* from Peninsular Malaysia and southern Thailand (1.0/100), together with three divergent lineages of *K. baleata* sensu lato (1.0/100) from the Sunda Islands, Sulawesi, Palawan, and southern Myanmar; (2) the second clade (0.99/99) comprises populations from eastern Indochina, including two unnamed candidate species (labelled *Kaloula* sp.), one from southern Vietnam (1.0/100) and one from central Laos (1.0/100), the latter being most closely related to *K. indochinensis* from central Vietnam (1.0/100).

Kaloula baleata sensu lato, which monophyly is robustly supported only in the ML analysis (0.83/98), features three geographically circumscribed mitochondrial lineages (see Fig. 2): (1) *K. baleata* 1 is distributed in Palawan Island of the Philippines and Sulawesi, Sumba, Bali, central Java, and southern Sumatra Islands of Indonesia; (2) *K. baleata* 2 is distributed in Sarawak and Sabah in Borneo and eastern Peninsular Malaysia, including the island of Tioman, as well as western Java, in sympatry with *K. baleata* 1; (3) *K. baleata* 3 is confirmed from a single locality in Tanintharyi Region, southern Myanmar (see Fig. 1).

Mitochondrial distances

The uncorrected genetic *p*-distances for the 16S rRNA gene fragment among members of the genus *Kaloula* are available in Table 2. Interspecific distances among *Kaloula* species varied from 1.43% (between *K. verrucosa* and *K. nonggangensis*) to 14.62% (between *K. rugifera* and an undescribed species, *Kaloula* sp. from Panay, Philippines). Intraspecific distances were high in several currently recognized species, including *K. kokacii* (up to 3.39%), but especially in *K. indochinensis* from the *K. baleata* complex, where populations from Khammouane Province, Laos, as well as from Dong Nai and Lam Dong provinces, Vietnam, showed *p*-distances from the closest taxon above 4.45% and 4.33%, respectively. These 16S distances are much higher than the proposed threshold of 3% for species-level divergence in anurans (Vieites et al. 2009), which was also recently supported to delimit species based on instances of reproductive isolation (Dufresnes et al. 2021). Accordingly, these distances are higher than the *p*-distances observed between some sister species pairs in the *K. baleata* complex, namely 3.0% between *K. baleata* and *K. latidisca*. We also report a high interspecific variation in *K. baleata*, namely 2.72% between *K. baleata* 1 and *K. baleata* 2.

Morphology

Given the limited number of morphometric characters provided in the original description of *K. indochinensis* in Chan et al. (2013), we did not incorporate their data in our analysis, which further avoids measurer bias. Consequently, the morphological data on *K. indochinensis* relies only on our specimens from near the type locality of this species in Kon Ka Kinh National Park in Gia Lai Province, central Vietnam, as well as in Yok Don National Park in Dak Lak Province, central Vietnam. These specimens were confirmed as *K. indochinensis* by the genetic analysis (Fig. 2). The first two principal components (PCs) of the PCA explain 48.72% (PC1: 28.68%, PC2: 20.04%) of the variation among specimens of *K. indochinensis*, *Kaloula* sp. from central Laos, and *Kaloula* sp. from southern Vietnam (Fig. 3). The specimens of each population/candidate species form distinct groups in the morphospace, without overlapping, and also with respect to sex (Fig. 3).

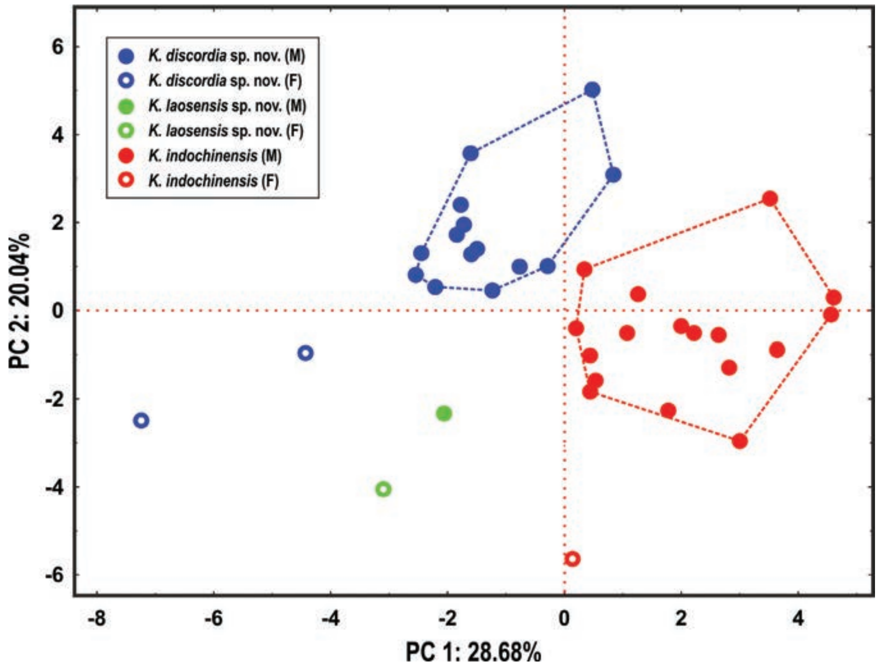

Figure 3. Scatterplot of first two PCs in the principal component analysis (PCA) on morphological data from *Kaloula indochinensis*, *Kaloula discordia* sp. nov. from southern Vietnam, and *Kaloula laosensis* sp. nov. from Khammouane, central Laos, overlayed by convex hull polygons; filled circles indicate male specimens, and empty circles indicate female specimens. The morphological data used in the analysis is presented in Table 3.

Table 2. Uncorrected average interspecific (below and above diagonal) and intraspecific (on the diagonal) genetic *p*-distances for the 16S rRNA mtDNA gene fragment (in percentage) are given for species of the genus *Kaloula*.

	Species	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**22**	**23**	**24**
1	*Kaloula discordia* sp. nov.	0.39	4.45	6.17	5.41	5.40	5.47	6.29	4.33	7.00	6.74	9.57	11.17	8.14	6.25	6.98	7.17	6.08	7.45	7.00	6.65	12.77	9.41	10.61	6.54
2	*Kaloula laosensis* sp. nov.	4.45	0.22	4.77	6.81	6.59	7.03	7.09	6.28	7.24	8.14	10.74	12.08	7.93	7.65	8.95	8.47	7.47	6.78	7.01	7.89	14.01	10.69	10.64	8.39
3	*K. indochinensis*	6.17	4.77	0.37	7.24	7.25	6.85	8.20	6.26	7.90	7.45	11.87	12.98	8.37	7.92	8.82	8.70	6.86	6.96	7.29	7.84	15.09	11.44	11.07	7.81
4	*K. baleata* 1	5.41	6.81	7.24	1.37	2.72	2.08	3.22	4.79	6.64	7.07	9.72	10.85	8.05	6.90	6.71	7.37	6.24	8.19	7.21	6.72	12.46	9.26	11.25	7.57
5	*K. baleata* 2	5.40	6.59	7.25	2.72	2.56	2.65	3.96	5.36	6.74	7.29	10.26	11.31	7.92	7.35	7.14	7.74	6.76	8.19	7.04	6.82	12.59	9.67	10.59	7.84
6	*K. baleata* 3	5.47	7.03	6.85	2.08	2.65	0.00	3.00	4.98	6.65	6.44	9.10	10.74	7.57	6.86	6.80	7.06	5.99	7.80	6.51	6.26	13.66	9.00	9.95	7.34
7	*K. latidisca*	6.29	7.09	8.20	3.22	3.96	3.00	1.45	6.04	7.28	7.47	9.71	11.01	8.89	7.41	7.26	7.74	7.13	8.43	7.82	7.24	14.05	9.41	11.83	7.66
8	*K. mediolineata*	4.33	6.28	6.26	4.79	5.36	4.98	6.04	0.55	5.89	5.43	8.74	10.49	6.12	5.70	6.95	6.05	5.61	6.81	5.46	5.39	12.30	8.49	10.02	4.97
9	*K. borealis*	7.00	7.24	7.90	6.64	6.74	6.65	7.28	5.89	0.00	7.43	10.12	11.18	2.07	7.07	7.92	7.46	6.44	2.76	1.93	6.78	13.40	9.91	10.11	7.75
10	*K. conjuncta*	6.74	8.14	7.45	7.07	7.29	6.44	7.47	5.43	7.43	1.39	8.37	9.78	8.18	5.08	8.59	4.56	3.53	7.70	8.00	3.56	11.65	8.05	7.06	2.57
11	*K. kalingensis*	9.57	10.74	11.87	9.72	10.26	9.10	9.71	8.74	10.12	8.37	2.07	6.18	10.05	9.39	11.22	8.23	7.55	9.74	9.43	7.52	10.16	4.43	11.20	7.78
12	*K. kokacii*	11.17	12.08	12.98	10.85	11.31	10.74	11.01	10.49	11.18	9.78	6.18	3.39	11.41	10.64	11.73	9.29	9.26	10.64	10.51	8.25	9.62	4.48	12.17	9.25
13	*K. nonggangensis*	8.14	7.93	8.37	8.05	7.92	7.57	8.89	6.12	2.07	8.18	10.05	11.41	0.00	7.73	8.60	8.77	8.05	2.76	1.43	8.35	14.14	10.02	11.05	8.14
14	*K. picta*	6.25	7.65	7.92	6.90	7.35	6.86	7.41	5.70	7.07	5.08	9.39	10.64	7.73	0.31	9.40	3.83	3.15	7.54	8.18	3.51	11.78	8.75	9.18	4.56
15	*K. pulchra*	6.98	8.95	8.82	6.71	7.14	6.80	7.26	6.95	7.92	8.59	11.22	11.73	8.60	9.40	0.68	8.88	8.98	8.56	7.93	9.27	14.15	10.63	12.93	8.41
16	*K. rigida*	7.17	8.47	8.70	7.37	7.74	7.06	7.74	6.05	7.46	4.56	8.23	9.29	8.77	3.83	8.88	1.90	2.73	8.56	7.99	2.45	9.90	7.82	7.74	3.58
17	*K.* cf. *rigida*	6.08	7.47	6.86	6.24	6.76	5.99	7.13	5.61	6.44	3.53	7.55	9.26	8.05	3.15	8.98	2.73	n/c	7.59	7.45	1.92	10.72	7.78	6.82	3.88
18	*K. rugifera*	7.45	6.78	6.96	8.19	8.19	7.80	8.43	6.81	2.76	7.70	9.74	10.64	2.76	7.54	8.56	8.56	7.59	0.00	1.98	8.12	14.62	9.33	10.70	7.98
19	*K. verrucosa*	7.00	7.01	7.29	7.21	7.04	6.51	7.82	5.46	1.93	8.00	9.43	10.51	1.43	8.18	7.93	7.99	7.45	1.98	0.27	7.48	13.70	9.19	10.44	7.68
20	*K. walteri*	6.65	7.89	7.84	6.72	6.82	6.26	7.24	5.39	6.78	3.56	7.52	8.25	8.35	3.51	9.27	2.45	1.92	8.12	7.48	0.30	9.38	6.72	6.61	3.19
21	*Kaloula* sp. Panay	12.77	14.01	15.09	12.46	12.59	13.66	14.05	12.30	13.40	11.65	10.16	9.62	14.14	11.78	14.15	9.90	10.72	14.62	13.70	9.38	0.00	8.51	12.80	10.47
22	*Kaloula* sp. East Luzon	9.41	10.69	11.44	9.26	9.67	9.00	9.41	8.49	9.91	8.05	4.43	4.48	10.02	8.75	10.63	7.82	7.78	9.33	9.19	6.72	8.51	1.85	10.44	7.51
23	*Kaloula* sp. Samar & Leyte	10.61	10.64	11.07	11.25	10.59	9.95	11.83	10.02	10.11	7.06	11.20	12.17	11.05	9.18	12.93	7.74	6.82	10.70	10.44	6.61	12.80	10.44	1.56	7.78
24	*Kaloula* sp. Sibuyan	6.54	8.39	7.81	7.57	7.84	7.34	7.66	4.97	7.75	2.57	7.78	9.25	8.14	4.56	8.41	3.58	3.88	7.98	7.68	3.19	10.47	7.51	7.78	1.66

Bioacoustics

The advertisement call of *Kaloula* sp. from southern Vietnam is documented based on the recordings of one calling male individual. Males of *Kalophrynus interlineatus* (Blyth, 1855), *Occidozyga martensii* (Peters, 1867), and *Microhyla mukhlesuri* Hasan, Islam, Kuramoto, Kurabayashi & Sumida, 2014, were also calling at the same habitat. The calls of *Kaloula* sp. are slow and low-pitched, resonant, booming sounds that resemble the sound of a bicycle horn to the human ear. Call parameters are shown in Table 3, and the sonograms and waveforms of the call are presented in Fig. 4. The call consists of a single note with 11–13 pulses with an average duration of 190.8 ms (144.8–260.6 ms, N = 23). Calls were repeated at a rate of one call per second, 56.5 (42–71) calls per minute, and had an average intercall interval of 1,222.9 ms (327.1–8,954.4 ms, N = 23). The fundamental frequency was not evident, and the average dominant frequency was 0.30–0.45 kHz. Three harmonics were detected at 1.0, 1.2, and 1.5 kHz (Table 3, Fig. 4). The call of the Laos population of *Kaloula* sp. was not recorded.

Systematics

The two populations of *Kaloula* spp. from Laos and southern Vietnam belong to the *K. baleata* species complex and are characterized by divergent mitochondrial lineages and distinctive morphologies. Their amount of 16S divergence (Table 2), together with the substantial morphological differentiation between them and the closely related species *K. indochinensis* (Fig. 3), suggests a species-level split. Based on our mitochondrial genealogy, the species from Khammouane Province of Laos is the sister species of *K. indochinensis*, while the species from southern Vietnam is the sister species of a clade including *K. indochinensis* and the Laos species (Fig. 2). In addition to diagnostic morphological differences (see below), the three Indochinese species of the *K. baleata* species complex also feature stable differences in coloration, as well as differences in bioacoustic characteristics for the studied Vietnamese species. The distribution of all members of the *K. baleata* species complex in Indochina, following our results, is detailed in Fig. 5. Given the congruence of morphological, bioacoustic, and molecular data in suggesting a deep divergence of the Laos and southern Vietnam *Kaloula* sp. populations from all currently recognized species in the genus, we herein describe them as two new species of *Kaloula*.

Kaloula discordia Poyarkov, Gorin, Bragin & Nguyen, sp. nov.

https://zoobank.org/3E487D92-32B2-447E-AB70-0059D2F504A9

Figs 2, 4, 6–8, 9C, Tables 3, 4

Chresonymy. *Kaloula baleata* [partim]—Orlov et al. (2002: 99); Nguyen et al. (2005: 43); Orlov and Ananjeva (2007: 148); Nguyen et al. (2009: 94).

Kaloula indochinensis [partim]—Chan et al. (2013: 334, 2014: 577); Chandramouli and Prasad (2018: 52); Poyarkov et al. (2021b: 39); Vassilieva (2021: 72–73); Holden (2023: 149).

Holotype. ZMMU A-8134 (field number NAP-14688), adult male, from a pond in Cat Tien National Park, along the road from park headquarters to the Bau Sau Lake, Dong Nai River valley, Dac Lua Commune, Tan Phu District, Dong Nai Province, Vietnam, collected by N.A. Poyarkov, A.M. Bragin, and V.A. Gorin on 15 June, 2024 (11.44121°N, 107.41312°E; elevation 137 m a.s.l.) (Figs 6, 7C).

Paratypes (n = 14). ZMMU A-8135–A-8140 (field numbers NAP-14689–14694), six adult males with collection data same as the holotype; ZMMU A-8141–A-8143 (field numbers NAP-14688–14694), three adult males with collection data same as the holotype; ZISP 15285–15287 (field numbers NAP-14687, NAP-14695–14696), three adult males with collection data same as the holotype; ZMMU A-4739 (field number NAP-01674), adult male from Cat Tien National Park, Dong Nai River valley, Dac Lua Commune, Tan Phu District, Dong Nai Province, Vietnam, collected by N.A. Poyarkov on June 1, 2011 (11.44576°N, 107.38673°E; elevation 128 m a.s.l.); ZMMU A-4642 (field number NAP-00630), adult female from Cat Tien National Park, Dong Nai River valley, Dac Lua Commune, Tan Phu District, Dong Nai Province, Vietnam, collected by N.A. Poyarkov and A.B. Vassilieva on June 2, 2009 (11.44576°N, 107.38673°E; elevation 128 m a.s.l.) (Fig. 8).

Referred materials (n = 3). ZMMU-A-4602-1–3 (field numbers NAP-02010, NAP-02086, and NAP-02122), adult female and two subadult females from Cat Tien National Park, Dong Nai River valley, Dac Lua Commune, Tan Phu District, Dong Nai Province, Vietnam, collected by E.A. Galoyan on August 22, 2011, September 15, 2011, and November 11, 2011 (11.419503°N, 107.426442°E; elevation 117 m a.s.l.).

