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Observation records of the Bangon Monitor Lizard, *Varanus bangonorum* (Squamata, Varanidae), with emphasis on behaviour and local threats, from the Municipality of Abra de Ilog, Occidental Mindoro, Philippines

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Abstract

Varanus bangonorum, the Bangon Monitor Lizard, is one of eleven Varanidae species endemic to the Philippines; its occurrence is restricted to dwindling habitats on the islands of Mindoro and Semirara. This lizard is still poorly known and is presently classified as “Least Concern” under the IUCN Red List in 2022. Observations recorded during eleven years (2011 to 2022) originate from the north-central Municipality of Abra de Ilog in Occidental Mindoro where this species was never before documented. We emphasise the primary factors that pose threats to the monitor lizard population, including insights gained from the knowledge of local community members. We present information about the lizards’ diet, for example, they can consume invasive toxic cane toads without harm. The result of our collective long-term observation records may serve as a baseline for further studies and contribute to evaluating the conservation status of this species.

Key Words

Bayawak, foraging behaviour, herpetofauna of the Philippines, monitor lizard, new locality records

Introduction

The Philippines is a tropical archipelago of 7641 islands (Kinser 2020) bordered by the Pacific Ocean to the east and the West Philippine Sea (South China Sea). Two major seasons determine the climate: a rainy season from June to November and a dry season from December to May, with noticeable temperature drops from December to February (Philippine Atmospheric, Geophysical, and Astronomical Services Administration 2022). Stated as one of the world’s eighteen mega-biodiverse countries (Convention on Biological Diversity 2022), the Philippines hosts a high percentage of endemic species, including eleven species of Varanidae (Auliya and Koch 2020). All are protected under national law and are classified as Critically Endangered (1), Vulnerable (2) and Other Threatened Species (8) by the Department of Environment and Natural Resources (2019).

One of the representatives of this family is *Varanus bangonorum* Bangon Monitor Lizard or Mindoro Water Monitor (Welton et al. 2014; Eidenmüller 2021); “Bayawak” in Tagalog and most local dialects.

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The Bangon Monitor Lizard was first described in 2014 as phenotypically similar, but not closely related to *Varanus marmoratus* and is assigned to subgenus *Soterosaurus* (Welton et al. 2014; Bucklitsch et al. 2016). The distinguishing features of this species are large dark spots in the gular fold (Auliya and Koch 2020), 136 ± 9 dorsal scales at mid-body and 160 ± 6 ventral scales (Welton et al. 2014). These lizards are found only on two islands: Mindoro and Semirara (Camina 2019). The insular distribution of the species is not well known (Suarez 2019), 17 specimens examined by Welton et al. (2014) from Mindoro Island were from Occidental Mindoro Province: Municipality of Sablayan, Municipality of Paluan and Municipality of San Jose; Oriental Mindoro Province: Municipality of Naujan and Mt Halcon; and only one locality from Semirara Island, Municipality of Caluya. The lizard's IUCN Red List status was recently updated from Not Evaluated (NT) to Least Concern (LC) (Cielo and Gaulke 2022).

Our report details visual encounters from the north-central Municipality of Abra de Ilog in Occidental Mindoro where observations of *Varanus bangonorum* have never been documented before. Observations in this region complete the gap in the distribution of this species on Mindoro Island. In general, observations of this monitor lizard species are rarely documented (Alviola et al. 2022).

Methods and materials

Observation area

Mindoro is the seventh largest island in the Philippines, located off the south-western coast of Luzon and north-east of Palawan and politically divided into two provinces: Occidental Mindoro and Oriental Mindoro. All our observations originate from the vicinity of Barangay Udalo, Municipality of Abra de Ilog, Province of Occidental Mindoro. The total observation area, locally known as Munting Buhangin, consists of 14.52 ha (145,200 m^2) of privately-owned real estate, subdivided into eighty residential lots and several common areas with currently fourteen finished vacation houses of various styles and sizes (Fig. 1).

Ridges with a maximum height of 155 m elevation form natural boundaries to the east, south and west; the northern border is an approximately 700-metre-long rocky coast interspersed with four beaches and coves (Suppl. material 1: fig. S1). This shoreline is closely fronted by a long stretch of coral reefs and forms part of the Verde Island Passage (or Verde Island Marine Corridor), internationally hailed as the "Center of the Center of Marine Biodiversity" (Carpenter and Springer 2005). There is no direct road access, hence no motorised traffic within this area. To protect the natural surroundings with its inhabiting wildlife, trespassing and harmful activi-