Diagnosis. *Kaloula discordia* sp. nov. is distinguished from its congeners by the following combination of morphological characters: (1) medium body size (SVL 42.9–56.2 mm); (2) eyes comparatively small (eye length comprising 60%–75% of snout length); (3) dorsally uniform dark olive-brown; (4) beige-gray ventrally with irregular white mottling on belly and limbs; (5) pale yellow or orange-brown 8-shaped patch on either side of the neck posterior to eyes; (6) yellowish to orange axillary patch present; (7) grayish to beige-yellow inguinal patch present edged with black markings; (8) from gray to beige spot on tibiotarsal articulation present; (9) dark interorbital bar absent; (10) enlarged, widened finger disks (3FDD 6.5%–7.5% of SVL), ca. 1.27 times wider than toe disks; (11) finger subarticular tubercle formula: 1:1:2:2; (12) toe subarticular tubercle formula: 1:1:2:2:2; (13) two metacarpal tubercles not in touch with each other; (14) two metatarsal tubercles, outer metatarsal tubercle rounded, smaller than elongated inner metatarsal tubercle.

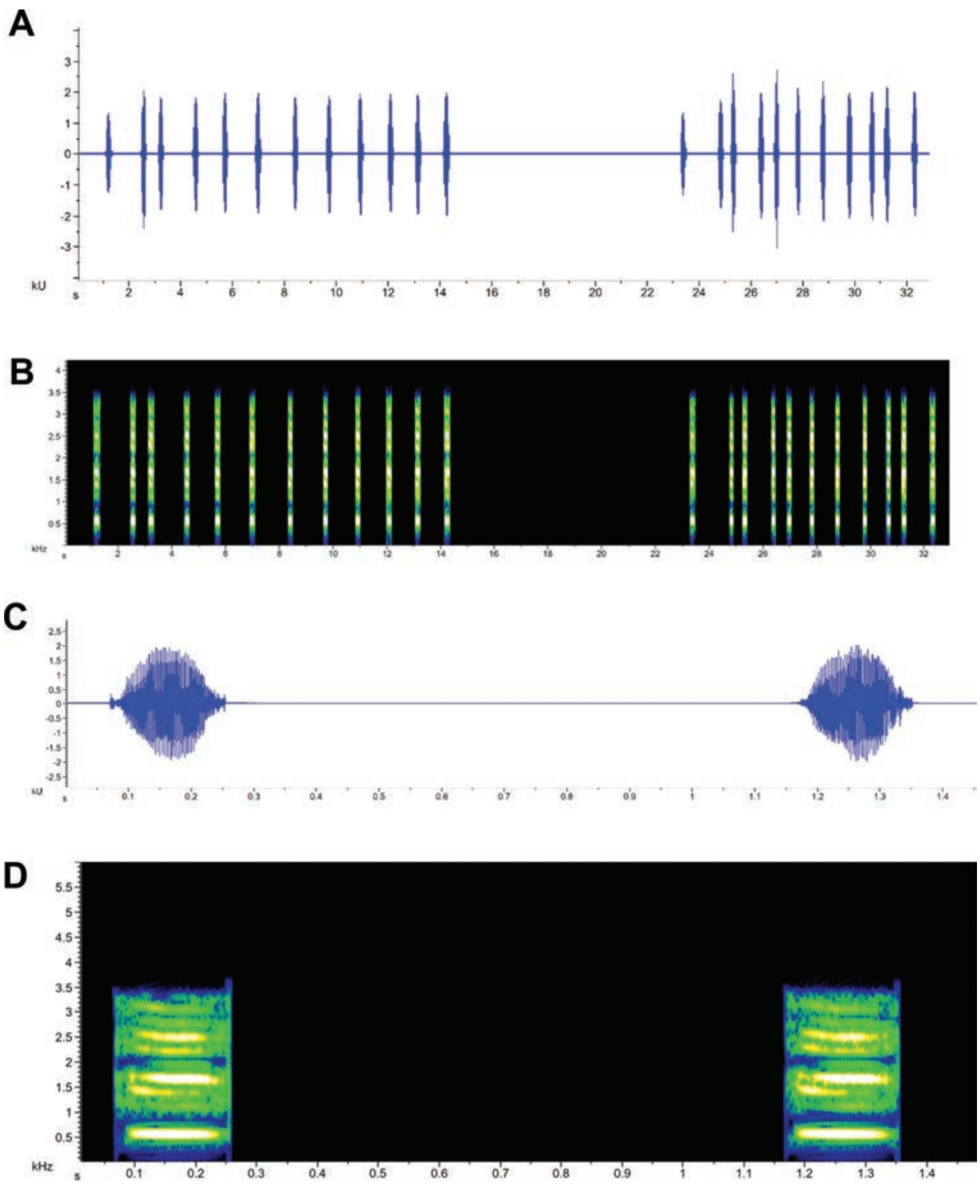

Figure 4. The call of *Kaloula discordia* sp. nov. from Cat Tien NP, Dong Nai Province, Vietnam. **A.** 33 s waveform of relative amplitude (Rel. amp.) and corresponding spectrogram over time; **B.** 1.5 s waveform and corresponding spectrogram of two calls, obtained from the last two calls in (**A**). Data on acoustic characters is summarized in Table 4.

Table 3. Comparison of male advertisement calls for *Kaloula discordia* sp. nov. and other members of the *K. baleata* species complex. NR: not recorded.

Species	*Kaloula discordia* sp. nov.	*Kaloula indochinensis*	*Kaloula baleata*	*Kaloula ghoshi*
Locality	Cat Tien NP, Tan Phu District, Dong Nai Province, Vietnam	Kon Von II village, Dak Roong Commune, K'Bang District, Gia Lai Province, Vietnam	Kinabalu, Sabah, Malaysia	Hut Bay, Little Andaman Island
Coordinates	11.411°N, 107.419°E	14.543°N, 108.414°E	NR	NR
Number of calls measured	23	40	NR	NR
Call duration (ms)	186.1 (144–214)	215.6 (194–250)	280–300	400 (320–640)
Intercall interval (ms)	1539.4 (851–8954)	789.3 (481–1627)	NR	300 (240–530)
Call repetition rate (call/s)	1.0 (0.75–1.21)	1.0 (0.76–1.25)	NR	NR
Call repetition rate (call/minute)	56.5 (42–71)	60.1 (45.9–75.2)	NR	NR
Notes/call	1	1	NR	NR
Pulse/call	11–13	1*	Described as pulsed	7–25
Dominant frequency (kHz)	0.38 (0.30–0.45)	0.38 (0.34–0.43)	1–3	1
2nd harmonic (kHz)	1.0	0.8	NR	NR
3rd harmonic (kHz)	1.2	1.2	NR	NR
4th harmonic (kHz)	1.5	1.37	NR	NR
Temperature (°C)	24.0	20.5	23.0	29.1
Source	this study	Nguyen et al. (2022)	Malkmus et al. (2002)	Chandramouli and Prasad (2018)

Table 4. Measurements (in mm) of specimens of *Kaloula discordia* sp. nov., *Kaloula laosensis* sp. nov.**,** and *K. indochinensis*. For character abbreviations, see the Materials and methods section. Abbreviations: F – adult female; M – adult male; SF – subadult female; SM – subadult male.

Species	Sample ID	Sex	Status	SVL	HL	SL	EL	N-EL	HW	IND	IOD	UEW	FLL	LAL	HAL	1FL	IPTL	OPTL	3FDD	HLL	TL	FL	OMTL	1TOEL	4TDD
Kaloula discordia **sp. nov.**	ZMMU-A-8134	**M**	holotype	51.5	11.7	5.5	4.1	3.9	14.8	3.2	5.3	3.0	31.9	26.4	15.7	8.1	3.0	3.5	3.6	60.0	14.7	30.2	2.1	7.9	2.3
K. discordia **sp. nov.**	ZMMU-A-4739	**M**	paratype	52.0	11.9	5.2	4.0	3.9	15.2	3.2	5.3	2.9	33.4	26.8	15.8	8.2	3.0	3.4	3.8	62.7	15.3	31.4	2.5	7.7	2.9
K. discordia **sp. nov.**	ZISP 15285	**M**	paratype	48.0	10.8	5.1	3.1	3.7	13.7	3.1	5.0	2.8	31.2	24.1	15.0	7.8	2.9	3.3	3.2	58.0	13.4	28.9	2.5	7.9	2.6
K. discordia **sp. nov.**	ZMMU-A-8135	**M**	paratype	49.6	11.3	5.3	3.4	3.8	14.0	3.2	4.9	2.7	31.5	25.3	15.3	7.9	2.6	3.3	3.5	59.0	14.1	29.7	2.4	7.6	2.8
K. discordia **sp. nov.**	ZMMU-A-8136	**M**	paratype	45.4	10.6	4.9	3.3	3.4	13.8	3.0	4.8	2.7	31.4	24.2	14.6	7.8	2.9	2.8	3.3	57.4	14.1	27.9	2.2	7.2	2.4
K. discordia **sp. nov.**	ZMMU-A-8137	**M**	paratype	42.9	10.3	4.9	3.0	3.0	13.7	3.0	4.5	2.6	30.8	24.0	14.9	7.8	2.3	3.1	3.0	56.3	13.1	28.5	2.0	7.1	2.4
K. discordia **sp. nov.**	ZMMU-A-8138	**M**	paratype	48.7	11.1	5.3	3.7	3.5	14.7	3.2	4.9	2.9	31.1	25.0	15.4	7.9	2.4	3.0	3.5	60.6	14.4	30.5	2.2	7.4	2.3
K. discordia **sp. nov.**	ZMMU-A-8139	**M**	paratype	49.5	11.2	5.1	3.1	3.5	15.0	3.1	4.7	3.0	31.7	24.9	14.2	7.4	2.6	3.1	3.3	60.5	13.8	31.0	1.9	7.5	2.7
K. discordia **sp. nov.**	ZMMU-A-8140	**M**	paratype	47.2	10.5	5.0	3.4	3.3	15.1	3.0	4.9	2.7	27.9	23.0	14.1	6.8	2.7	3.3	3.5	57.6	14.7	27.4	1.9	6.9	2.8
K. discordia **sp. nov.**	ZISP 15286	**M**	paratype	52.6	12.2	5.8	3.8	3.8	15.7	3.2	5.6	2.8	32.9	26.3	15.6	7.9	3.2	3.6	4.0	61.9	15.1	31.1	2.3	8.2	3.4
K. discordia **sp. nov.**	ZISP 15287	**M**	paratype	49.1	10.6	5.3	3.6	3.5	14.5	3.1	5.0	2.7	31.2	24.6	15.4	8.1	2.8	3.1	3.4	60.6	14.3	29.7	2.6	8.2	2.6
K. discordia **sp. nov.**	ZMMU-A-8141	**M**	paratype	50.0	11.3	5.5	3.5	3.7	15.3	3.2	5.0	3.0	31.8	25.2	15.2	7.5	2.6	3.0	3.3	60.5	14.3	31.0	1.9	7.4	2.6
K. discordia **sp. nov.**	ZMMU-A-8142	**M**	paratype	52.2	11.7	5.1	3.6	3.2	15.3	3.2	5.0	2.8	31.9	25.1	15.6	7.9	2.5	3.0	3.6	61.0	14.1	30.8	1.9	7.2	2.7
K. discordia **sp. nov.**	ZMMU-A-8143	**M**	paratype	51.5	11.7	5.5	3.4	3.6	15.0	3.2	5.1	2.7	32.1	25.4	15.2	7.9	2.9	3.1	3.7	58.5	13.4	29.9	2.4	7.5	2.6
K. discordia **sp. nov.**	ZMMU-A-4642	**F**	paratype	56.2	12.8	6.2	4.1	4.2	17.0	3.8	5.9	3.0	35.9	28.2	16.3	9.3	3.2	3.7	4.0	67.4	16.7	33.3	3.0	9.0	3.0
K. discordia **sp. nov.**	ZMMU-A-4602-1	**F**		56.4	13.0	5.4	4.2	4.0	16.9	3.7	6.0	3.0	35.8	28.6	16.6	10.0	3.4	3.4	3.8	67.6	17.2	33.7	3.0	9.1	2.9
K. discordia **sp. nov.**	ZMMU-A-4602-2	**SF**		25.3	7.0	3.1	2.5	2.8	7.5	1.9	3.2	1.5	15.5	11.9	7.0	3.8	2.0	1.8	1.7	30.2	7.4	13.9	-	3.5	1.5
K. discordia **sp. nov.**	ZMMU-A-4602-3	**SF**		35.0	8.3	3.8	3.0	2.8	10.2	2.6	3.6	1.9	23.2	14.5	10.3	5.9	2.2	2.2	2.6	40.2	9.6	20.4	1.4	4.5	2.0
Kaloula laosensis **sp. nov.**	ZISP 15284	**M**	holotype	44.0	10.6	4.6	3.3	3.2	12.6	2.7	4.3	2.4	27.6	21.7	13.0	7.5	2.7	2.8	3.4	49.3	11.6	25.4	2.0	7.3	2.2
K. laosensis **sp. nov.**	ZMMU-A-8144	**F**	paratype	54.8	12.4	5.3	5.0	5.2	16.5	3.6	5.7	3.0	33.8	28.3	17.8	10.4	3.2	3.7	4.3	66.9	16.4	32.5	2.8	8.4	2.8
Kaloula indochinensis	ZMMU-A-8147	**M**		45.3	11.0	4.9	3.9	3.3	14.7	3.1	5.0	2.8	31.5	23.7	13.9	8.1	3.1	3.4	3.8	55.2	14.0	27.2	2.19	7.2	2.3
K. indochinensis	ZMMU-A-8148	**M**		50.9	11.6	5.3	3.4	3.8	15.1	3.0	4.5	2.5	32.5	24.8	14.6	7.9	2.9	3.1	3.3	57.3	14.5	27.5	2.72	8.1	2.4
K. indochinensis	ZMMU-A-8149	**M**		42.3	9.8	4.8	3.4	3.3	12.0	2.9	4.5	2.4	28.1	20.4	12.6	7.8	2.4	2.6	2.9	47.9	10.9	24.9	2.15	6.2	2.2
K. indochinensis	ZMMU-A-8150	**M**		46.3	10.7	4.9	3.5	3.5	13.0	3.1	4.5	2.6	29.9	22.4	13.9	7.9	2.6	3.0	3.4	50.7	12.2	26.2	2.59	7.0	2.4
K. indochinensis	ZMMU-A-8151	**M**		48.5	11.2	5.2	3.9	3.5	15.7	3.1	4.7	2.7	31.1	24.0	14.6	8.2	3.2	3.3	3.7	55.1	12.9	27.6	2.44	7.4	2.7
K. indochinensis	ZMMU-A-8152	**M**		49.1	11.9	5.3	4.0	3.8	15.0	3.3	5.1	2.8	30.6	24.4	14.9	8.7	3.1	3.2	3.6	55.9	12.7	27.5	2.35	7.4	2.3
K. indochinensis	ZMMU-A-8153	**M**		48.8	11.8	4.9	4.0	3.6	15.3	3.0	5.0	2.7	31.3	24.3	14.5	7.8	2.9	3.3	3.6	53.0	12.7	26.2	2.80	6.6	2.5
K. indochinensis	ZMMU-A-8154	**M**		49.8	11.9	5.5	3.9	3.5	16.3	3.4	5.0	2.7	32.0	24.3	15.1	8.4	2.7	3.1	3.8	54.5	12.9	27.5	2.62	7.4	2.5
K. indochinensis	ZMMU-A-6316	**M**	topotype	50.4	11.7	5.2	4.0	3.7	14.9	3.2	4.9	2.9	28.1	22.8	14.5	8.6	3.2	3.3	3.1	54.7	12.4	27.0	2.75	6.8	2.3
K. indochinensis	ZMMU-A-6317	**M**	topotype	47.3	10.8	5.1	3.8	3.3	14.1	3.0	4.9	2.9	26.7	22.0	13.9	8.0	2.7	2.9	2.8	52.2	12.2	25.7	2.89	5.9	1.8
K. indochinensis	ZMMU-A-6318	**M**	topotype	51.1	11.6	5.4	3.9	3.5	16.2	3.3	5.2	2.7	31.7	25.2	15.5	8.9	3.4	3.4	3.5	55.8	13.0	27.5	2.84	7.1	2.2
K. indochinensis	ZMMU-A-6319	**M**	topotype	59.6	12.7	5.8	4.1	4.0	17.7	3.4	5.8	3.1	34.3	28.6	18.1	10.4	3.9	4.4	4.2	63.9	15.0	31.6	2.79	8.5	2.8
K. indochinensis	ZMMU-A-6320	**M**	topotype	51.3	12.3	5.3	4.0	3.8	15.9	3.4	5.3	2.9	30.5	24.9	15.7	9.2	3.3	3.2	3.4	55.4	13.1	27.3	2.79	7.7	2.2
K. indochinensis	ZMMU-A-6321	**M**	topotype	50.0	12.3	5.3	4.0	3.7	16.1	3.4	5.4	3.2	28.4	24.0	14.7	8.8	2.9	3.4	3.4	55.7	13.4	27.5	3.22	7.0	2.1
K. indochinensis	ZMMU-A-6322	**M**	topotype	50.5	12.4	6.0	4.2	4.2	16.5	3.6	5.7	3.0	30.4	24.9	15.7	9.4	3.5	3.5	3.9	57.8	13.9	28.8	2.88	7.8	2.4
K. indochinensis	ZMMU-A-6323	**M**	topotype	51.7	11.4	5.4	4.0	3.7	15.0	3.6	5.9	3.3	31.3	25.3	15.8	9.2	3.6	3.9	3.2	58.0	14.1	28.0	2.85	7.4	2.4
K. indochinensis	ZMMU-A-6324	**M**	topotype	50.2	12.4	5.5	4.0	3.5	15.7	2.9	5.0	3.1	29.1	23.6	14.6	8.2	3.3	3.4	3.2	55.7	13.6	26.5	2.97	7.0	2.2
K. indochinensis	ZMMU-A-6325	**M**	topotype	48.2	10.7	5.1	3.9	3.7	14.3	3.3	5.1	3.0	28.8	23.1	14.4	7.8	2.7	2.9	3.4	53.9	12.4	26.3	2.65	6.7	2.1
K. indochinensis	ZMMU-A-6315	**F**	topotype	55.2	13.1	6.2	4.3	4.4	17.3	3.4	5.8	3.1	32.9	27.9	18.0	9.8	3.2	3.8	4.0	62.7	14.8	31.9	3.08	8.4	2.5
K. indochinensis	ZMMU-A-8155	**SF**		36.1	8.7	4.4	3.4	2.9	11.6	2.6	3.9	2.1	24.8	18.8	11.7	5.9	2.2	2.4	3.0	41.6	10.4	21.3	1.69	5.7	2.1

Figure 5. Known distribution of the *Kaloula baleata* species complex members in Indochina. Dots within symbols denote type localities. Symbol colors correspond to mitochondrial DNA lineages (Figs 1, 2. Note that the tentative assignment of the populations that were not sequenced is based on geographic proximity and/or examination of morphological or photographic data from these populations). Symbol numbers correspond to locality numbers given in Appendix 1.

Description of the holotype (Fig. 6). Adult male in a good state of preservation, habitus robust, head wider than long (HW/HL 1.27), snout projecting beyond lower jaw, gently rounded in lateral view (Fig. 6C); truncated in dorsal view; top of head flat; upper eyelid lacking supraciliary tubercles; eye length less than snout length (EL/SL 0.74) and less than interorbital distance (EL/IOD 0.76); pupils round; nostrils rounded, placed more towards the lateral sides of the snout, located closer to tip of snout than to eye, relatively close to each other (IND/IOD 0.60); supratympanic fold flat, glandular, rather thin; tympanum not visible (Fig. 6C); dorsal surfaces of body and limbs with sparse tubercules, getting denser backwards; ventral surfaces of body and limbs with flat tubercules (Fig. 6A, B). Cloacal opening unmodified, directed posteriorly. Forelimbs relatively long, more than a half of hind limb length (FLL/HLL 0.53); hand long, comprising more than a half of lower arm length (HAL/LAL 0.60) and almost half of forelimb length (HAL/FLL 0.49); fingers rather robust, notably flattened in cross section; relative finger lengths: I<II<IV<III; fingers free of webbing; terminal digits flattened forming wide transversely expanded disks (Fig. 6E); finger subarticular tubercles distinct, large and round, finger subarticular tubercle formula 1:1:2:2; inner metacarpal tubercle elongate; outer metacarpal tubercule triangular-shaped, dilated, bigger than inner metacarpal tubercle (OPTL/IPTL1.18), two metacarpal tubercles not in touch with each other (Figs 6E, 9C); hindlimbs robust, relatively short, not much longer than body length (HLL/SVL 1.16); relative toe lengths: I<II<V<III<IV; tarsal fold on inner surface of tarsus absent; tips of all toes widened, forming rounded terminal disks; all toe disks having dorso-terminal groves; the disk on toe IV the largest (Figs 6D, 9C); toe webbing well developed between all toes, reaching disks at all toes except toe IV; webbing formula I1-2II1.5-2.5III1.5-3IV3-1.5V; toe subarticular tubercules distinct, rounded; toe subarticular formula 1:1:2:2:2; two metatarsal tubercles, inner metatarsal tubercle elongated, oval; outer metatarsal tubercle smaller, rounded (Figs 6D, 9C).

Coloration. In life, the dorsal surfaces of the head and body olive-brown, dorsal surfaces of the limbs grayish-olive with grayish-white spots (Fig. 6A); flanks of the body and lateral sides of the head grayish olive; orange-brown 8-shaped patches on the neck posterior to eyes; bright-orange axillary patch continuing on elbows (Fig. 7C); grayish-white inguinal patch; small grayish-beige spots near tibiotarsal articulation; ventral surfaces of head and body gray, darker on the throat near the jaw; belly and ventral surfaces of limbs pinkish with weak white mottling (Fig. 6B). Iris golden with brown reticulations ventrally and dorsally from the pupil (Figs 6C, 7C). In preservation after one year of storage in ethanol, dorsal coloration faded to dark gray, light patches became less pronounced, and they faded to light gray or grayish-beige, though the pattern generally remained unchanged.

Measurements of the holotype (in mm): SVL 51.5; HL 11.7; HW 14.8; SL 5.5; EL 4.1; N-EL 3.9; IND 3.2; IOD 5.3; UEW 3.0; FLL 31.9; LAL 26.4; HAL 15.7; HLL 60.0; TL 45.0; FL 30.2; IPTL 3.0; OPTL 3.5; 1FL 8.1; 1TOEL 7.9; OMTL 2.1; 3FDD 3.6; 4TDD 2.3.

Variation. Table 4 presents the morphometric variation of the type series. Fig. 8 displays the variation in dorsal coloration of the paratypes. In general, all paratypes agree well with the description of the holotype, differing only in the brightness of light inguinal and axillary markings. In male paratypes ZISP 15285 and ZMMU A-8138, the right foot is poorly developed; in male paratype ZMMU A-8139, the right hand is poorly developed.

Tadpole morphology. Vassilieva (2021) provided morphometric data and a detailed description of tadpoles of *Kaloula discordia* sp. nov. (as *K. indochinensis*). Vassilieva (2021) noted that tadpoles of '*K. indochinensis*' from Cat Tien NP, Dong Nai Province, southern Vietnam (corresponding to *Kaloula discordia* sp. nov.) differ from *K. indochinensis* sensu stricto from Kon Ka Kinh NP, Gia Lai Province, and Chu Mon Ray NP, Kon Tum Province, central Vietnam, by having comparatively longer spiracle tubes (markedly longer than the vent tubes), a narrower mouth relative to body width, more developed tail musculature (tail base width more than a third of the body width), and a rather contrasting tail coloration.

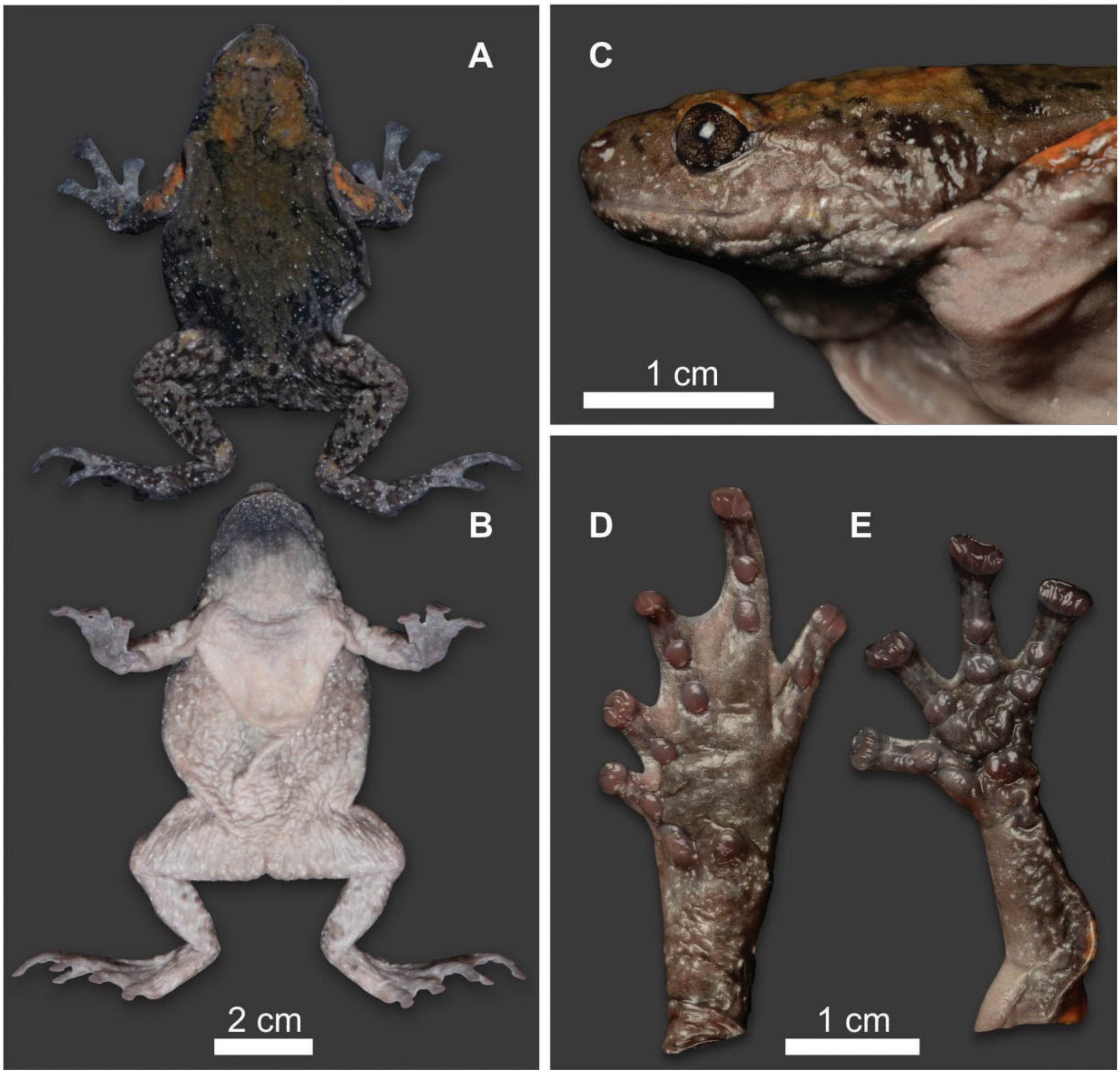

Figure 6. Holotype of *Kaloula discordia* sp. nov. (ZMMU A-8134), adult male. **A.** Dorsal view; **B.** Ventral view; **C.** Head in a lateral view; **D.** Plantar view of left foot; **E.** Volar view of left hand. Photographs by N.A. Poyarkov.

Distribution and natural history. *Kaloula discordia* sp. nov. is currently reliably known from Dong Nai, Lam Dong, Tay Ninh, and Binh Phuoc provinces of Southern Vietnam and was also reported from the adjacent Mondulkiri Province of eastern Cambodia; the known distribution of the new species is shown in Fig. 5, and the locality information is detailed in Appendix 1. The single Cambodian record of the new species from Phnom Prich Wildlife Sanctuary in Mondulkiri Province, originally reported as *K. indochinensis* by Chan et al. (2013) and Holden (2023), can be confidently assigned to *Kaloula discordia* sp. nov. based on the external morphology and coloration of the recorded specimen and the geographic proximity of the locality to the population of *Kaloula discordia* sp. nov. in Binh Phuoc Province of southern Vietnam. The new species was recorded mostly from relatively low elevations of ca. 70–300 m a.s.l.; in Bao Loc forestry of Lam Dong Province, the new species was recorded at elevations up to 550 m a.s.l. *Kaloula discordia* sp. nov. is restricted to lowland and hilly, seasonally dry, semi-deciduous, and evergreen monsoon forests of southern Vietnam (see Vassilieva et al. 2016). Male frogs were recorded calling from small temporary pools and flooded areas from May to November (Fig. 7A, B); the peak of the breeding season coincides with heavy rains in June–August. The new species is semi-fossorial and quite elusive and is usually active only after periods of heavy rains; frogs are aestivating during dry periods in leaf litter, underground borrows, or tree hollows (Vassilieva et al. 2016; Holden 2023). Diet consists of ants and beetles (Vassilieva et al. 2016); the new species is a good climber; males can call when perching a few meters above ground level (Holden 2023). The actual distribution of *K. discordia* sp. nov. is still insufficiently known, but we suppose that this species

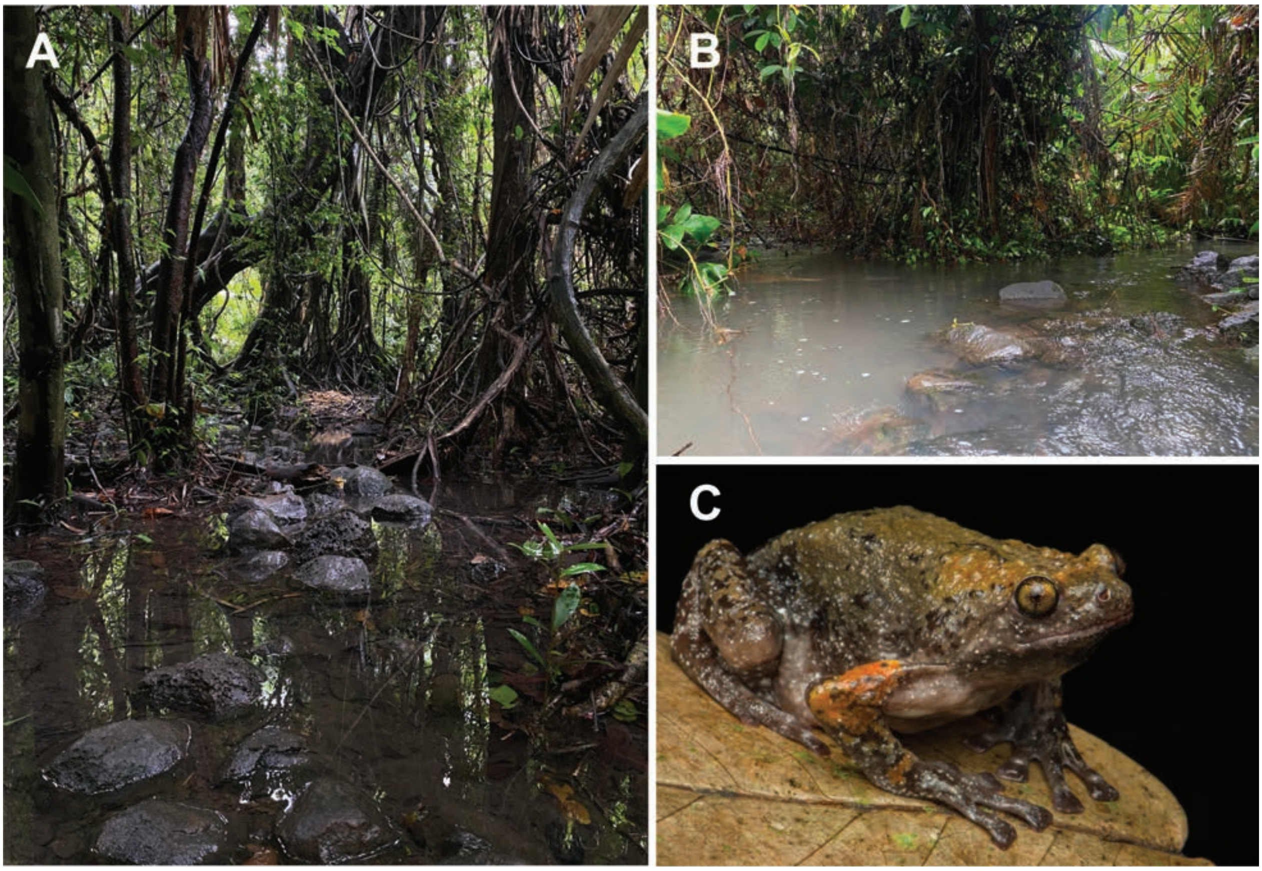

Figure 7. Breeding habitats of *Kaloula discordia* sp. nov. (**A, B**) and lateral view of holotype of *Kaloula discordia* sp. nov. in situ (ZMMU A-8134) (**C**). Photographs by A.M. Bragin.

is likely to occur in those provinces of southern Vietnam where forests similar in composition are still preserved, including Dak Nong, Binh Duong, Binh Thuan, and Ba Ria-Vung Tau provinces. Syntopic species of amphibians included *Microhyla butleri* Boulenger, 1900; *M. heymonsi* Vogt, 1911; *M. mukhlesuri*; *Kaloula pulchra*; *Fejervarya limnocharis* (Gravenhorst, 1829); *Occidozyga martensii*; *Polypedates megacephalus* Hallowell, 1861; and *Rhacophorus annamensis* Smith, 1924.