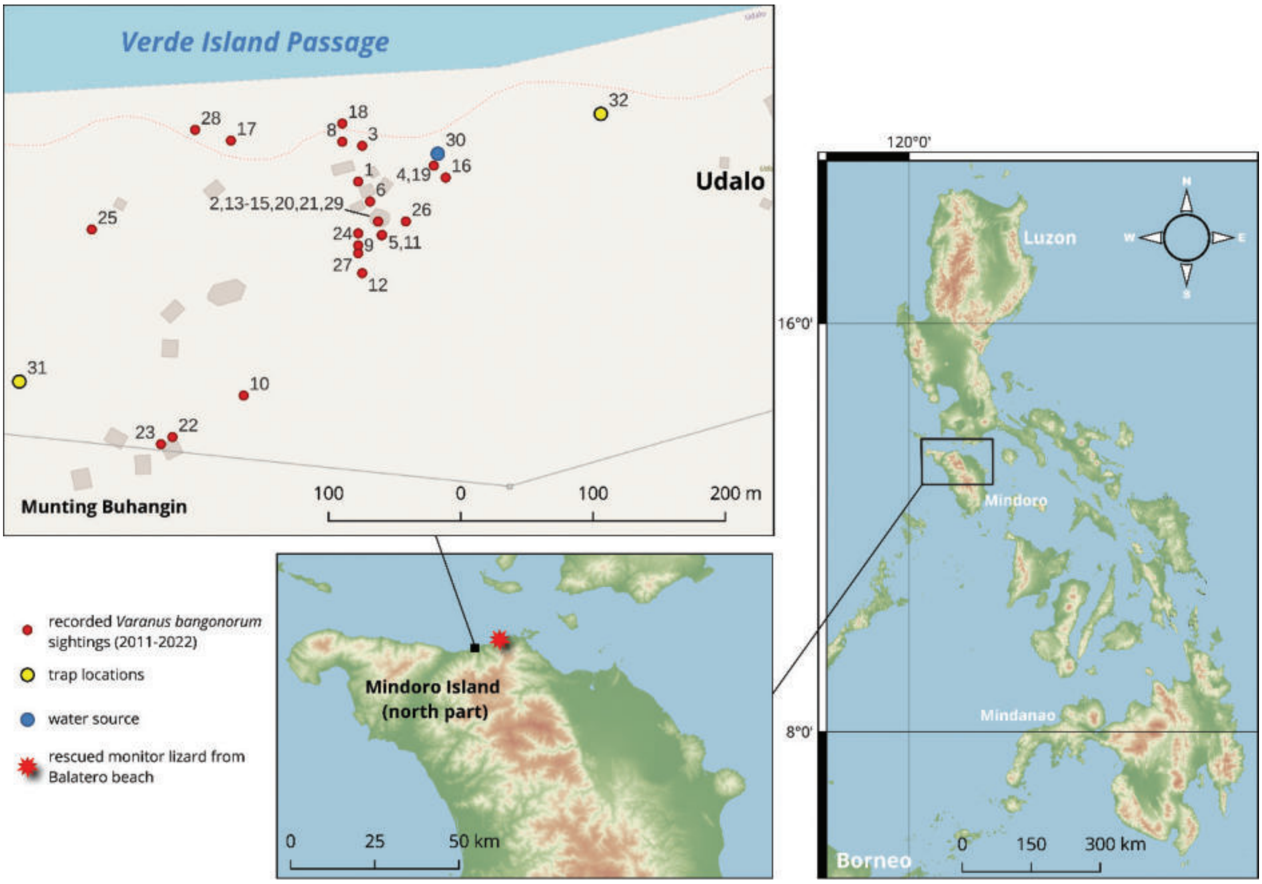

Figure 1. Map of the Philippines indicating the position of the records of *V. bangonorum*. Base maps: Open Street Map and data from http://srtm.csi.cgiar.org.

ties, including all forms of hunting, plant/fruit collection, littering, logging and kaingin (slash-and-burn), are prohibited and privately monitored within the boundaries of Munting Buhangin. Approximately three-quarters of the area has remained undisturbed since 1998, resulting in a partly very dense secondary tropical lowland forest with areas of semi-closed canopy.

Dominant flora includes members of the Dipterocarpaceae, other native non-hardwood tree and shrub species, vining plants (*Anamirta* sp.) and climbing ferns (*Lygodium* sp.) and old fruit trees (*Mangifera indica*, *Cocos nucifera*) (Suppl. material 1: fig. S2). Typical beach vegetation includes species of *Barringtonia*, *Terminalia*, *Caesalpinia* and *Ipomoea* (Primavera and Sadaba 2012).

No rivers or creeks run through the property. The only constant freshwater source is a small oval-shaped drip-catch basin of approximately 100 cm × 50 cm with a maximum depth of only 5 cm, located above one of the beaches at 31 m elevation close to the eastern boundary. Especially during the dry season, there are gatherings of Cane Toads (*Rhinella marina*) and other amphibian species. These may provide easy prey for lizards and snakes (Suppl. material 1: fig. S3).

Species observation

Our report is based on citizen-science data, contributed mainly by the co-author and collected over eleven years from 2011 to 2022. Each animal observed was photographed or video recorded; the summary includes only clearly identified records. We selected 29 clear and identified examples to represent sightings of the lizards during this period (Table 1).

We then mapped out the area, based on the sightings of *Varanus bangonorum*. Depending on the contributor, the use of recording equipment varied, mostly Panasonic DMC-TZ8 and TZ71, lately Sony RX10 IV and occasionally cell phone camera (Realme C11). We also verified the geographical coordinates of the sighting localities by using a hand-held navigator Garmin GPSmap 62s and added our corresponding field notes as a short reference (Table 1; 1–29). Coordinates and elevation for other points of interest are additionally included, for example, water sources and traps (Table 1; 30–32).

We gathered information, particularly on the local hunting methods, through informal interviews with local community members from Udalo (Tagalog) and Latag (Iraya). The interviews conducted were not standardised. The interviews did not last long, the basic questions being about the observation of monitor lizards in given locations and the attitude of the local population towards them and potential threats.

All photo- and video-graphic evidence is based on chance encounters within Munting Buhangin. We did not handle lizards for any purpose other than rescue and release (Table 1; 7, 9, 12, 13).

Table 1. Recorded sightings of *Varanus bangonorum* with geographical coordinates and field notes. Each location represents a unique sighting/individual (see Fig. 1).

Observation	Date	Latitude, Longitude	Elévation (m)	Notes
1	24-11-2011	13.477778°N, 120.816861°E	27	In-house, observation by H.D. Prüßner
2	17-02-2012	13.4775°N, 120.817°E	31	In-house, the smallest specimen
3	21-01-2012	13.478028°N, 120.816889°E	19	Resting close to the beachfront
4	20-12-2013	13.477889°N, 120.817389°E	46	Rescued from a dog attack; died later
5	02-06-2014	13.477404°N, 120.817025°E	29	Hunting and consuming insect
6	11-10-2015	13.477639°N, 120.816944°E	20	Basking next to the wall
7	16-06-2016	13.500131°N, 120.881394°E	3	Purchased from the captor and released
8	24-09-2016	13.478056°N, 120.81675°E	17	Thermoregulating ≥ 3 m above ground
9	16-09-2017	13.477417°N, 120.816861°E	30	In-house, caught and released
10	09-09-2018	13.476286°N, 120.816061°E	73	Near a residence, observation by A. Richard
11	21-05-2019	13.477406°N, 120.817028°E	19	Basking on a wooden trunk
12	23-05-2019	13.477139°N, 120.816889°E	17	In-house: 1 of 4 - caught and released
13	25-05-2019	13.4775°N, 120.817°E	31	In-house: 2 of 4 - caught and released
14	26-05-2019	13.4775°N, 120.817°E	31	In-house: 3 of 4 remained in hiding
15	28-05-2019	13.4775°N, 120.817°E	31	In-house: 4 of 4 died, no visible injuries
16	02-06-2019	13.477806°N, 120.817472°E	27	Largest specimen, digging for food
17	11-10-2019	13.478064°N, 120.815972°E	11	Beach front, foraging
18	09-03-2020	13.478183°N, 120.81675°E	7	Beach front: basking on a tree trunk, ≤ 6 m
19	13-09-2020	13.477889°N, 120.817389°E	46	Hiding amongst leaf litter in a ravine
20	18-09-2021	13.4775°N, 120.817°E	31	Near the house, sleeping in a tree
21	19-09-2021	13.4775°N, 120.817°E	31	Basking after a heavy rain > 12 hours
22	23-01-2022	13.475997°N, 120.815564°E	79	1 of 3, observation by G. Aguirre
23	12-02-2022	13.475947°N, 120.815483°E	80	2 of 3, with prey (Cane Toad)
24	10-07-2022	13.477333°N, 120.816861°E	36	In-house, help with the escape
25	27-07-2022	13.477444°N, 120.815°E	32	3 of 3, observation by N. Bandico
26	03-08-2022	13.4775°N, 120.817194°E	40	Foraging in a bush
27	11-09-2022	13.477278°N, 120.816861°E	36	Foraging in the forest litter
28	15-09-2022	13.478139°N, 120.815722°E	shore	Beachfront, hidden in the rocks
29	22-10-2022	13.4775°N, 120.816944°E	19	Observation of defecating lizard
Other relevant points				
30	Water source	13.477972°N, 120.817417°E	31	100 cm × 50 cm × 4 to 5 cm depth
31	Traps	13.476383°N, 120.814494°E	79	The western slope, dead in trap, by-catch: rodent
32	Traps	13.47825°N, 120.818556°E	30	Eastern slope, traps and spread-out fishnet