Comparisons. *Kaloula discordia* sp. nov. most closely resembles other frogs of the *K. baleata* species complex in overall morphology; most specifically, it is similar to *K. indochinensis*, with which it was previously confused. From *K. indochinensis*, the new species can be distinguished by having generally smaller eye size in males (EL mean 3.5 ± 0.3 mm [n = 14] vs. mean 3.9 ± 0.2 mm [n = 18]; EL/SVL 0.071 ± 0.004 vs. 0.079 ± 0.004); smaller finger I in males (1FL mean 7.8 ± 0.4 mm [n = 14] vs. mean 8.5 ± 0.7 mm [n = 18]; 1FL/SVL 0.16 ± 0.01 vs. 0.17 ± 0.007); generally smaller inner palmar tubercules in males (IPTL mean 2.8 ± 0.3 mm [n = 14] vs. mean 3.1 ± 0.4 mm [n = 18]; IPTL/SVL 0.056 ± 0.004 vs. 0.062 ± 0.005); and generally smaller outer palmar tubercules in males (OPTL mean 3.2 ± 0.2 mm [n = 14] mm vs. mean 3.3 ± 0.4 mm [n = 18]; OPTL/SVL 0.064 ± 0.004 vs. 0.067 ± 0.005); generally longer hindlimbs in both sexes (HLL mean 59.6 ± 1.9 mm [n = 14] in males, mean 67.5 ± 0.2 mm [n = 2] in females vs. mean 55.2 ± 3.3 mm [n = 18] in males; 62.7 mm [n = 1] in female; HLL/SVL 1.21 ± 0.04 vs. 1.12 ± 0.03 in both sexes); smaller metatarsal tubercule in males (OMTL mean 2.2 ± 0.3 mm [n = 14] vs. mean 2.7 ± 0.3 mm [n = 18]); by having two metacarpal tubercles, see Fig. 9C (vs. three metacarpal tubercles, see Fig. 9B); by having two subarticular tubercles on toe IV, see Fig. 9C (vs. three well-developed subarticular tubercles, see Fig. 9B); and by having olive dorsal coloration (vs. chocolate-brown to dark grayish-brown).

From *K. baleata* (morphological data taken from Chan et al. 2013), *Kaloula discordia* sp. nov. can be distinguished by having generally smaller eyes in both sexes (EL mean 3.5 ± 0.3 mm [n = 14] in males, mean 4.2 ± 0.01 mm [n = 2] in females vs. mean 4.1 ± 0.4 mm [n = 10] in males, mean 4.5 ± 1.7 mm [n = 2] in females); larger distance between nares in both sexes (IND mean 3.1 ± 0.1 mm [n = 14] in males, mean 3.7 ± 0.1 mm [n = 2] in females vs. mean 2.7 ± 0.4 mm [n = 10] in males, mean 3.1 ± 0.30 mm [n = 2] in females); wider finger III disks in both sexes (3FDD mean 3.5 ± 0.3 mm [n = 14] in males, mean 3.9 ± 0.1 mm [n = 2] in females vs. mean 1.7 ± 0.2 mm [n = 10] in males, mean 2.1 ± 0.6 mm [n = 2] in females); smaller metatarsal tubercule in males (OMTL mean 2.2 ± 0.3 mm [n = 14] vs. mean 3.2 ± 0.2 mm [n = 10]); and by having two subarticular tubercles on toe IV, see Fig. 9C (vs. three well-developed subarticular tubercles).

Figure 8. Variation in dorsal coloration in paratypes of *Kaloula discordia* sp. nov. Scale bar equals 5 cm. Photographs by N.A. Poyarkov.

From *K. latidisca* (morphological data taken from Chan et al. 2014), the new species can be distinguished by having shorter head in males (HL mean 11.2 ± 0.6 mm [n = 14] vs. mean 14.4 ± 0.8 mm [n = 4]); narrower head in males (HW mean 14.7 ± 0.7 mm (n = 14) vs. mean 18.7 ± 1.1 mm [n = 4]); smaller distance between nares in males (IND mean 3.1 ± 0.1 mm [n = 14] vs. mean 4.0 ± 0.1 mm [n = 4]); smaller distance between eyes in males (IOD mean 5.0 ± 0.2 mm [n = 14] vs. mean 5.7 ± 0.4 mm [n = 4]); smaller eye in males (EL mean 3.5 ± 0.3 mm [n = 14] vs. mean 4.6 ± 0.3 mm [n = 4]); smaller metatarsal tubercle in males (OMTL mean 2.2 ± 0.3 mm [=14] vs. mean 3.4 ± 0.1 mm [n = 4]); and by having two subarticular tubercles on toe IV, see Fig. 9C (vs. three subarticular tubercles).

Furthermore, *Kaloula discordia* sp. nov. differs from *K. aureata* Nutphand, 1989, by having an olive dorsum with no dark reticulations (vs. golden dorsum with dark brown reticulations); from *K. borealis* by having wider finger disks (vs. finger tips slightly dilated but not forming wide disks); by yellowish blotches on flanks absent (vs. present); and by olive dorsal coloration (vs. gray-brown). The new species is further diagnosed from *K. conjuncta* by the stratified coloration on flanks absent (vs. present) and by having distinct outer metatarsal tubercle (vs. weak or indistinct). *Kaloula discordia* sp. nov. differs from *K. ghoshi* by having axillary and inguinal light spots (vs. absent); tubercles on dorsum and venter (vs. smooth skin or with small granules dorsally); and olive dorsal coloration (vs. orange-brown). The new species further differs from *K. kalingensis* by having tubercles on the dorsum (vs. dorsum smooth); by having a distinct outer metatarsal tubercle (vs. indistinct); yellow or orange axillary and inguinal spots (vs. usually absent or small and red if present); lacking light pericloacal ring (vs. present); from *K. kokacii* by tuberculated dorsum (vs. smooth); axillary and inguinal spots (vs. absent); from *K. mediolineata* by lacking dorsolateral stripes (vs. present); sacral medial stripe absent (vs. present); and finger disks widened (vs. finger disks slightly dilated but not forming wide disks). *Kaloula discordia* sp. nov. is distinguished from *K. nonggangensis* by lacking protuberant tubercles on the upper surface of finger tips (vs. present); from *K. picta* by lacking dorsolateral stripes (vs. present); by the stratified coloration on flanks absent (vs. present); and by having wide finger disks (vs. finger disks slightly dilated but not forming wide disks). The new species further differs from *K. pulchra* by the absence of dorsolateral stripes (vs. always present); by having axillary and inguinal spots (vs. absent); from *K. rigida* by having wide finger disks (vs. finger disks slightly dilated but not forming wide disks); by the stratified coloration on flanks absent (vs. present); by the absence of dorsolateral stripes (vs. present); and by having axillary and inguinal spots (vs. absent). *Kaloula discordia* sp. nov. differs from *K. rugifera* by having wide finger disks (vs. slightly expanded small finger disks); by having axillary and inguinal spots (vs. absent); from *K. verrucosa* by having wide finger disks (vs. slightly expanded small finger disks); by having axillary and inguinal spots (vs. absent); from *K. walteri* by having wide finger disks (vs. slightly expanded small finger disks); by the stratified coloration on flanks absent (vs. present); having axillary and inguinal spots (vs. absent); and by having a distinct outer metatarsal tubercle (vs. indistinct or absent). *Kaloula discordia* sp. nov. is geographically isolated from other members of the *K. baleata* species complex and most of the other congeners, except *K. pulchra*, with which it occurs in sympatry everywhere throughout its range, and, possibly, *K. mediolineata*, with which the new species might co-occur in the Tay Ninh, Binh Phuoc, and Ba Ria-Vung Tau provinces of southern Vietnam.

Acoustic data. For a comparison of the male advertisement calls of *Kaloula discordia* sp. nov. with *K. indochinensis* sensu stricto, see Table 3; data for *K. indochinensis* are taken from Nguyen et al. (2022). The power call parameters of *Kaloula discordia* sp. nov. and *K. indochinensis* are quite similar, with the dominant frequency of the call being the same in the two species (0.38 kHz, range 0.34–0.43 kHz vs. 0.38 kHz, range 0.30–0.45 kHz, respectively) (Table 3). At the same time, *Kaloula discordia* sp. nov. had a slightly shorter call duration (186.1 ms, range 144–214 ms) than *K. indochinensis* (215.6 ms, range 194–250 ms) and a significantly longer intercall interval: 1539.4 ms (range 851–8954 ms) in *Kaloula discordia* sp. nov. vs. 789.3 ms (range 481–1627 ms) in *K. indochinensis*. These differences have to be taken cautiously as the records of calling of the two species were taken with different ambient temperatures (20.5 °C for *K. indochinensis* and 24.0 °C for the new species), which also could contribute to the observed differences in call parameters between the two species. Nguyen et al. (2022) did not calculate the number of pulses per call in *K. indochinensis* and noted the presence of one pulse per call in this species (Nguyen et al. 2022: Table 2), while we recorded 11–13 pulses per call in *Kaloula discordia* sp. nov. These differences actually result from the different terminology used by Nguyen et al. (2022) and our study, as the waveform oscillograms in Nguyen et al. (2022) clearly show the presence of several pulses in each call.

Etymology. The specific epithet "*discordia*" is a noun in apposition, in the nominative case, given in reference to the Roman mythological goddess Discordia. According to the poet Hesiod, this goddess personified not only strife and discord but also competition and labor (Hesiod, Theogony: 20–24, 226–230; see Most 2006). The duality of this name echoes the two aspects of the discovery of the new species. The first aspect is the authors' hard work and laborious approach in collecting data for the description of the new species. The second challenge pertains to the authors' internal struggle to choose a politically correct and neutral name for the new species. In modern taxonomy, international teams often face the common challenge of strife and competition; however, this can also lead to overall scientific progress. We recommend "*South Vietnamese Painted Frog*" as the common name in English, "*Yuzhnovietnamskiy Bychiy Uzkorot*" as the common name in Russian, and "*Ễnh ương Nam bộ*" as the common name in the Vietnamese language.

Conservation status. At present, the new species is reliably known only from four localities in southern Vietnam and a single locality in eastern Cambodia (Fig. 5; Appendix 1). The main threats to this species in Vietnam are habitat loss and degradation. The new species is restricted to the lowland monsoon tropical forests of southern Vietnam; it should be noted that these forests during the last 40 years have been subjected to greater anthropomorphic conversion (including logging, agriculture, road construction, and other human activities) than other areas in Vietnam (e.g., De Koninck 1999; Kuznetsov and Kuznetsova 2011; Laurance 2007; Meijer 1973; Meyfroidt and Lambin 2008). The range of the new species covers several nature conservation areas of southern Vietnam and Cambodia, including Cat Tien NP (Dong Nai Biosphere Reserve), Bu Gia Map NP, Lo Go-Xa Mat NP (Vietnam), and Phnom Prich Wildlife Sanctuary (Cambodia). Given the lack of comprehensive studies on the adjacent territories, we suggest *Kaloula discordia* sp. nov. be classified as Data Deficient (DD) according to the IUCN's Red List categories (IUCN 2019).

Kaloula laosensis Poyarkov, Orlov, Gorin & Milto, sp. nov.

https://zoobank.org/BF91FDDB-DE05-4518-96E5-E9151DDDF87F

Figs 2, 9A, 10–12, Table 4

Chresonymy. *Kaloula baleata* [partim]—Orlov and Ananjeva (2007: 148); Nguyen et al. (2009: 94).

Kaloula indochinensis [partim]—Chan et al. (2013: 334, 2014: 577); Teynié et al. (2014: 29); Chandramouli and Prasad (2018: 52); Poyarkov et al. (2021b: 39); Holden (2023: 148).

Holotype. ZISP 15284 (field label ZISP 199), adult male, Na Home Village, Bouphala District, near the border of Nakai-Nam Theun National Park, Khammouane Province, Laos, collected by N.L. Orlov, S.N. Nguyen, and K.D. Milto on June 18, 2009 (17.544528°N, 105.695278°E; elevation 174 m a.s.l.).

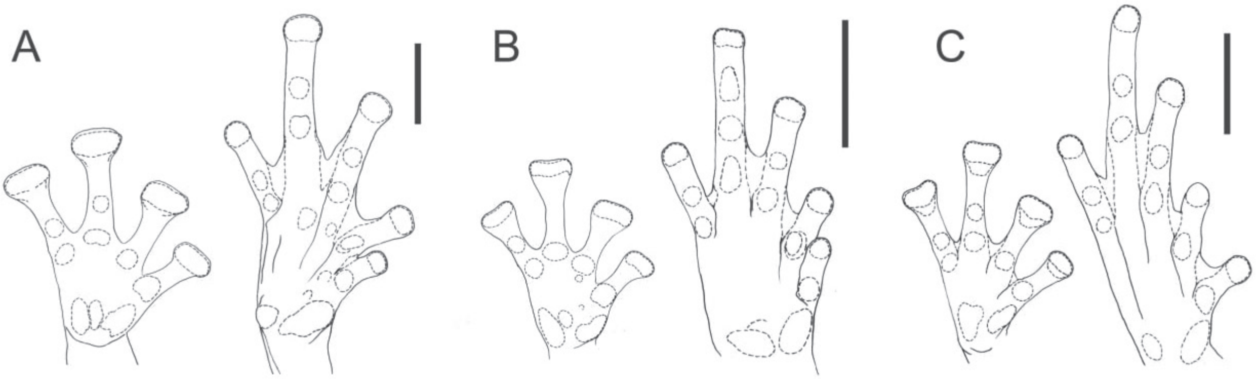

Figure 9. Morphological details of feet and hands in the three species of the *Kaloula baleata* species complex of eastern Indochina. **A.** Volar view of the left hand and plantar view of the right foot of *Kaloula laosensis* sp. nov.; **B.** Volar view of the left hand and plantar view of the right foot of *K. indochinensis*; **C.** Volar view of the left hand and plantar view of the right foot of *Kaloula discordia* sp. nov. Scale bar equals 5 mm. Drawings by N.A. Poyarkov.

Paratype (n = 1). ZMMU A-8144 (field label ZISP 200), adult female from the same location and with the same collection data as the holotype.

Diagnosis. *Kaloula laosensis* sp. nov. is distinguished from its congeners by the following combination of morphological attributes: (1) medium size (SVL ranging 44.0–54.8 mm); (2) eyes comparatively large (eye length comprising 75%–100% of snout length); (3) dark-brown dorsally, with numerous irregular dark blotches forming pericloacal ring; (4) gray or beige ventrally with dense white mottling on belly and limbs; (5) orange triangular patch on either side of the neck posterior to eyes; (6) bright-orange axillary patch present; (7) orange inguinal patch present; (8) bright-orange butterfly-shaped blotch above cloaca present; (9) gray spot on tibiotarsal articulation present; (10) dark interorbital bar present; (11) enlarged, widened finger disks (7.8%–7.9% of SVL), ca. 1.53 times wider than toe disks; (12) finger subarticular tubercle formula: 1:1:2:2; (13) toe subarticular tubercle formula: 1:1:2:2:2; (14) three metacarpal tubercles, median metacarpal tubercle in contact with inner metacarpal tubercle; (15) two metatarsal tubercles, inner metatarsal tubercle ca. three times larger than outer metatarsal tubercle; (16) three small supernumerary tubercles at the basis of toes I, III, IV.

Description of the holotype (Fig. 10). Adult male in a good state of preservation, habitus robust; head wider than long (HW/HL 1.19); snout projecting beyond lower jaw, truncated in dorsal and lateral views (Fig. 10C); top of head flat; upper eyelid lacking supraciliary tubercles; eye length less than snout length (EL/SL 0.75) and less than interorbital distance (EL/IOD 0.77); pupils round; nostrils rounded, placed more towards the lateral sides of snout, located closer to tip of snout than to eye, relatively close to each other (IND/IOD 0.63); supratympanic fold flat, tubercular; tympanum not visible (Fig. 10C); dorsal surfaces of body and limbs with sparse tubercules, getting denser backwards; ventral surfaces of body and limbs almost smooth (Fig. 10A, B). Cloacal opening unmodified, directed posteriorly. Forelimbs relatively long, more than a half of hind limb length (FLL/HLL 0.63); hand long, comprising more than a half of lower arm length (HAL/LAL 0.6) and almost half of forelimb length (HAL/FLL 0.47); fingers rather robust, flattened in cross section; relative finger lengths: I<II<IV<III; no webbing between fingers; terminal digits flattened into very wide transversely expanded T-shaped disks (Fig. 10E); finger subarticular tubercles distinct, protuberant, large and round, finger subarticular formula: 1:1:2:2; metacarpal tubercles three (Figs 9A, 10E), inner metacarpal tubercle elongate, flattened; outer metacarpal tubercule oval, dilated, slightly bigger than inner (OPTL/IPTL 1.05), median metacarpal tubercle the smallest, oval-shaped, contacting both the inner and outer metacarpal tubercles (Figs 9A, 10E); hindlimbs robust, relatively short, not much longer than body length (HLL/SVL 1.12); relative toe lengths: I<II<V<III<IV; tarsal fold on inner surface of tarsus absent; tips of all toes widened, forming terminal oval-shaped disks (Figs 9A, 10D); all toe disks having dorso-terminal groves; the disk on toe IV the largest; toe webbing well developed between all toes, reaching disks at all toes except toe IV; webbing formula ɪ1.5-2ɪɪ1.5-2.5ɪɪɪ1.5-3ɪᴠ3-2ᴠ; toe subarticular tubercules distinct, rounded, protruding; toe subarticular formula: 1:1:2:2:2; two metatarsal tubercles, inner metatarsal tubercle elongated, oval, shovel-shaped; outer metatarsal tubercle smaller, rounded; three small supernumerary tubercles at the basis of toes I, III, IV (Figs 9A, 10D).

Coloration. In life, dorsal surfaces of head and body dark brown with contrasting black spots and blotches; black interorbital bar between the upper eyelids; irregular black blotch on the snout; a series of black blotches forming a L-shaped chevron pattern in the scapular region; elongated black blotches on mid-dorsum, body flanks, and sacral area, forming a black pericloacal ring with irregular borders (Figs 2, 11); dorsal surfaces of limbs grayish brown with dark-brown to black blotches; flanks of body and lateral sides of head grayish brown; on lateral sides of belly getting dark-brown with white spots and blotches; pale orange patches on the neck posterior to eyes; bright reddish-orange axillary patch edged with dark brown; bright reddish-orange inguinal patch edged

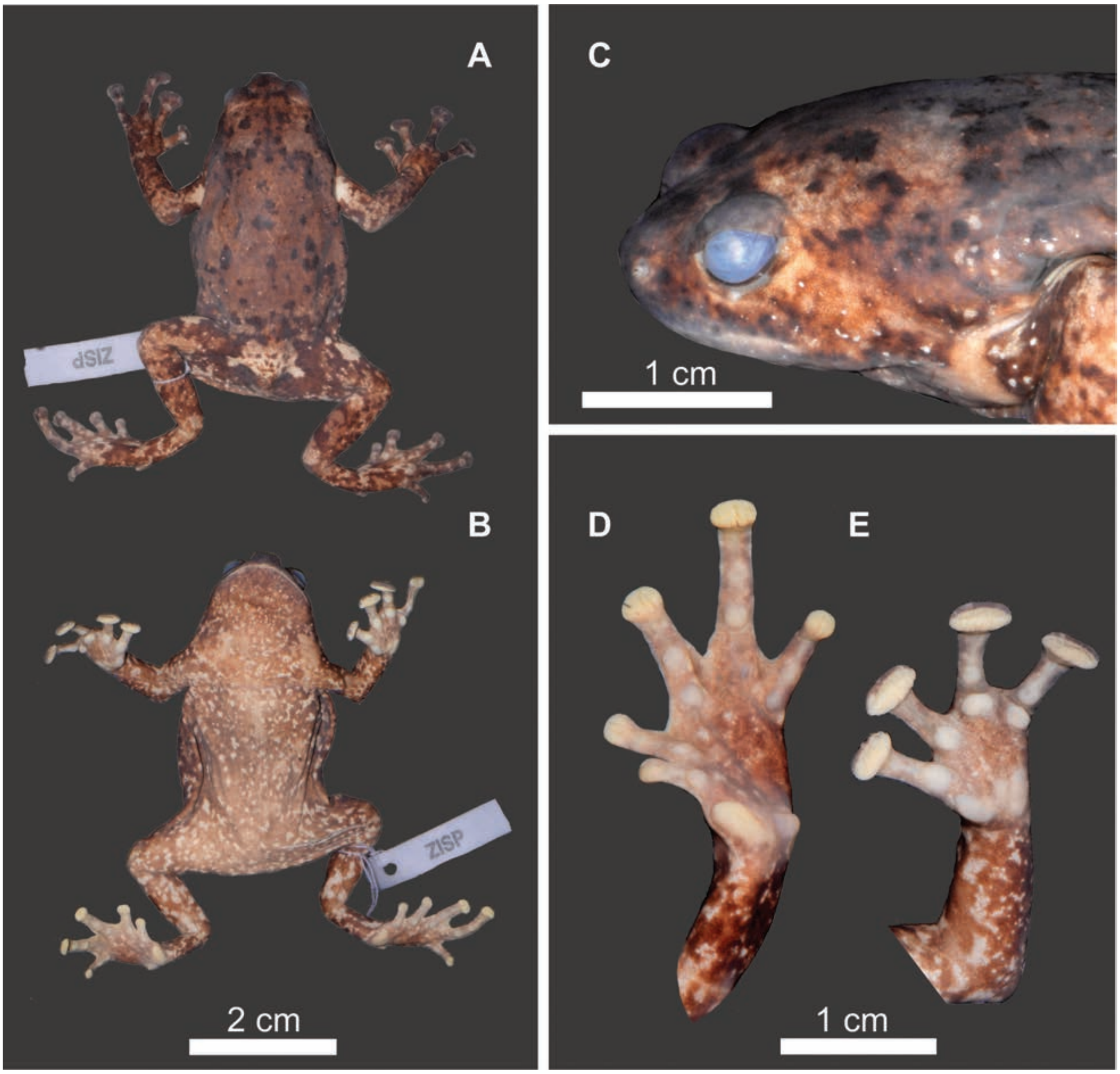

Figure 10. Holotype of *Kaloula laosensis* sp. nov. (ZISP 15284), adult male. **A.** Dorsal view; **B.** Ventral view; **C.** Head in a lateral view; **D.** Plantar view of left foot; **E.** Volar view of left hand. Photographs by V.A. Gorin.

with black; bright reddish-orange patch above cloaca in the center of dark pericloacal ring (Fig. 2); small grayish-beige spots near tibiotarsal articulation; ventral surfaces of head and body gray, darker near the jaw; belly and ventral sides of limbs densely mottled with white spots and dots (Fig. 10); iris chocolate brown with copper sparkles dorsally and ventrally (Fig. 11). In preservation after 15 years of storage in ethanol, dorsal coloration faded to dark grayish-brown; dark markings on dorsum are well descernible; light patches became less pronounced and faded to yellowish or beige in color, though the coloration pattern generally remained unchanged (Fig. 10).

Measurements of the holotype (in mm): SVL 44.0; HL 10.6; HW 12.6; SL 4.6; EL 3.3; N-EL 3.2; IND 2.7; IOD 4.3; UEW 2.4; FLL 27.6; LAL 21.7; HAL 13.0; HLL 49.3; TL 37.0; FL 25.4; IPTL 2.7; OPTL 2.8; 1FL 7.5; 1TOEL 7.3; OMTL 2.0; 3FDD 3.4; 4TDD 2.2.

Variation. Morphometric variation of the type series is presented in Table 4. A dorsal view of a female paratype specimen is presented in Fig. 12. In general, the female paratype specimen agrees well with the description of the holotype, diverging only in body size, which is larger than in the holotype (SVL 54.8 mm). Female paratype ZMMU-A-8144 has comparatively fewer dark markings on the dorsum (Fig. 12), but generally, the coloration pattern is similar to that of the holotype. Holden (2023: 148, fig. 152) published a photo of the new species from Laos as '*K. indochinensis*,' which has bright-red axillary, inguinal, and pericloacal patches and lighter grayish-brown dorsal coloration.

Tadpole morphology. Currently, data on the larval morphology of *Kaloula laosensis* sp. nov. are lacking.

Distribution and natural history. *Kaloula laosensis* sp. nov. is currently reliably known only from two localities in the Khammouane Province of Laos; the distribution

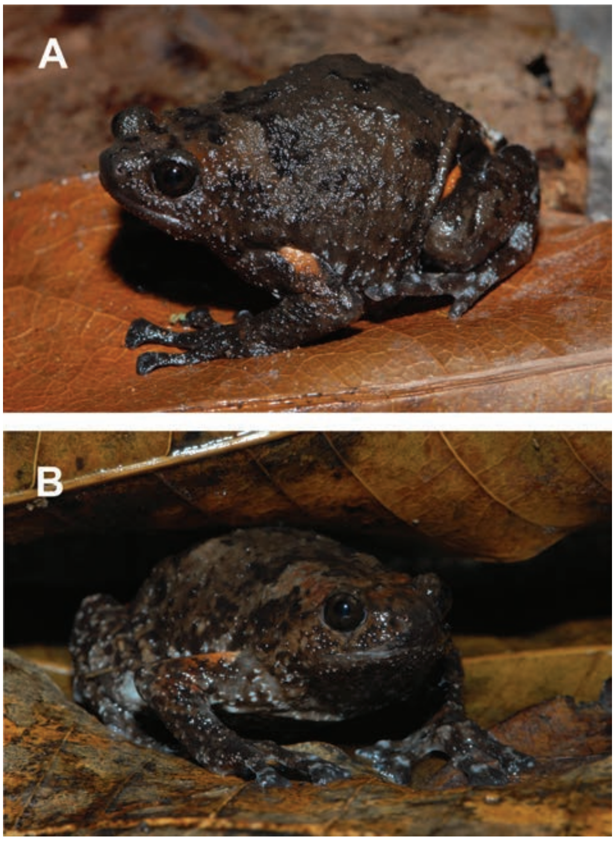

Figure 11. Dorsolateral (**A**) and frontal (**B**) views of the holotype of *Kaloula laosensis* sp. nov. in situ (ZISP 15284, male). Photographs by N.L. Orlov.

of the new species is shown in Fig. 5, and the locality information is detailed in Appendix 1. In Nakai-Nam Theun NP and in Hin Nam No NP, Khammouane Province, Laos, the new species was recorded from limestone evergreen tropical forests at relatively low elevations of ca. 100–300 m asl. The data on the natural history of *Kaloula laosensis* sp. nov. is scarce; it inhabits primarily lowland forests and is quite secretive, emerging only after heavy rains from June to September, when depressions in the forest floor are flooded and form temporary pools. The new species is likely associated with limestone karst landscapes of central Laos; the actual distribution of *Kaloula laosensis* sp. nov. is unknown, but we suppose that this species is likely to occur in adjacent provinces of Vietnam (Ha Tinh and Quang Binh), which harbor limestone forests similar in composition. Details about the new species' reproductive biology and diet are unknown.

Comparisons. *Kaloula laosensis* sp. nov. most closely resembles other members of the *K. baleata* species complex in overall morphology, and comparison with these species appears to be the most pertinent. Most specifically, the new species superficially resembles *K. indochinensis*, with which it was previously confused. From *K. indochinensis*, *Kaloula laosensis* sp. nov. can be distinguished by having relatively longer head in males (HL/SVL 0.24 [n = 1] by our data or mean 0.25 ± 0.1 [n = 4] reported by Chan et al. 2013) vs. mean 0.23 ± 0.1 [n = 18] by our data or [n = 25] reported by Chan et al. 2013); relatively wider disk of finger III in males (3FDD/HAL 0.26 ± 0.1 [n = 1] vs. mean 0.23 ± 0.1 [n = 18]); relatively longer first toe in males (1TOEL/FL 0.29 ± 0.1 [n = 1] vs. mean 0.26 ± 0.1 [n = 18]); by having three metacarpal tubercles with median metacarpal tubercle touching both the inner and outer metacarpal tubercles, see Fig. 9A (vs. median metacarpal tubercle small, not touching the inner metacarpal tubercle, see Fig. 9B); by having two subarticular tubercles on toe IV, see Fig. 9A (vs. three well-developed subarticular tubercles, see Fig. 9B); by having supranumerary tubercles at the basis of toes I, III, and IV, see Fig. 9A (vs. supranumerary tubercles absent, see Fig. 9B); by the presence of numerous contrasting black markings on dorsum (vs. absent or indistinct); and brownish dorsal coloration (vs. chocolate to dark grayish-brown).

From *K. baleata*, *Kaloula laosensis* sp. nov. can be distinguished by having a relatively larger distance between nares in males (IND/HW 0.21 [n = 1] by our data or [n = 6] reported by Chan et al. (2013) vs. 0.18 ± 0.1 [n = 10] reported by Chan et al. (2013)); wider finger III disks in males (3FDD 3.4 ± 0.1 mm [n = 1] by our data) or mean 3.1 ± 0.3 mm [n = 6] reported by Chan et al. (2013) vs. 1.7 ± 0.2 mm [n = 10] reported by Chan et al. (2013)); by having two subarticular tubercles on toe IV, see Fig. 9A (vs. three well-developed tubercles); and by having supranumerary tubercles at the basis of toes I, III, and IV, see Fig. 9A (vs. supranumerary tubercles absent).

From *K. latidisca*, *Kaloula laosensis* sp. nov. can be distinguished by having relatively shorter heads in males (HL/SVL 0.24 [n = 1] by our data or mean 0.25 ± 0.1 [n = 4] reported by Chan et al. (2013) vs. mean 0.27 ± 0.1 [n = 4] reported by Chan et al. (2014)); relatively smaller distance between eyes in males (IOD/HW 0.34 [n = 1] by our data or mean 0.33 ± 0.1 [n = 6] reported by Chan et al. (2013) vs. mean 0.30 ± 0.1 [n = 4] reported by Chan et al. ((2014); by the presence of numerous black markings on the dorsum (vs. black markings on dorsum absent or few); and by having two subarticular tubercles on toe IV (vs. three well-developed tubercles).

From *Kaloula discordia* sp. nov. (described above), the new species can be distinguished by having a relatively longer head in males (HL/SVL 0.24 [n = 1] by our data or mean 0.25 ± 0.2 [n = 6] reported by Chan et al. (2013) vs. mean 0.22 ± 0.2 [n = 14]); relatively shorter snout in males (SL/HL 0.43 [n = 1] by our data or mean 0.40 ± 0.1 [n = 6] reported by Chan et al. (2013) vs. mean 0.47 ± 0.1 [n = 14]); relatively narrower head in males (HW/HL 1.19 [n = 1] vs. mean 1.31 ± 0.2 [n = 14]); wider finger III disks (3FDD/HAL 0.26 [n = 1] vs. mean 0.23 ± 0.10 [n = 14]); shorter toe I (1TOEL/FL 0.29 [n = 1] vs. mean 0.25 ± 0.1 [n = 14]); by having three metacarpal tubercles, see Fig. 9A (vs. two metacarpal tubercles, see Fig. 9C); by having supranumerary tubercles at the basis of toes I, III, and IV, see Fig. 9A (vs. supranumerary tubercles absent, see Fig. 9C); by the brown coloration of the dorsum (vs. olive); and by the presence of numerous black markings on the dorsum (vs. black markings on dorsum absent or few).

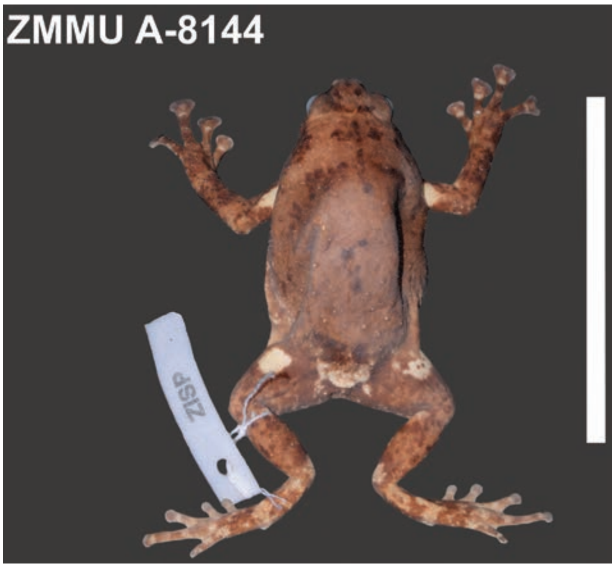

Figure 12. Dorsal coloration in a single paratype of *Kaloula laosensis* sp. nov. (ZMMU-A-8144, female) Scale bar equals 5 cm. Photograph by V.A. Gorin.