Results

Observations of *V. bangonorum*

These lizards are diurnal, leading a mixed life between terrestrial and arboreal habitats. On 18-09-2021, we observed a young individual resting and sleeping for more than 12 hours overnight, approximately two metres above the ground. It did not seek shelter from the continuous heavy rainfall (Fig. 2A, Table 1; 20 and 21). The lizards also chose higher vegetation for basking on tree trunks at three to six metres height (Fig. 2B, Table 1; 8, 18, 21, Suppl. material 1: fig. S4).

The by-far largest (estimated SVL of more than 50 cm) monitor lizard was observed for several minutes while foraging for rodents (Muridae). Using alternate movements of the front legs for digging, it expanded a hole in the ground in a slightly sloped area (Table 1; 16). Throughout this activity, the lizard constantly remained alert by frequently withdrawing its head from the hole, looking around and flicking its tongue (Suppl. material 2). This specimen's impressive size was visible when it finally ran off with a captured rodent in its snout.

The catching of the notorious invasive Cane Toads (*Rhinella marina*) was most frequently observed (Suppl. material 3). The same adult animal, distinguished by its remaining neck snare, was sighted three weeks earlier within the same observation area (Table 1; 22). The third and last encounter happened six months later when this individual was observed roaming around in a different location, still wearing the remains of the neck sling (Fig. 3, Table 1; 25).

We observed a young specimen catching and chewing on a large insect, likely a member of the order Coleoptera (Table 1; 5, Suppl. material 1: fig. S5). The unusual observation was when, after seeing the observer, the lizard squatted and then excreted faeces before disappearing into the undergrowth. A closer examination of the faeces showed chitinous insect parts' remains, suggesting that some insects' exoskeleton cannot be completely digested, confirming insectivorous behaviour amongst young individuals (Table 1; 29, Suppl. material 1: fig. S6).

Figure 2. A. A juvenile specimen is sleeping on a shrub (*Plumeria* sp.) in a characteristic position- holding the branch close to the body, with four limbs and a tail along the tree trunk on the night of 18 September 2021 at 23:52 h.; **B.** Adult monitor lizard (Table 1; 18) basking on a palm tree trunk (*Cocos nucifera*). Photographs by Michaela S. Webb.

Figure 3. Adult lizard carrying a hunted Cane Toad (*Rhinella marina*) (12-02-2022). The same individual was observed six months later (27-07-2022). Photographs by Gerlyn D. Aguirre (left), Nhayume A. Bandico (right).

Local threats

Before description of the Mindoro endemic species *Varanus bangonorum* in 2014 (Welton et al. 2014), we observed the public trading of monitor lizards during occasional visits to Puerto Galera (a neighbouring municipality in the Province of Oriental Mindoro) between 1998 and 2002: animals were bound and hung alive from tree limbs by the roadside (Brgy. Sto. Niño; main road from Balatero pier to town proper).

The last encounter (on 16-06-2016) with a captive monitor lizard happened on the verge of departure from Balatero pier by private outrigger boat (banka) back to Munting Buhangin. We observed a person with a monitor lizard on a "leash" (a string looped around the neck) while walking with the lizard along Balatero beach. After some difficult discussions, he finally agreed to part with the animal in exchange for PHP 200 (Table 1; 7, Suppl. material 1: fig. S7).

The hunting of local Monitor Lizards is seasonal during dry months. It is done by setting different kinds of simple, yet very effective traps hidden within leaf-littered grounds. Foot and neck snares are being used to trap the lizards (e.g. Suppl. material 1: figs S8–S10). Depending on location and positioning, empty traps may be challenging to detect by the untrained eye unless the string used as a snare is of bright colour. In 2022, we successfully destroyed more than thirty traps on the Western and Eastern sloping borders at Munting Buhangin (Fig. 4, Table 1; 31 and 32).

Discussion and conclusion

Our observations confirm that *V. bangonorum* successfully hunts and feeds on invasive toads without harm (Table 1; 23, 25, Suppl. material 3). When threatened, Cane Toads release toxins from their parotid glands that may be lethal to some Varanidae species (Pettit et al. 2020, 2021a; Harvey et al. 2022), but genetic testing suggests that four varanids of the subgenera *Soterosaurus* are physiologically resistant to toad toxins (Pettit et al. 2021b).

According to a 30-year (1989–2018) review study, no online trading was traced to originate from the island of Mindoro (Sy and Lorenza II 2020). Since our single observation (Table 1; 7, Suppl. material 1: fig. S7), there are no indications yet of *Varanus bangonorum* being purposely captured for trade or trafficking, online or direct, in Munting Buhangin. The fact suggesting this state is that the seller we met was neither aware of the species' status nor the reptile's value in the pet trade at that time. In comparison, other endemic Philippine monitor lizard species are traded at up to PHP 100,000 (*Varanus bitatawa*) (Sy 2012).

Figure 4. On 27-02-2022 remains of a strangled juvenile Bangon Monitor Lizard (*Varanus bangonorum*) were discovered and removed; all traps were destroyed and snares were collected. The western slope, Munting Buhangin. Photographs by Michaela S. Webb.

Seemingly overlooked and not yet addressed are the online encouragements to hunt "Bayawak", with several YouTube channels proudly presenting trapping methods with instructions on how to prepare the monitor lizard meat for consumption (e.g. Totskey TV 2021). Although the local Tagalog regard the Bangon Monitor Lizard more as a predator than as a food source, they do not collect monitor lizards' eggs for consumption either. Our interviews include reports of these reptiles sneaking into the villages to catch young chickens or to steal eggs. The described hunting methods have gone on for decades without monitoring by the Government; at least for the indigenous people, they are integrated into their way of life. We conclude that deliberate hunting by setting traps is one of the major threats in this area and probably across the entire Philippine islands, especially as most of the captured animals appear to be young specimens.

Another threat is the irreversible loss of natural habitats through a wide range of human activities, such as development (e.g. housing, infrastructure, small-scale mining, river channelling), (illegal) logging and the expansion of slash-and-burn practices (kaingin) into more remote and previously-untouched areas (Tapper 2006; Smith et al. 2012; Wagner et al. 2015).

A possible solution might be to actively involve the local people in a long-term 'Bayawak Project' similar to 'Pawikan Patrol' - a sea turtle conservation project in Barangay Udalo (Pawikan Patrol 2022). Such a project could raise awareness and instil pride in possession and protection towards these lizards and other endemic animals instead of exploiting them. Local knowledge would be a valuable resource and contribute to further research projects. Another great example to follow is workshops regarding wildlife conservation, as presented by Bhattacharya et al. (2019), showing monitor lizards and their usefulness in the ecosystem.

Considering the observations presented herein, we strongly encourage conservationists to pay more attention to the threats to *Varanus bangonorum* (Suppl. material 1: fig. S11). Conducting observations on a broader long-term scale would help in their conservation.

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Supplementary material 1

Photographic documentation of the observations

Authors: Przemysław Zdunek, Michaela S. Webb

Data type: Adobe PDF file

Link: https://doi.org/10.3897/herpetozoa.36.e96841.suppl1

Supplementary material 2

Large adult lizard digging in search of rodents on the slope by the forest on 02/06/2019

Authors: Przemysław Zdunek, Michaela S. Webb

Data type: MP4 file

Link: https://doi.org/10.3897/herpetozoa.36.e96841.suppl2

Supplementary material 3

Varanus bangonorum catching an invasive Cane Toad (*Rhinella marina*)

Authors: Przemysław Zdunek, Michaela S. Webb

Data type: MP4 file

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A new species of saxicolous *Lepidophyma* (Squamata, Xantusiidae) from Tamaulipas, Mexico

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Abstract

We describe a new saxicolous species of *Lepidophyma* from the Sierra Madre Oriental, in the state of Tamaulipas, Mexico and provide morphological and molecular data to support the novelty of this species. The new species is most closely related to members of the *L. sylvaticum* group; however; it is a flattened form specialised for a saxicolous lifestyle and it can be distinguished from all other members by a combination of meristic characters. Genetic analysis suggests that several populations of *L. sylvaticum* might represent additional novel taxa, while the validity of *L. micropholis* is questioned. We discuss conservation priorities of the new species.

Resumen

Describimos una nueva especie saxícola de *Lepidophyma* de la Sierra Madre Oriental, en el estado de Tamaulipas, México, y proporcionamos datos morfológicos y moleculares para apoyar la validez de esta especie. La nueva especie está más estrechamente relacionada con los miembros del grupo *L. sylvaticum*, sin embargo es de un morfotipo aplanado especializado para una vida saxícola y se puede distinguir de todos los demás miembros por una combinación de caracteres merísticos. Los análisis genéticos sugieren que varias poblaciones de *L. sylvaticum* podrían representar nuevos taxones adicionales, mientras que se cuestiona la validez de *L. micropholis*. Se discuten las prioridades de conservación de la nueva especie.

Kurzfassung

Wir beschreiben eine neue Felsen bewohnende Art von *Lepidophyma* aus der Sierra Madre Oriental im Bundesstaat Tamaulipas (Mexiko) und legen morphologische und molekulare Daten vor, die die Hypothese, dass es sich um eine neue Art handelt, unterstützen. Die neue Art ist am nächsten mit der *L. sylvaticum*-Gruppe verwandt, hat jedoch eine abgeflachte Morphologie, die zeigt, dass sie auf Felsen spezialisiert ist. Sie lässt sich durch einer Kombination meristischer Merkmale von allen anderen Mitgliedern der Gattung unterscheiden. Genetische Analysen deuten an, dass mehrere Populationen von *L. sylvaticum* weitere neue Arten darstellen könnten, während die Gültigkeit von *L. micropholis* in Frage gestellt wird. Wir empfehlen Prioritäten für den Schutz der neuen Art.

PENSOFT.

Key Words
conservation, night lizards, reptiles, Sierra Madre Oriental, systematics, taxonomy

Palabras Clave
conservación, Lagartijas Nocturnas, Reptiles, Sistemática, Taxonomía

Schlüsselwörter
Nachteidechsen, Naturschutz, Reptilien, Sierra Madre Oriental, Systematik, Taxonomie

Introduction

The lizard genus *Lepidophyma* (Xantusiidae) ranges from Colima on the Pacific coast and Nuevo León and Tamaulipas on the Atlantic, south along both versants to Costa Rica and Panama, but is mostly absent from the Yucatan Peninsula (Bezy and Camarillo 2002). Predominately a lowland genus, some species range into the mountains to elevations of at least 2100 m a.s.l. Members of *Lepidophyma* are generally restricted to mesic habitats, although several species (e.g. *L. cuicateca*, *L. gaigeae*, *L. occulor*, *L. radula*) have been found in semi-arid interior valleys which are covered by low growing tropical deciduous forest and semi-desert (Bezy and Camarillo 2002; Canseco-Márquez et al. 2008).