Furthermore, *Kaloula laosensis* sp. nov. differs from *K. aureata* by having a brownish dorsum with no reticulations (vs. golden dorsum with dark brown reticulations); from *K. borealis* by having wide finger disks (vs. finger tips dilated but not forming wide disks); by the absence of yellowish blotches on flanks (vs. present); an by brownish dorsal coloration (vs. gray-brown). *Kaloula laosensis* sp. nov. further differs from *K. conjuncta* by the stratified coloration on flanks absent (vs. present); by having distinct outer metatarsal tubercle (vs. weak or indistinct); from *K. ghoshi* by the presence of axillary and inguinal spots (vs. axillary and inguinal spots absent); by tuberculated dorsum and venter (vs. smooth or with small flat granules dorsally); by brownish dorsal coloration (vs. orange-brown). The new species differs from *K. kalingensis* in that it has tubercles on the dorsum (vs. dorsum smooth), a distinct outer metatarsal tubercle (vs. indistinct), and yellow or orange axillary and inguinal spots (vs. usually absent or small and red if present). *Kaloula laosensis* sp. nov. further differs from *K. kokacii* by the presence of dorsal tubercles, axillary and inguinal spots, and a light pericloacal ring (vs. absent). The new species differs from *K. mediolineata* by dorsolateral stripes and sacral stripe absent (vs. present); by having enlarged, wide finger disks (vs. finger tips slightly dilated but not forming wide disks); from *K. nonggangensis* by protuberant tubercles on the upper surface of finger tips absent (vs. present); from *K. picta* by the absence of dorsolateral stripes and stratified coloration on flanks (vs. present); by having enlarged, wide finger disks (vs. finger tips slightly dilated but not forming wide disks). The new species can be readily diagnosed from *K. pulchra* by the absence of dorsolateral stripes (vs. present) and by having axillary and inguinal spots (vs. absent). *Kaloula laosensis* sp. nov. further differs from *K. rigida* by having enlarged, wide finger disks (vs. finger tips slightly dilated but not forming wide disks), by the absence of dorsolateral stripes and stratified coloration on flanks (vs. present), and by having axillary and inguinal light spots (vs. absent). The new species differs from *K. rugifera* by having enlarged, wide finger disks (vs. slightly dilated small finger disks) and by having axillary and inguinal spots (vs. absent) and is further different from *K. verrucosa* by having very wide finger disks (vs. small finger disks) and by having axillary and inguinal spots (vs. absent). The new species differs from *K. walteri* by having very wide finger disks (vs. slightly dilated small finger disks), by the absence of stratified coloration on flanks (vs. presence), by having axillary and inguinal light spots (vs. absent), and by having a distinct outer metatarsal tubercle (vs. indistinct or absent). Except for *K. pulchra*, which can be found in central Laos in syntopy with the new species, *Kaloula laosensis* sp. nov. is geographically separated from most of its relatives.

Acoustic data. The male advertisement call of *Kaloula laosensis* sp. nov. has not been recorded, and the bioacoustic data on this species is absent.

Etymology. The specific epithet "*laosensis*" is an adjective in the nominative case, given in reference to the new species' distribution in central Laos. The name also mirrors the specific epithet of *K. indochinensis*, with which the new species was previously confused. We recommend "*Laotian Painted Frog*" as the common English name, "*Laosskiy bychiy uzkorot*" as the common name in Russian, "*Ễnh ương Lào*" as the common name in Vietnamese, and "ອຶງຢາງລາວ" ("*Ung Yang Lao*") as the common name in Lao languages.

Conservation status. At present, the new species is known only from two localities in Khammouane Province of Laos (Fig. 5). The main threats to this species in Laos are habitat loss and degradation due to intensified logging and deforestation, namely Nakai-Nam Theun NP and Hin Nam No NP. We propose that the IUCN's Red List categories (IUCN 2019) classify *Kaloula laosensis* sp. nov. as Data Deficient (DD) due to the absence of comprehensive studies in the adjacent territories.

Comments. We assume, based on the distribution of the new species, that several specimens from the type series of *K. indochinensis* in the original description by Chan et al. (2013), collected in Khammouance Province of Laos and labeled as '*K. indochinensis* Laos' or 'Laos OTU' throughout the text, actually represent *Kaloula laosensis* sp. nov. These specimens include six adult males stored in the Field Museum of Natural History (FMNH), Chicago, USA, under voucher numbers FMNH 270360–65. The lack of genetic information on these specimens in the original description of *K. indochinensis* prevented Chan et al. (2013) from recognizing their taxonomic distinctiveness.

Discussion

Until recently, *K. baleata* sensu lato was reported to inhabit a vast area in Southeast Asia, including territories of Indonesia, Malaysia, India, Thailand, Laos, and Vietnam (Blackburn et al. 2013; Chandramouli and Prasad 2018). This was until Blackburn et al. (2013), who published the first comprehensive phylogeny of the genus, revealed that

K. baleata is a complex of (presumably) cryptic species, which motivated morphological investigations and taxonomic revisions (Chan et al. 2013, 2014). Our discovery of *Kaloula discordia* sp. nov. from Southern Vietnam and *Kaloula laosensis* sp. nov. from Central Laos follows the recent descriptions of two new species from Indochina: *K. indochinensis* (see Chan et al. 2013) and *K. latidisca* (see Chan et al. 2014), as well as the elevation of *K. ghoshi* to full species status (see Chandramouli and Prasad 2018). It is quite remarkable that the populations from southern Vietnam and central Laos, which we herein describe as two new species, were well-known to the authors of previous taxonomic studies of this group (Chan et al. 2013, 2014). In fact, Chan et al. (2013) included six specimens from central Laos (FMNH 270360–65) in their morphological study, assigning them to *K. indochinensis* and including them in the type series of this species, but without assessing their genetic divergence from topotypic specimens of *K. indochinensis* from central Vietnam. Similarly, Chan et al. (2013) did not include specimens of the *K. baleata* complex members from southern Vietnam or adjacent Cambodia in their analysis. However, they included these localities in the proposed distribution range of '*K. indochinensis*,' likely based on photo records, likewise assuming their conspecificity. In essense, our study thus unravelled diversity in the *K. baleata* complex that was "hidden in plain sight" and thereby emphasized the need for careful genetic and phenotypic surveys in presumably widespread species of Southeast Asian amphibians. For example, the common practice of including specimens from different localities in a type series may be misleading. Here, the type locality of *K. indochinensis* described by Chan et al. (2013) from Gia Lai Province of Vietnam and the type locality of *Kaloula laosensis* sp. nov. in Central Laos are located in biogeographically and climatically different parts of Indochina, separated from each other by a straight distance of over 500 km (see Poyarkov et al. 2021b, 2023). Hence, including specimens from both areas in a type series without DNA-barcoding at least some representative specimens from each locality appears an unnecessary gamble.

Different research groups have developed a tradition of providing extensive datasets of morphological characters for Asian anurans (e.g., Poyarkov et al. 2014, 2020b, 2021a; Meegaskumbura et al. 2015; Garg et al. 2019; Biju et al. 2020; Suwannapoom et al. 2018; Brakels et al. 2023). However, some researchers continue to present limited datasets of seemingly handpicked morphological characters. In the description of *K. walteri* by Diesmos et al. (2002), authors provided data on a total of 21 morphometric characters, including complete measurements of fore- and hindlimbs. Mo et al. (2013) reduced the number of studied characters to 14 and lacked data on fingers, toes, and respective disks in their description of *K. nonggangensis*. In the description of *K. indochinensis* by Chan et al. (2013), the number of studied characters further decreased to 11, with no morphometric data on forelimbs and only partial data on hindlimbs. In the following paper, Chan et al. (2014) described *K. latidisca* and provided the same set of 11 characters. Finally, in the most recent taxonomic paper on the genus *Kaloula* (Chandramouli and Prasad 2018), the authors provided measurements on a set of 17 characters of *K. ghoshi*. None of the respective papers, however, explains or at least mentions the observed reduction in the number of studied morphometric characters. The lack of data on the morphology of these relatively newly described species makes it difficult to compare datasets from different studies and hampers further research into species diversity in *Kaloula* frogs (see the Morphology and Comparisons subsections in the Results section). Since amphibian taxonomy relies heavily on morphological descriptions, we emphasize the utmost importance of providing comprehensive and complete datasets for newly discovered and documented species that can then be re-used in future integrative studies.

Our discovery of two new species of the *K. baleata* complex from Indochina corroborates the idea that many species of Southeast Asian amphibians currently considered to be widely distributed may instead still encompass unrecognized diversity, hidden by a lack of comprehensive field surveys and/or the aforementioned deficiency of comprehensive morphological and genetic data (e.g., Chen et al. 2018; Hasan et al. 2019; Gorin et al. 2020; Suwannapoom et al. 2020; Poyarkov et al. 2020a; Lyu et al. 2023; Liu et al. 2024; Trofimets et al. 2024). Here, the new species of *Kaloula* from the Cat Tien National Park in southern Vietnam appears particularly remarkable. Cat Tien National Park covers one of the few remaining massifs of lowland monsoon forests in Vietnam and likely represents one of the best herpetologically studied areas in Vietnam (Yushchenko et al. 2023b). Herpetological exploration of this territory has continued for over 40 years with numerous expeditions and long-term monitoring programs by Vietnamese, Russian, German, USA, British, and Canadian herpetologists, the results of which were summarized in a monograph (Vassilieva et al. 2016). With 45 species of amphibians and 107 species of reptiles recorded in the park, Cat Tien NP is unanimously recognized as an important local center of herpetofaunal diversity (Geissler et al. 2011, 2015; Vassilieva et al. 2013, 2016; Holden and Poyarkov 2021; Yushchenko et al. 2023a,b). The new *Kaloula* species from Cat Tien NP thus illustrates the need for herpetological surveys even in presumably well-known Southeast Asian regions and species.

Moreover, our study provides new information on the evolution, diversity, and distribution of other members of the *K. baleata* species complex, specifically *K. latidisca* and *K. baleata*. Chan et al. (2014) described *K. latidisca* from Kedah, northern Peninsular Malaysia, based on phylogenetic data from Blackburn et al. (2013). In Chan et al.'s (2014) phylogenetic reconstruction, *K. latidisca* is suggested as a sister lineage to *K. baleata* sensu lato, which included samples from Java, Palawan, Borneo, Sulawesi, and southern Peninsular Malaysia. Chan et al. (2014) noted that the populations of southern Peninsular Malaysia and Borneo may not be conspecific with either *K. baleata* or *K. latidisca*, but they refrained from further taxonomic revisions and did not discuss the diversity and extent of distribution of members of the *K. baleata* complex in the

Thai-Malay Peninsula. First, our data showed relatively low, although species-level, sequence differentiation at the 16S rRNA gene between *K. latidisca* and the different lineages of *K. baleata* (p = 3.00%–3.96%; Table 2). Second, the monophyly of *K. baleata* with respect to *K. latidisca* is only moderately supported in our analyses (0.83/98, see Fig. 2). Finally, the mitochondrial distribution of *K. latidisca* and the three lineages of *K. baleata* partially overlap with each other, which requires investigating and potentially updating their respective ranges (see Fig. 1). Specifically, we hypothesize the following.

1. Our mitochondrial data suggests that *K. latidisca* inhabits southern peninsular Thailand, including Satun and Suratthani provinces (see Fig. 1); numerous localities of the *K. baleata* complex from the Isthmus of Kra in the north to the Thai-Malay national border in the south likely belong to this species, which is penetrating to Malaysia only in the state of Kedah (see Fig. 5).
2. The populations of the *K. baleata* complex from Tioman Island in southern Peninsular Malaysia, as well as from the Terengganu and Pahang states in northern Peninsular Malaysia, all belong to the *K. baleata* lineage 1 and thus appear conspecific with the populations from western Java based on their lack of mitochondrial differentiation. It appears that the Titiwangsa Mountain Range separates the *K. baleata* clade 1 and *K. latidisca* ranges in Peninsular Malaysia and southern Thailand.
3. The *K. baleata* population from the Tanintharyi Region of southern Myanmar features distinct mtDNA (lineage 3) that is genealogically closer to the mtDNA lineage(s) found in *K. baleata* sensu stricto (lineage 2) than to *K. latidisca* (Fig. 2). At the same time, this lineage likely extends to the Tenasserim Mountains/Tanintharyi Region north of the Isthmus of Kra (Figs 1, 5). This would imply that *K. latidisca* effectively separates the ranges of the *K. baleata* mtDNA lineages restricted to Sundaland and the Tenasserim Mountains north of the Isthmus of Kra.

These distribution patterns, combined with low genetic divergence in the 16S rRNA gene and unclear morphological differentiation between *K. latidisca* and *K. baleata,* stress the need for further integrative studies to clarify the distribution of these species and the taxonomic relevance of their mitochondrial diversity.

Our new data on the diversity and distribution in the *K. baleata* species complex may have important biogeographic implications. In eastern Indochina, the three *Kaloula* species (*K. indochinensis, Kaloula laosensis* sp. nov., and *Kaloula discordia* sp. nov.) group into a clade that is sister to the clade including all other populations of the complex, from the Thai-Malay Peninsula and Southeast Asian islands. The distribution areas of the three Indochinese species correspond well to the main biogeographic regions elaborated by Poyarkov et al. (2021b, 2023). Specifically, the range of *K. indochinensis* is associated with the Central Annamites region, while the newly described *Kaloula laosensis* sp. nov. and *Kaloula discordia* sp. nov. are restricted to the Northern Annamites and the Central-South Vietnam Lowlands, respectively. In the *K. baleata-K. latidisca* clade, the distribution of *K. baleata* lineage 1 encompasses Wallacea (Sulawesi, Sumba, and Palawan islands), Java, and Sumatra, while *K. baleata* lineage 2 extends over Borneo and eastern Peninsular Malaysia but also on Java (although it may be a result of a human-associated dispersal). At the same time, the range of *K. baleata* lineage 3 is restricted to the North Tenasserim Region, namely north of the Isthmus of Kra, while *K. latidisca* likely inhabits the South Tenasserim south of Kra and the South Thai-Malayan Lowlands Region. The diversification of these lineages and its association with paleographic events shall greatly benefit from phylogeographic analyses that implement nuclear markers, first to be able to assess their genomic divergence and to test for potential hybridization and introgression in areas of mitochondrial sharing.

Acknowledgements

The fieldwork in Vietnam was completed within the frameworks and with partial financial support from the research project E-1.2 of the Joint Vietnam-Russia Tropical Science and Technology Research Center for 2024 (Task No. 3). Permission to conduct fieldwork in Vietnam was granted by the Bureau of Forestry, the Ministry of Agriculture and Rural Development of Vietnam, and local administrations. Permission to conduct fieldwork and collect specimens in Cat Tien National Park was granted by Agreement #37/HD on the scientific cooperation between Cat Tien National Park and the Joint Vietnam-Russia Tropical Science and Technology Research Centre. Permissions to conduct fieldwork and collect specimens were granted by the Department of Forestry, Ministry of Agriculture and Rural Development of Vietnam (permit numbers #547/TCLN-BTTN; #432/TCLN-BTTN; #822/TCLN-BTTN; #142/SNgV-VP; #1539/TCLN-DDPH; #1700/UBND.VX); the Forest Protection Departments of the People's Committees of Gia Lai Province (permit numbers #530/UBND-NC; #1951/UBND-NV), Phu Yen Province (permit number #05/UBND-KT); by the Department of Forestry, Ministry of Agriculture and Forestry of Lao PDR (permit number #271/08, form #511); and by the Institute of Animals for Scientific Purpose Development (IAD), Bangkok, Thailand (permit number U1-01205-2558). Specimen collection protocols and animal operations followed the Institutional Ethical Committee of Animal Experimentation of the University of Phayao, Phayao, Thailand (certificate number UP-AE61-01-04-0022). We thank Valentina F. Orlova and Roman A. Nazarov (ZMMU) for permission to examine specimens under their care. We thank the members of MSU HerpLab, including T. Matsukoji, S. S. Idiiatullina, N. S. Kliukin, D. V. Arkhipov, and E. N. Solovyeva, for their support and assistance. NAP and AMB are grateful to Andrei N. Kuznetsov, Svetlana P. Kuznetsova, Hoi Dang Nguyen, and Leonid P. Korzoun for their support and organization of fieldwork. We express our sincere gratitude to Peter Mikulíček and two anonymous reviewers for numerous helpful comments and suggestions, which allowed us to improve the previous version of the manuscript.

This work was supported by the Russian Science Foundation to N.A. Poyarkov (Grant No. 22-14-00037, specimen collection and preservation, molecular and morphological analyses, data analyses) and the Thailand Science Research and Innovation Fund and the University of Phayao (Unit of Excellence 2025 on Aquatic animals biodiversity assessment (Phase I)) to C. Suwannapoom.

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Appendix 1

Table A1. List of localities of the *Kaloula baleata* complex members in Indochina appearing on Fig. 5. **Symbols:** (1) = Locality number on Fig. 5; (2) = Verified by morphology data (yes/no); (3) = Verified by molecular data (yes/no).