The taxonomy and systematics of *Lepidophyma* has received extensive attention for many decades (Smith 1973; Bezy 1973, 1984, 1989; Bezy et al. 1982; Bezy and Camarillo 1992, 1999, 2002). Despite this commendable effort, it appears that the diversity in the genus remains underestimated and new species continue to be discovered. Currently, 22 species are recognised as valid, of which five were described in the last two decades (Canseco-Márquez et al. 2008; García-Vázquez et al. 2010; Palacios-Aguilar et al. 2018; Arenas-Moreno et al. 2021; Lara-Tufiño and Montes de Oca 2021). Molecular analyses have shown that additional taxa might need to be recognised (Noonan et al. 2013).

Here, we describe a new species from the Sierra Madre Oriental of Tamaulipas, based on molecular and morphological evidence and compare it to closely related forms related to the widespread *Lepidophyma sylvaticum*.

Materials and methods

Taxonomic sampling

Between 2009 and 2020, we collected multiple specimens of night lizards of the genus *Lepidophyma* from the Sierra Madre Oriental of eastern Mexico. We photographed all live lizards, including dorsal, lateral and ventral profiles and euthanised 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.

The material collected was deposited at the Instituto de Investigaciones sobre los Recursos Naturales (**INIRENA**) of the Universidad Michoacana de San Nicolás de Hidalgo (**UMSNH**) in Morelia, Mexico; and the Museo de Zoología, Facultad de Ciencias (**MZFC**) of the Universidad Nacional Autónoma de México (**UNAM**) in Mexico City. Although we formally accessioned the specimens we collected, several specimens examined from both the MZFC and Herpetology Collection, University of Texas at Arlington (**UTA**) collections have not been catalogued, in which case we list the original field numbers and the respective museum in which they were deposited. Original field number abbreviations are as follows: **CIG** (Christoph I. Grünwald); **JAC** (Jonathan A. Campbell), **JRV** (Jacobo Reyes-Velasco) and **RLB** (Robert. L. Bezy). Museum acronyms throughout follow Sabaj (2020). Specimen numbers for all material examined are provided in Appendix 1. We were not able to measure type specimens of some previously described taxa, so we used the measurements of the type specimens provided in the original descriptions and other published literature (Bezy 1984, 1989; Bezy and Camarillo 2002).

Specimens examined and used for scale counts and comparison of character states are listed in Appendix 1. Distribution maps were generated, based on the GBIF database (www.gbif.org), which includes both museum records as well as distribution records from the Inaturalist platform (inaturalist.org). Additional localities mapped were taken from taxonomic works (Bezy 1984, 1989; Bezy and Camarillo 1992, 1999, 2002) and recent state lists for Nuevo León (Lemos-Espinal et al. 2018), Querétaro (Dixon and Lemos-Espinal 2010), San Luis Potosí (Lemos-Espinal and Dixon 2013) and Hidalgo (Ramírez-Bautista 2014). Additionally, we mapped all localities of the specimens collected by us.

Measurements and character states

Our measurements and character states follow Savage (1963) for all Xantusiidae and Bezy and Camarillo (2002) for *Lepidophyma*. Measurements and character states were taken from data published in Bezy's succession of work on Mexican *Lepidophyma* (Bezy 1973, 1984, 1989; Bezy et al. 1982; Bezy and Camarillo 1992, 1999, 2002). Numbers taken from Bezy (1984) for *L. sylvaticum* were taken for the ¨Wet Environs" populations only, as these populations include the type locality of *L. sylvaticum*. We do not consider the "Arid Environs *L. sylvaticum*" (*sensu* Bezy 1984) to be *L. sylvaticum* sensu stricto. These populations appear to be a composite of different taxa (see results below). Additional comparisons between species were enhanced with data from Bezy and Camarillo (2002), Canseco-Márquez et al. (2008), García-Vázquez et al. (2010), Arenas-Moreno et al. (2021) and Lara-Tufiño and Montes de Oca (2021).

Abbreviations used in the text and tables were adopted from Bezy and Camarillo (2002) and are as follows: snout-vent length (**SVL**), tail length (**TL**), total length (**TotL**), head length (**HL**), head width (**HW**), head height (**HH**), orbit diameter (**ED**), fourth toe length (**4TL**), total femoral pores (**FPT**), lateral tubercle rows (**LTR**), gulars (**GUL**), dorsal scales along the mid-dorsal line between the post-parietals and a point above the vent (**DOR**), fourth toe lamellae (**FTL**) and paravertebral row (**PVR**).

Scale counts were performed with the aid of a dissecting microscope. Measurements were taken with a ruler or digital calipers (General, USA) under a dissecting microscope. Bilateral characters were scored on both left and right sides and given in that order, separated by a slash (/). Head length was measured from the tip of the snout to the posterior end of the occipitals, head width was measured at the widest point of the head at the posterior part of the jaw, while head depth was measured at the highest point of the head, at the level of the orbit. All scale dimensions were measured at their maximum.

We have included a high resolution PDF of photographs of the head scalation of the new species, as well as several related forms for comparison, as Suppl. material 1: plate S1.

DNA extraction, amplification and molecular analysis

We sequenced genetic data from two nuclear (C-mos, Rag-1) and three mitochondrial loci (16s, cytb, ND4), using the primers listed in Noonan et al (2013). These loci were shown to be informative for estimating evolutionary relationships in the family Xantusiidae (Noonan et al. 2013). We extracted DNA from tissue samples stored in 96% ethanol with the use of standard potassium acetate protocol (Sokolov et al 1989) and performed Polymerase Chain Reaction (PCR) in total volumes of 48 µl (+ 2 µl of DNA) with the use of regular Taq (Invitrogen), under the conditions specified in Noonan et al. (2013) and references therein. We shipped the unpurified PCR products for sequencing at BGI Tech Solutions (Hong Kong).

We manually trimmed and edited the raw chromatograms using the programme Geneious v. 9.1.6 (Biomatters Ltd., Auckland, NZ). We included additional sequences of members of the family Xantusidae obtained from GenBank in order to infer the phylogenetic relationships of the new samples. We deposited all new sequences in GenBank (Suppl. material 2: table S1). Each locus was individually aligned in MAFFT version 7 (Katoh et al. 2017), with the Q-INS-I option and we concatenated all sequences in FASconCAT v.1.04 (Kück and Longo 2014). We included an additional five nuclear loci used by Noonan et al. (2013); however, we did not sequence these loci for our new taxa. The final alignment consisted of 6819 base pairs and included 66 individuals of the family Xantusidae: 60 individuals of *Lepidophyma*, five individuals of *Xantusia*, as well as one individual of *Cricosaura typica* (Suppl. material 2: table S1). We then performed Maximum Likelihood analysis of the concatenated dataset in IQ-Tree (Nguyen et al. 2015) using the IQ-Tree web server (Trifinopoulos et al. 2016), with an Auto substitution model and 1,000 bootstrap replicates to assess support. Additional analyses were carried out including only nuclear and only mitochondrial loci and they show very similar topology and, thus, they are not included here. We have included all sequences used in this study in a table which is included as Suppl. material 2: table S1.

Results

Molecular phylogenetic results

The phylogenetic relationships of *Lepidophyma*, based on our Maximum Likelihood analysis are, in general, similar to those of Noonan et al. (2013), which is not surprising, as the majority of sequences are shared between the two studies; thus, the general evolutionary relationships of the group will not be addressed here. The sequences of the new species from the vicinity of Jaumave (Tamaulipas) are nested within a group of species consisting of *Lepidophyma occulor*, *L. sylvaticum* and *L. micropholis* (Fig. 1). Our new species appears to be most closely related to an individual of *Lepidophyma* from San Roque, Nuevo León. These two taxa are sister to *L. sylvaticum* + *L. micropholis*, with strong support (bootstrap value = 100). In fact, *L. micropholis* appears to be nested within *L. sylvaticum*, while *L. sylvaticum* might in fact represent several taxa, something that had previously been shown by Noonan et al. (2013).

Systematic account

Lepidophyma jasonjonesi sp. nov.

https://zoobank.org/7389A805-0166-4D07-9D18-02372E324B4E

Figs 2, 3

Proposed standard English name: Jones' Night Lizard

Proposed standard Spanish name: Lagartija Nocturna de Jones

Type material. ***Holotype*** (Fig. 2). INIRENA 2817 (Original field number CIG-0969). Adult male collected in a rock crevice near a small seep, 28 km NNE of Jaumave, on Federal Highway 101 to Ciudad Victoria, Municipio de Victoria, Tamaulipas, Mexico

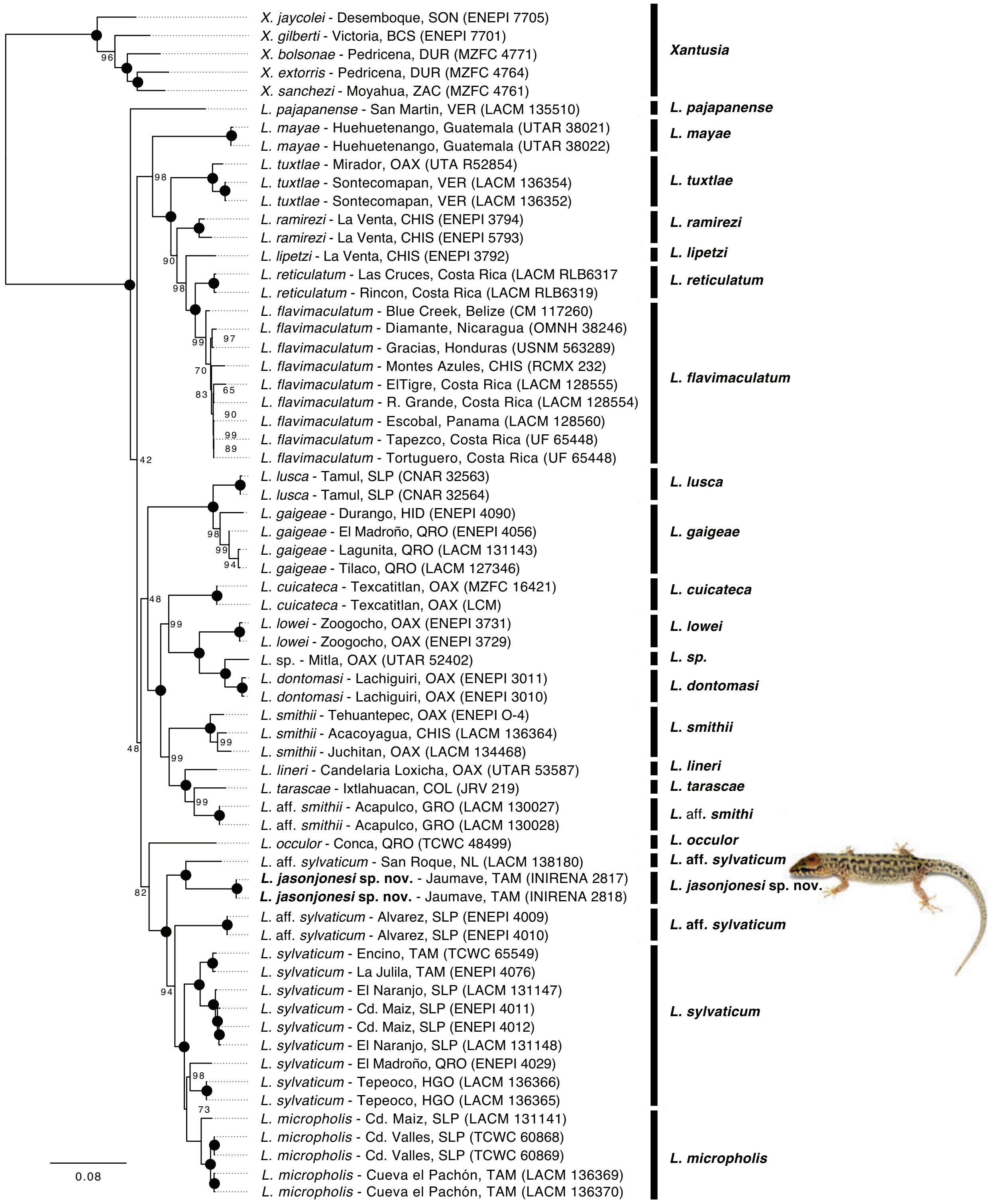

Figure 1. Maximum Likelihood phylogenetic inference of members of the Xantusiid genera *Lepidophyma* and *Xantusia*, based on four mitochondrial and seven nuclear loci. All nodes with bootstrap values equal to 100 are marked with a black dot.

(23.617489, -99.285573, 1,005 m a.s.l.; datum = WGS84) (Fig. 3), by Jason M. Jones and Ivan Ahumada-Carrillo on 26 July 2016.

***Paratypes* (Fig. 4).** INIRENA 2818 (Original field number CIG-0970). Adult male, with the same collection data as the Holotype.