Species	(1)	(2)	(3)	Location	Sources
K. laosensis sp. nov.	1	yes	yes	Nakai-Nam Theun NP, Khammouan, Laos (type locality)	Chan et al. (2013); our data
K. laosensis sp. nov.	2	yes	no	Hin Nam No NP, Khammouane, Laos	Luu et al. (2016)
K. indochinensis	3	yes	no	Kiat Ngong, Xepian NBCA, Champasak, Laos	Chan et al. (2013)
K. indochinensis	4	yes	no	Chu Mom Ray NP, Kon Tum, Vietnam	Jestrzemski et al. (2013)
K. indochinensis	5	yes	yes	Kon Chu Rang NR, Gia Lai, Vietnam (type locality)	Chan et al. (2013); our data
K. indochinensis	6	yes	yes	Kon Ka Kinh NP, Gia Lai, Vietnam	Vassilieva et al. (2016); our data
K. indochinensis	7	yes	no	An Khe, Gia Lai, Vietnam	Chan et al. (2013); our data
K. indochinensis	8	yes	no	Song Hinh, Phu Yen, Vietnam	Do et al. (2017)
K. indochinensis	9	yes	no	Tuy Hoa, Phu Yen, Vietnam	Do et al. (2017)
K. indochinensis	10	yes	yes	Yok Don NP, Dak Lak, Vietnam	our data
K. discordia sp. nov.	11	yes	yes	Phnum Prech WS, Mondulkiri, Cambodia	Chan et al. (2013)
K. discordia sp. nov.	12	yes	no	Bu Gia Map NP, Binh Phuoc, Vietnam	Vassilieva et al. (2016); our data
K. discordia sp. nov.	13	yes	yes	Bao Lam, Lam Dong, Vietnam	Vassilieva et al. (2016); https://www.inaturalist.org/observations/212830145
K. discordia sp. nov.	14	yes	yes	Cat Tien NP, Dong Nai, Vietnam (type locality)	our data; https://www.inaturalist.org/observations/168961087
K. discordia sp. nov.	15	yes	no	Lo Go-Xa Mat NP, Tay Ninh, Vetnam	Vassilieva et al. (2016); our data
K. baleata Clade 3	16	yes	no	Thungyai Naresuan East, Umphang, Tak, Thailand	our data
K. baleata Clade 3	17	yes	no	Thong Pha Phum, Kanchanaburi, Thailand	https://www.inaturalist.org/observations/180645435
K. baleata Clade 3	18	yes	no	Suan Phueng, Ratchaburi, Thailand	our data
K. baleata Clade 3	19	yes	no	Kaeng Krachan NP, Phetchaburi, Thailand	https://www.inaturalist.org/observations/153399059; https://www.inaturalist.org/observations/176926007
K. baleata Clade 3	20	no	yes	Yeybu, Tanintharyi, Myanmar	Zug et al. (2018)
K. baleata Clade 3	21	yes	no	Saphan, Prachuap Khiri Khan, Thailand	https://www.inaturalist.org/observations//218052579
K. latidisca	22	yes	no	Saeng, Surathani, Thailand	Chan-ard et al. (2011); our data
K. latidisca	23	yes	no	Khao Sok NP, Surat Thani, Thailand	https://www.inaturalist.org/observations/70379448; https://www.inaturalist.org/observations/191660698
K. latidisca	24	yes	yes	Kapong, Phangnga, Thailand	https://www.inaturalist.org/observations/161810790
K. latidisca	25	yes	no	Khao Lak-Lam Ru NP, Phang Nga, Thailand	Pauwels et al. (2000); https://www.inaturalist.org/observations/194115334
K. latidisca	26	yes	no	Thalang, Phuket, Thailand	https://www.inaturalist.org/observations/202954715
K. latidisca	27	yes	no	Khao Phanom Bencha NP, Krabi, Thailand	our data
K. latidisca	28	yes	no	Ban Na San, Surat Thabi, Thailand	https://www.inaturalist.org/observations/83178477
K. latidisca	29	yes	no	Tha Sala, Nakhon Si Thammarat, Thailand	https://www.inaturalist.org/observations/190990980
K. latidisca	30	yes	no	Khao Luang NP, Nakhon Si Thammarat, Thailand	Chan-ard et al. (2011); our data
K. latidisca	31	yes	no	Khao Pu-Khao Ya NP, Phatthalung, Thailand	https://www.inaturalist.org/observations/92061585
K. latidisca	32	yes	no	Namtok Khao Chong, Trang, Thailand	Chan-ard et al. (2011); our data
K. latidisca	33	yes	no	Yan Ta Khao, Trang, Thailand	https://www.inaturalist.org/observations/55886801
K. latidisca	34	yes	yes	Ton Nga Chang WS, Songkhla, Thailand	https://www.inaturalistchancc.org/observations/114582513
K. latidisca	35	yes	yes	Tha Le Ban NP, Satun, Thialnd	our data
K. latidisca	36	yes	no	Tarutao Island, Satun, Thialnd	Nidup et al. (2013); our data
K. latidisca	37	yes	no	Bang Lang NP, Yala, Thailand	https://www.inaturalist.org/observations/165320389
K. latidisca	38	yes	yes	Gubir, Kedah, Malaysia (type locality)	Chan et al. (2014)
K. baleata Clade 1	39	yes	yes	Kampung Merapunh Lama, Pahang, Malaysia	Chan et al. (2014)
K. baleata Clade 1	40	yes	no	Hulu Terengganu, Terengganu, Malaysia	Badli-Sham et al. (2023)
K. baleata Clade 1	41	yes	no	Kuala Tahan, Pahang, Malaysia	https://www.inaturalist.org/observations/116005614
K. baleata Clade 1	42	yes	yes	Tioman Island, Pahang, Malaysia	Grismer et al. (2006)

ÖGH Herpetozoa
Austrian Herpetological Society

Herpetozoa 37: 421–428 (2024)
DOI 10.3897/herpetozoa.37.e134923

Vocalizations of *Atelopus ignescens* (Cornalia, 1849): The repertoire of a resilient species that whispers not to disappear

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Academic editor: Bibiana Rojas ♦ **Received** 17 August 2024 ♦ **Accepted** 3 December 2024 ♦ **Published** 18 December 2024

Abstract

The *Atelopus* (or harlequin frogs) have become a symbol of the ongoing efforts to conserve biodiversity in Ecuador over the past few decades. *Atelopus ignescens,* also known as jambato, was previously considered to be an extinct species until it was rediscovered in the Andean locality of Angamarca, Ecuador. This study presents and describes the calls of *Atelopus ignescens* for the first time. These are low intensity sounds that comprise a repertoire of three distinct call types: tonal, short and pulsed call. They have different spectral and temporal characteristics. The most notable difference in their structure is the presence or absence of pulses and the frequency modulation observed in tonal calls. In terms of spectral characteristics, the mean dominant frequency is 1.7 kHz, with up to 11 harmonics visible. A peculiar feature of the Jambato is the absence of a tympanic middle ear, a characteristic observed in the majority of *Atelopus* species. The absence of these structures, however, does not mean that harlequin toads are deaf since they seem to have extratympanic mechanisms of audition. It is our hope that our contribution will stimulate further research into the bioacoustics and behavior of this endangered and relatively unknown group of anurans.

Key Words

acoustic communication, bioacoustics, harlequin frogs, jambato, vocal repertoire,

Atelopus ignescens (Cornalia, 1849) is a species of diurnal anuran endemic to Ecuador that belongs to the family Bufonidae. The species is characterized by a medium size, a black coloration, and a reddish belly (Coloma et al. 2000). It is more commonly known by its vernacular name, "jambato," which is derived from the Kichwa terminology utilized by indigenous communities to refer to any members of this group of anurans as "sapo" or "rana" (Coloma and Guayasamin 2021). *Atelopus ignescens* was regarded as the most common, abundant, and widely distributed of the high Andean species in Ecuador (Peters 1973; Coloma et al. 2000). However, in the early 1980s, the species experienced a precipitous decline in population, and it was believed to reach extinction (Ron et al. 2003). This decline is not unique to this species. Throughout the Neotropics, a critical decline in populations of *Atelopus* species has been reported as a result of multiple impacts (e.g. infectious diseases caused by the *Batrachochytrium dendrobatidis* fungus, destruction of their habitats due to the expansion of the agricultural frontier and the overpopulation of introduced species such as *Oncorhynchus mykiss*) (La Marca et al. 2005; Pounds et al. 2006; Scheele et al. 2019; Forero Rodríguez et al. 2024). The history of the Jambato is not solely defined by this alarming and catastrophic disappearance. There are also fantastic accounts of its rediscovery and resilience amid a complex environmental crisis that continues to affect amphibians globally (Coloma 2016; Jaynes et al. 2022; Vega-Yánez et al. 2024; Yánez-Muñoz 2024).

The behavior of the *Atelopus* species is characterized by multimodal communication, which integrates auditory and visual signals (Cocroft et al. 1990; Lötters 1996). Acoustic communication has been described for 24 out of 100 species of its total diversity (Lötters et al. 2019; Rueda-Solano et al. 2020). In contrast to information on taxonomy, conservation, status and certain aspects of natural history (e.g. Coloma et al. 2010; Yánez-Muñoz et al. 2010; Tapia et al. 2017; Ortega-Andrade et al. 2021; Lötters et al. 2023; Vega-Yánez et al. 2024), the existing knowledge on the bioacoustics and behavioral characteristics of the *Atelopus* species of Ecuador is notably scarce.

The majority of *Atelopus* species lack a tympanic middle ear (Lötters 1996), which renders acoustic communication enigmatic, and perhaps explains the scarcity of studies in this subject (Womack et al. 2016, 2017, 2018). In the present study, we describe the vocal repertoire of *Atelopus ignescens*, the most emblematic anuran in Ecuador.

We recorded vocalizations of the jambato from the Andean locality of Angamarca, province of Cotopaxi, Ecuador (1°7'51.31"S, 78°54'16.85"W; 2962 m; Fig. 1). The area is situated in the western foothills of the An-

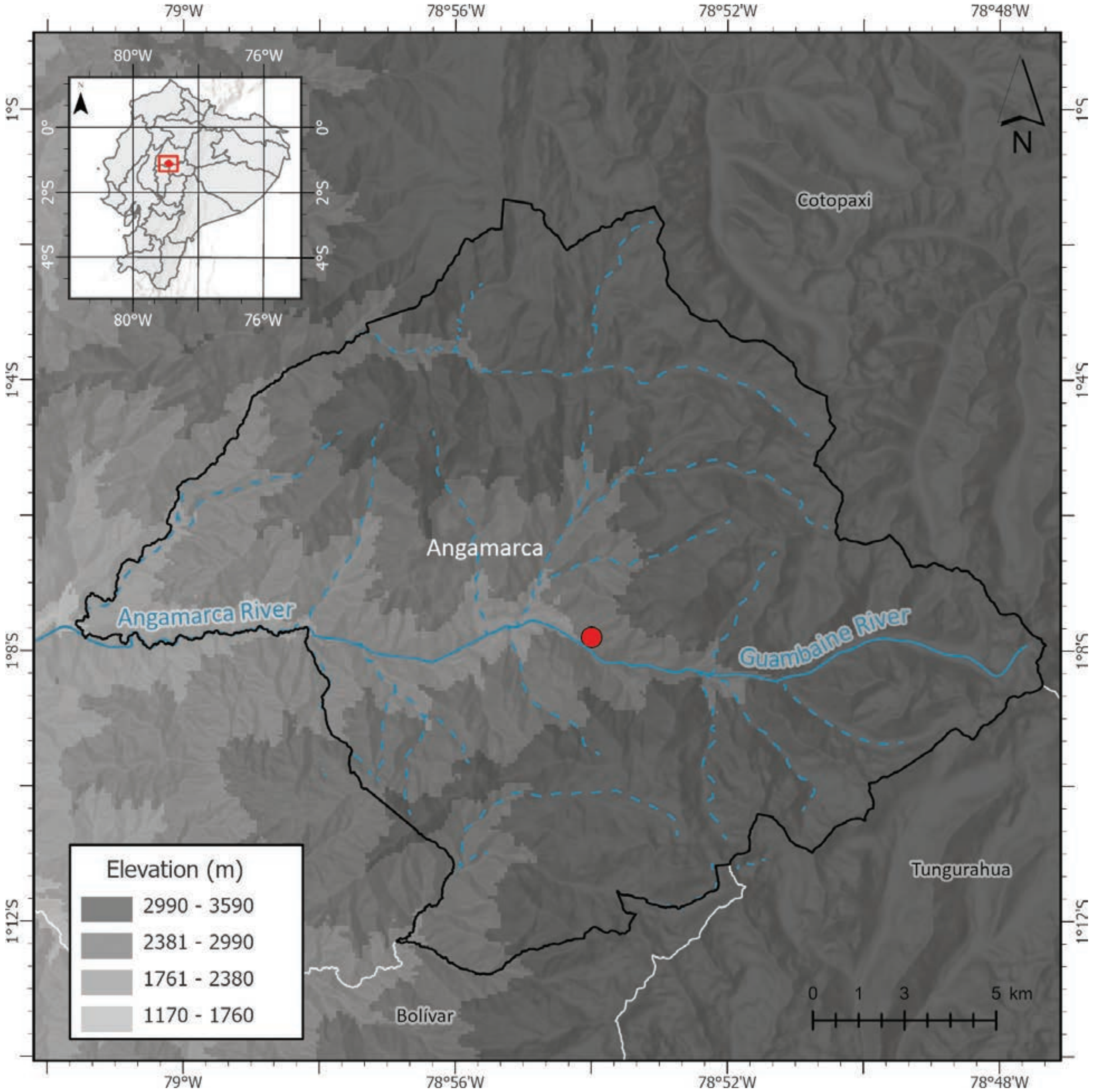

Figure 1. Map of the location of Angamarca (Ecuador), where the recordings of *Atelopus ignescens* were made.

des Mountains, belonging to the evergreen shrubland and paramo grassland ecosystem. The habitat is predominantly characterized by agricultural mosaic areas, grasslands and páramo vegetation (Vega-Yánez et al. 2024).

The vocalizations of an uncollected adult male of *A. ignescens* were recorded between 10:00–12:00 on December 8, 2021. The male was calling from grasslands and herbaceous vegetation at ground level. The calls were recorded using a cell phone, saving the audio files in FLAC format at a sampling rate of 44.1 kHz and 24 bits resolution. Prior to analysis, the audio files were converted to the WAV format and edited using the Adobe Audition CS6 software. The recordings are deposited at the Laboratorio de Biología Evolutiva de la Universidad San Francisco de Quito (LBE) and the Fonoteca Zoológica (www.fonozoo.com) del Museo Nacional de Ciencias Naturales (CSIC), Madrid, Spain (Appendix 1). The research was conducted under permits granted by the Ministerio de Ambiente, Agua y Transición Ecológica No. MAATE-DBI-CM-2022-0245.

The spectral and temporal parameters of calls were analyzed with the software Raven 1.6 (K. Lisa Yang Center for Conservation Bioacoustics at the Cornell Lab of Ornithology 2024). We used a Hann window with 256 samples of the Fast Fourier Transformation (FFT), a 90% overlap, and a frequency grid with 512 samples of the Discrete Fourier Transformation (DFT). The analyzed parameters were: Call duration (**CD**)**,** Rise time (**RT**), Decay time (**DT**) Intervals between calls (**IC**), Call rate (**CR**) Notes/call (**NC**), Note Duration (**ND**), Intervals between notes (**IN**), Note rate (**NR**) Pulse/note (**PN**), Pulse duration (**PD**) Intervals between pulses (**IP**), Pulse rate (**PR**), Dominant frequency (**DF**), Minimum frequency (**MinF**), Maximum frequency (**MaxF**), Frequency modulation (**FM**), Number of visible harmonics (**NH**), Harmonic frequency range, series of values that correspond to multiples of the fundamental frequency, with the value of the second and last harmonic taken as the reference (**HRF**). Definitions, terminology, and measurements of acoustic parameters follow the terms of Cocroft and Ryan (1995), Köhler et al. (2017), and Sueur (2018). The definition of call structure and the calculation of frequency modulation were based on Emmrich et al. (2020). In addition, to define the call types of the genus *Atelopus*, we follow the classification proposed by Cocroft et al. (1990). The oscillogram and spectrogram were obtained using R software (R Core Team, 2024), with the Seewave package version 2.2.3 (Sueur et al. 2008). To create the figures, we used a Hann window at 99% overlap with a size of 512 samples of the fast Fourier transform (FFT). The audio files in WAV format were imported with the tuneR package version 1.4.7 (Ligges et al. 2024). For each acoustic parameter, we calculated central tendency (means) and dispersion measures (maximum, minimum, and standard deviation).

The vocalizations of *Atelopus ignescens* comprises three distinct call types, ranging from non-pulsed to pulsed calls (Fig. 2, Table 1). They are low-intensity sounds, rendering them difficult to discern at close range. Intriguingly, their sounds remind us of rodent whispering (e.g. the vocalizations of *Rhipidomys albujai* described in Brito et al. (2017)). The mean call duration across these

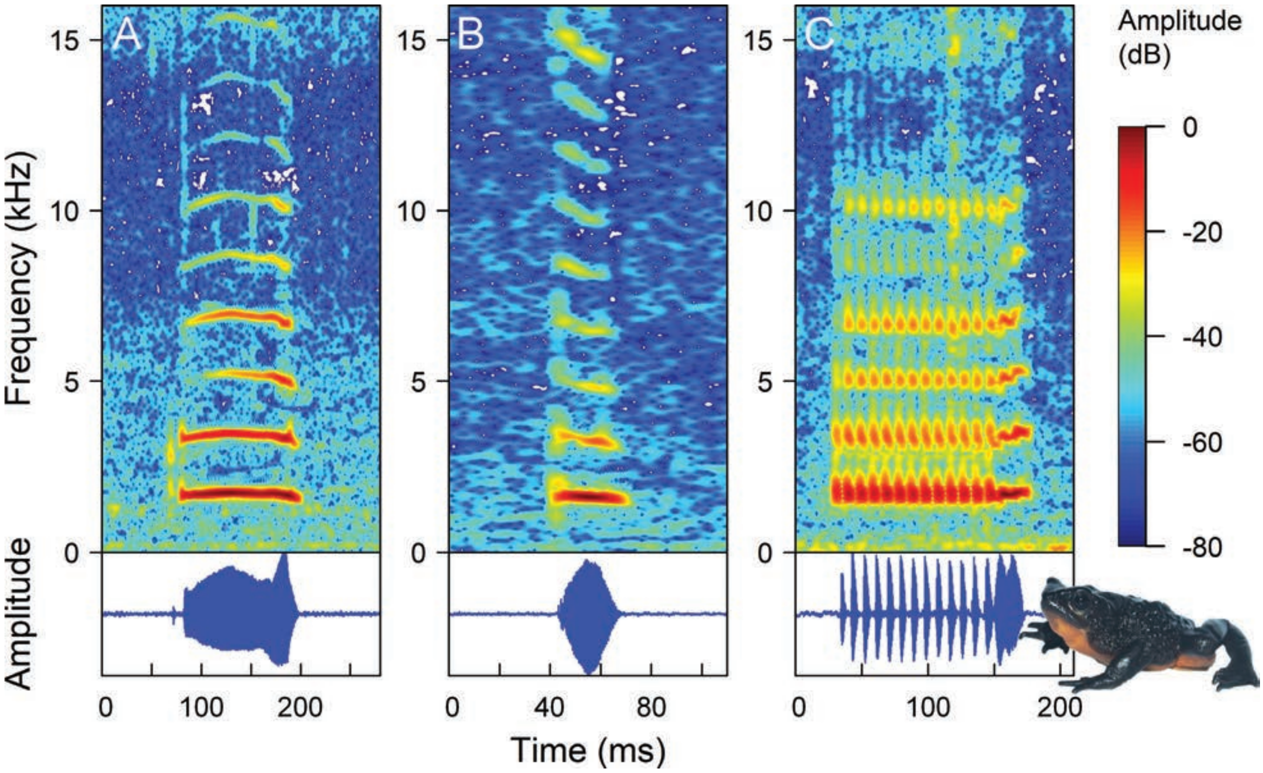

Figure 2. Call types present in *Atelopus ignescens* vocalization. **A.** Tonal call (Tc); **B.** Short call (Stc); **C.** Pulsed call (Pc). Spectrograms obtained using the Hann window at 99% overlap and 512 samples of FFT siz

Table 1. Spectral and temporal values of *Atelopus ignescens*. The abbreviations used in the parameters correspond to: DF = Dominant frequency; MinF = Minimum frequency; MaxF = Maximum frequency; FM = Frequency modulation; NH = Number of visible harmonics; HRF = Harmonic Frequency Range; CD = Call duration; RT = Rise time; DT = Decay time; IC = Intervals between calls; CR = Call rate; NC = Notes/call; ND = Note duration; IN = Intervals between notes; NR = Note rate; PN = Pulses/Note; PN = Pulse duration; IP = Interval between pulses; PR = Pulse rate. The abbreviations used in the call types correspond to: Tc = Tonal call; Stc = Short call; Pc = Pulsed call. The abbreviations used in units of measurement correspond to: kHz = kilohertz; ms = milliseconds; s = seconds; /min = per minute; /s = per second. We indicate: number of specimens/calls/notes/pulses.

Parameters	Call (general)	Call Types		
	n = 1/15/15/27	Tc	Sc	Pc
DF (kHz)	1.5–1.8 (1.7 ± 0.07)	1.64–1.81 (1.71 ± 0.06)	1.46–1.81 (1.60 ± 0.09)	1.55–1.72 (1.68 ± 0.05)
MinF (kHz)	1.03–1.64 (1.50 ± 0.12)	1.55–1.72 (1.64 ± 0.08)	1.29–1.55 (1.44 ± 0.06)	1.03–1.64 (1.51 ± 0.14)
MaxF (kHz)	1.55–2.07 (1.78 ± 0.12)	1.64–1.89 (1.71 ± 0.10	1.55–1.98 (1.70 ± 0.10)	1.72–2.07 (1.83 ± 0.12)
FM (Hz/ms)	0–14.38 (5.64 ± 4.26)	0.27–1.56 (0.91 ± 0.48)	1.71–14.38 (7.93 ± 3.34)	0–0.92
NH	5–11 (8.86 ± 1.63)	6–10 (9.67 ± 0.52)	8–11 (9.76 ± 0.83)	5–10 (8.11 ± 1.80)
HRF (kHz)	2.93–17.14	3.27–17.05	2.93–17.05	3.93–17.14
CD (ms)	27–1716 (370 ± 460.98)	111–322 (209.33 ± 81.94)	21–51 (33.82 ± 10.20)	189–137
RT %	18–91 (42.68 ± 16.44)	18–52 (35.75 ± 14.2)	30–70 (47.63 ± 11.13)	20–67 (35.83 ± 12.25)
DT%	9–82 (57.32 ± 16.44)	48–82 (64.25 ± 14.2)	30–70 (52.38 ± 11.13)	33–80 (64.17 ± 12.25)
IC (s)	2283–19178 (7465.54 ± 5193.34)	–	–	–
CR (/min)	3.13–25.97 (11.38 ± 7.19)	–	–	–
NC	1–4 (1.56 ± 0.89)	–	–	–
ND(ms)	21–266 (66.33 ± 72.83)	–	–	–
IN (ms)	41–612 (418.11 ± 170.87)	–	–	–
NR (/s)	1.57–10.87 (2.87 ± 3.01)	–	–	–
PN	13–15	–	–	–
PD (ms)	3–21 (7.67 ± 4.26)	–	–	–
IP (ms)	2–7 (4.76 ± 1.33)	–	–	–
PR (/s)	62.5–142.86 (90.20 ± 24.74)	–	–	–

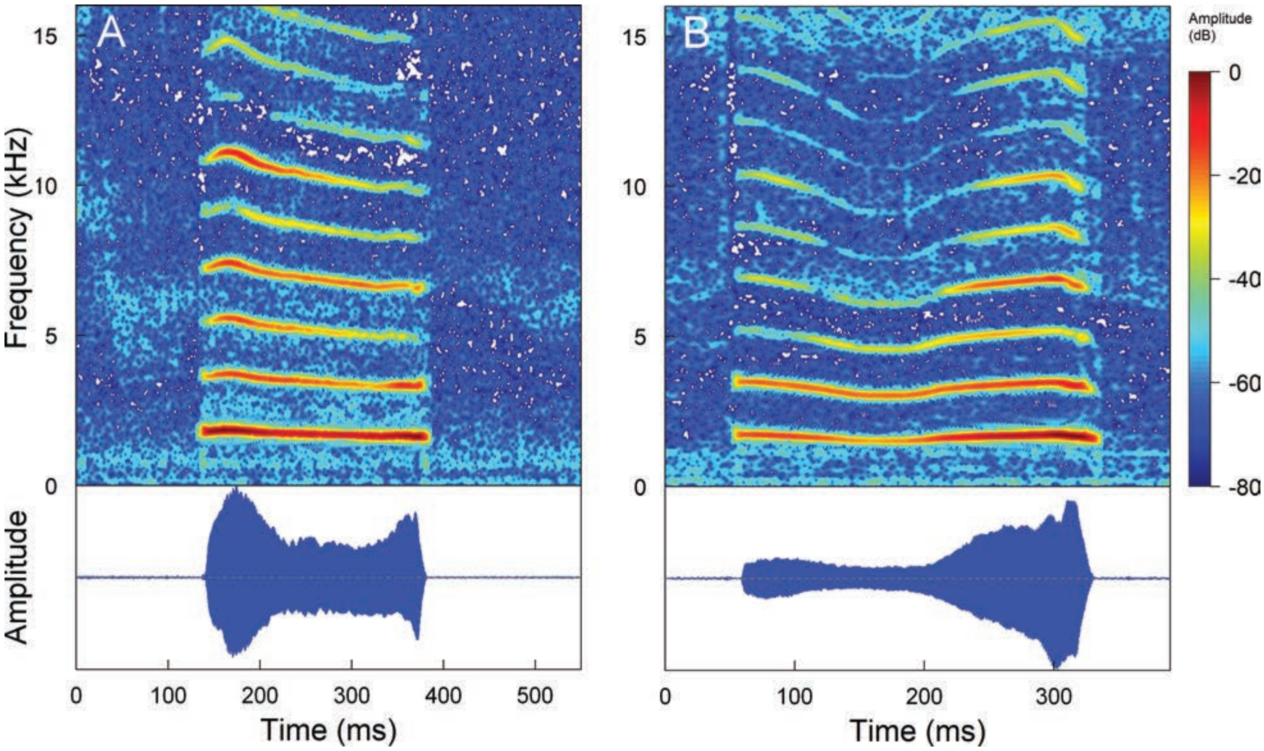

Figure 3. Variability of the tonal call of Atelopus ignescens, based on frequency modulation patterns **A.** Downward modulation tonal call; **B.** Complex modulation tonal call (upward-downward). Spectrograms obtained using the Hann window at 99% overlap and 512 samples of FFT size.

vocalizations in general is 370 ± 460.98 ms, emitted at mean intervals of 7465.54 ± 5193.34 ms, with a mean rate of 11.38 ± 7.19 calls/minute. The calls are composed of a mean of 1.56 ± 0.89 notes. The mean note duration is 66.33 ± 72.83 ms, emitted at mean intervals of 418.11 ± 170.87 ms, with a mean rate of 2.87 ± 3.01 notes/second. Some calls are composed of 13–15 pulses. The mean pulse duration is 7.67 ± 4.26 ms, emitted at mean intervals of 4.76 ± 1.33 ms, with a mean rate of 90.20 ± 24.74 pulses/second. The calls are downward frequency modulated (non-frequency modulated in pulsed calls), with a mean frequency modulation of 1.66 ± 0.07 Hz/ms. The mean dominant frequency is 1.66 ± 0.07 kHz. The mean minimum frequency is 1.50 ± 0.12 kHz, while the mean maximum frequency is 1.78 ± 0.12 kHz. Up to 11harmonics are visible, with the second having a mean frequency of 3.32 ± 0.15 kHz (ranging from 2.93–3.62 kHz) and the eleventh a mean frequency of 16.71 ± 0.34 kHz (ranging from 16.37–17.05 kHz) (Fig. 2, Table 1).

The vocalizations of *Atelopus ignescens* lack a stereotyped structural pattern. They are classified into three distinct types of calls based on their structural and temporal characteristics. Two of the types of vocalizations are non-pulsed calls. 1) *Tonal calls* (Tc; Fig. 2A) are defined as having a duration exceeding 100 ms. The majority of calls exhibit a downward modulated frequency, which in some instances can be a complex modulation between upward-downward (Fig. 3). The amplitude envelope demonstrates a mean rise of 35.75% and a mean decay of 64.25% of the total call time. This indicates that this particular type of call reaches its maximum amplitude in a relatively short period of time, with a subsequent progressive decay. 2) *Short calls* (Sc; Fig. 2B) are calls characterized by a duration not exceeding 100 ms and a downward frequency modulation. The amplitude envelope demonstrates a mean rise of 47.63% and a mean decay of 52.38% of the total call time. This indicates that in this type of call, the time to reach its maximum amplitude and subsequent decay is progressive. 3) *Pulsed calls* (Pc; Fig. 2C). In contrast, this type of call is characterized by repetitive and continuous bursts of emissions or pulses, with a duration of no more than 21 ms. The amplitude envelope exhibits a mean rise of 35.83% and a mean decay of 64.17% over the total time. This suggests that the pulses reach their maximum amplitude in a relatively short time, with a progressive decay.

In this study we present for the first time the description of the spectral and temporal parameters of *Atelopus ignescens* calls. A repertoire composed of three structural calls types (i.e., tonal call, short call, pulsed call). The vocalizations described in previous studies indicate that species of the genus *Atelopus* have more than one call type in their repertoire (e.g. Lötters et al. 1999; Granda-Rodríguez et al. 2020; Jorge et al. 2020; Rueda-Solano et al. 2020). Conversely, few species emit only one type of call (see Lötters et al. 2019). The *Atelopus ignescens* repertoire is comprised of three temporally distinct call types. While the classification system initially proposed by Cocroft et al. (1990) was considered, the *Atelopus ignescens* call is frequency modulated and thus does not have pure tones. Consequently, the classification criteria were developed based on calls that exhibited both tonal and pulsed characteristics. Although an emission pattern has been defined and characterized in the calls of the genus *Atelopus*, which is useful for unifying criteria for comparison, the behavioral contexts of each of its species have been poorly studied, defined, and understood (Lötters et al. 2019).

The vocalizations of *Atelopus* species from Ecuador remain largely unknown, with only those of *Atelopus exiguus* having been described (Coloma et al. 2000) out of the 25 species in Ecuador (Centro Jambatu 2020). Notably, *A. ignescens* was the first amphibian species described in Ecuador, and had its call formally documented only after 175 years (Cornalia 1849). This lack of information, perhaps related to declining populations (La Marca et al. 2005; Scheele et al. 2019; Lotters et al. 2023) can lead to misconceptions about species' vocalization capabilities (Batallas and Brito 2022). The absence of a tympanic middle ear in *Atelopus* (McDiarmid 1971) may further reinforce the notion that they might be deaf and that acoustic communication is insignificant (Cocroft et al. 1990). The *Atelopus* calls are characterized as inconspicuous and unremarkable (Cocroft et al. 1990; Rivera-Correa et al. 2021). In fact, the short non-pulsed call was not discernible at the time of recording the vocalizations of *A. ignescens*. This former type of call is consistent with the anecdotal description by Peters (1973), who describes them as a slight, low sound that is difficult to hear even at close range. However, more studies are needed to understand extra tympanic mechanisms for hearing in the absence of a middle ear and the relative importance of acoustic communication in this group (e.g. Boistel et al. 2011; Womack et al. 2018).

It is noteworthy that the Jambato, like the majority of the genus *Atelopus*, is distinguished by the absence of a tympanic middle ear (McDiarmid 1971). This trait renders their acoustic communication particularly intriguing, as they would be considered deaf subjectively. The limited knowledge of these endangered anurans presents opportunities for diverse studies on their behavior and communication.

Atelopus ignescens has become the most representative species for conservation in Ecuador and the Neotropics. Beyond behavioral characteristics, we endow this iconic species with an acoustic identity. We hope that this contribution will establish a baseline for acoustic knowledge of this species, thus facilitating future monitoring and discovery of new populations using passive acoustic methods. Furthermore, acoustic data may serve as a crucial component in evaluating the potential taxonomic issues that remain unresolved in this species (see Guayasamin et al. 2010). This knowledge is incorporated into the efforts to comprehend and preserve jambato, ensuring that its subtle calls are not lost on truly deaf ears.

Acknowledgments

First of all, we thank the community of Angamarca for their collaboration and interest in the conservation of the Jambato. We thank the members of the Alianza Jambato, with whom we had the opportunity to embark on fieldwork and engage in shared experiences. To Mario Yánez, for his valuable stories and advice on the conservation of this species, which gave us enough encouragement to shape this work. Special thanks to Laura González of the Fonoteca Zoológica de Madrid for her assistance in the assignment of collection numbers and the generation of QR codes. We thank Bibiana Rojas and two anonymous reviewers for their valuable and constructive comments that helped to improve the quality of this manuscript. This study was partially funded by Universidad San Francisco de Quito USFQ (Grants HUBI 5466, 16871, 17857). We also extend our appreciation to all the researchers around the globe for their efforts over the past decades to prevent the extinction of this species.

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Appendix 1

Recordings of an uncollected specimen of *Atelopus ignescens*. The associated codes correspond to three recordings, which were deposited at the Laboratorio de Biología Evolutiva (LAB) and are available at the Fonoteca Zoológica de Madrid (FZ):

A **B** **C**

Figure A1. Codes correspond to three recordings, available at the Fonoteca Zoológica de Madrid (FZ). **A.** LBE-C 061, FZ 14845, https://lc.cx/La_JkA; **B.** LBE-C 062; FZ 14846; https://lc.cx/G2D01L; **C.** LBE-C 063; FZ 14847; https://lc.cx/AGXuN4.