Diagnosis. *Lepidophyma jasonjonesi* sp. nov. can be distinguished from all its congeners by the following combination of characters: flattened head and body, head height 31–32% of head length; anterior pretympanic plate large, similar size as anterior supratemporal; 52–54 gular scales; 0–1 gular scales contacting first pair of infralabials;

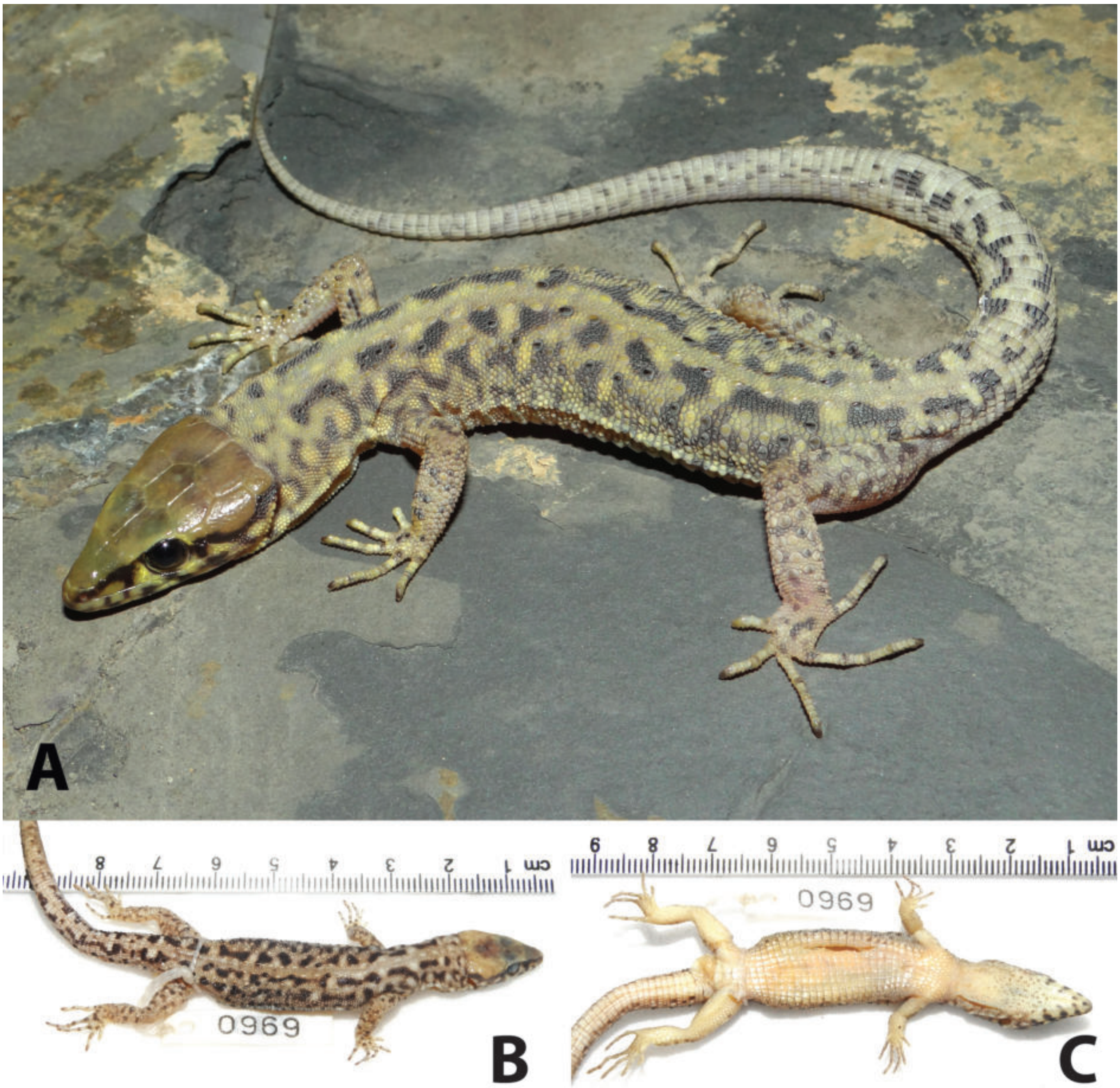

Figure 2. *Lepidophyma jasonjonesi* sp. nov. Holotype (INIRENA 2817). (**A**) Dorso-lateral perspective in life; (**B**) dorsal perspective in preservative; (**C**) ventral perspective in preservative.

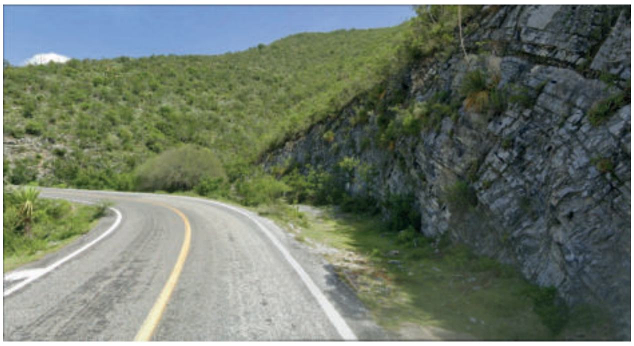

Figure 3. Photo of the locality and habitat at the type locality of *Lepidophyma jasonjonesi* sp. nov. (Photo from Google Earth).

180–182 dorsal scales mid-ventrally between occipitals and rump; 17–18 enlarged tubercles in paravertebral row between axilla and groin, 16 enlarged tubercles in second vertebral row between axilla and groin; 37–40 total femoral pores; 27–30 lamellae on fourth toe of foot, with 10–15 divided mid-ventrally; 35–38 ventral scale rows, with 10 longitudinal ventral scale rows; iris brown.

Comparisons. One of the most striking characteristics of this species which distinguish it amongst known *Lepidophyma* is the flattened head and body shape. While this character is not very evident from photos, it is evident when a specimen is in hand. We have included detailed photos of the head, including from a lateral profile (Fig. 5; Suppl. material 1: plate S1), which best portrays this unique flattening of the head. This species can be distinguished from most *Lepidophyma*, except *L. chicoasense*, *L. lipetzi*, *L. flavimaculatum*, *L. ramirezi* and *L. zongolica* by the high number (37–40) of total femoral pores, vs. 14–36 in the other species. It further differs from most species of *Lepidophyma*, except *L. smithii* and *L. tarascae*, by

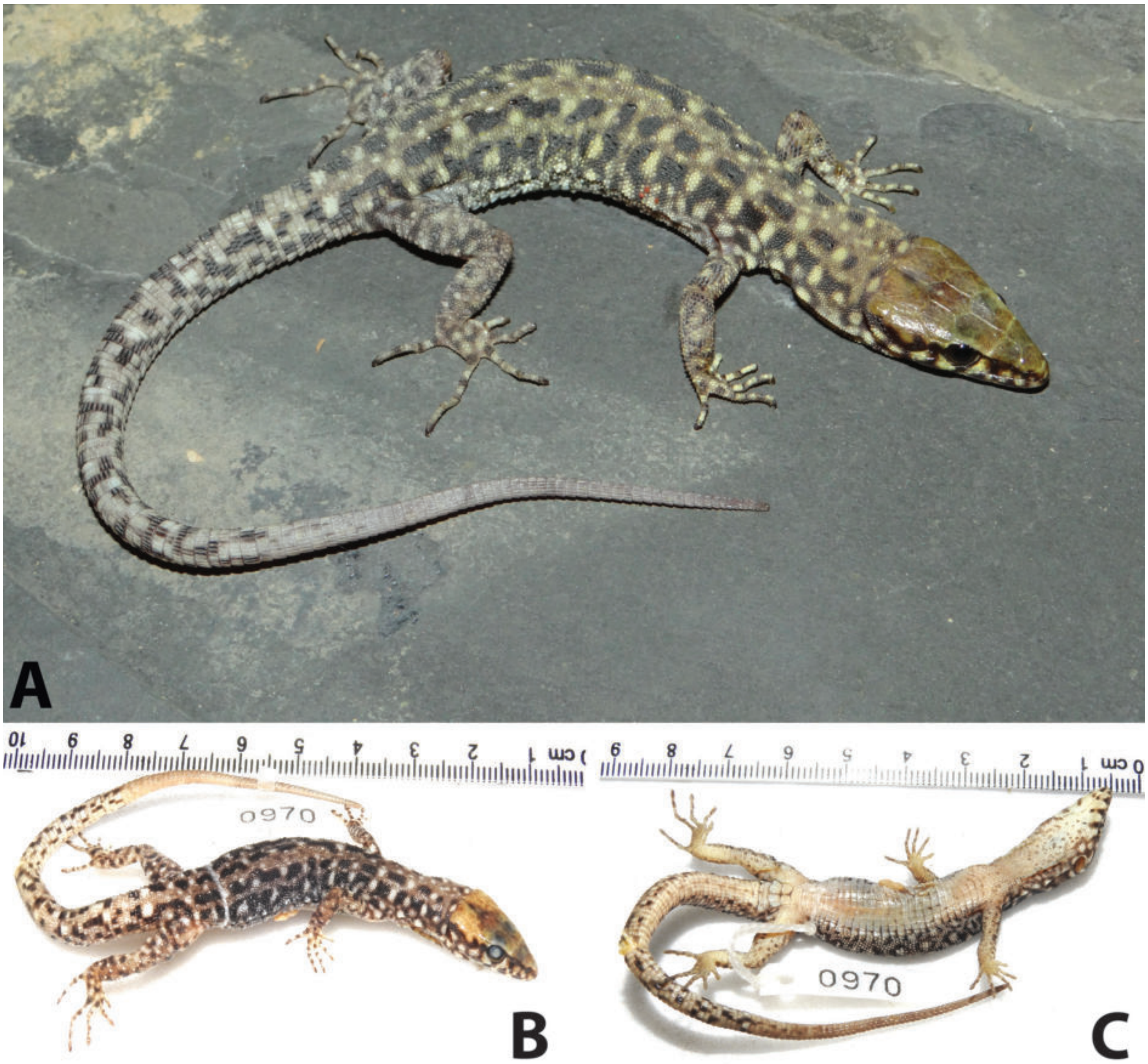

Figure 4. *Lepidophyma jasonjonesi* sp. nov. Paratype (INIRENA 2818). (**A**) Dorso-lateral perspective in life; (**B**) dorsal perspective in preservative; (**C**) ventral perspective in preservative.

possessing 16 lateral tubercle rows between the axilla and the groin, vs. 21–73 in the other species. It can be distinguished from members of the *L. gaigeae* group as defined by Bezy and Camarillo (1992, 2002), Canseco-Márquez et al. (2008) and Arenas-Moreno et al. (2021) by possessing enlarged tubercles interspersed amongst the small granular scales, including on the anterior and lateral portions of the body, vs. no enlarged tubercles in *L. cuicateca*, *L. dontomasi*, *L. gaigeae*, *L. lowei*, *L. lusca* and *L. radula*. *L. jasonjonesi* sp. nov. is most closely related to *L. sylvaticum* and *L. micropholis* (see results below). *L. jasonjonesi* sp. nov. differs from *L. micropholis* in the following manner (*L. micropholis* character states in parenthesis): a more flattened head and body build (vs. not flattened), HH/HL ratio of 0.31–0.32% (vs. 0.35 –0.37), larger dorsal scales in 180–182 dorsal scale rows (vs. smaller dorsal scales in 231–251 dorsal scale rows), 37–40 femoral pores (vs. 28–36), 16 lateral rows of tubercles (vs. 27–35), 52–54 gulars (vs. 55–68).

L. jasonjonesi sp. nov. differs from *L. sylvaticum* in the following manner (*L. sylvaticum* character states in parenthesis): a more flattened head and body build (vs. not flattened), HH/HL ratio of 0.31–0.32 (vs. 0.41–0.45), tympanum with upper part tilted approximately 20° posteriorly, allowing for a more flattened head-shape (vs. tympanum not tilted posteriorly), lacking enlarged tubercles on the lateral portions of the body, giving a smooth appearance (vs. enlarged lateral tubercles present, rugose appearance), 37–40 femoral pores (vs. 24–36). *L. jasonjonesi* sp. nov. can be distinguished from arid-land populations assigned to *L. sylvaticum* by Bezy (1984) by possessing more 180–182 mid-dorsal scales (vs. 150–178), by possessing one pretympanic scale separating postocular from second postorbital supralabial (vs. 2–3), by possessing 52–54 gulars (vs. 42–49) and by possessing 17–18 enlarged tubercles in the paravertebral row (vs. 15–17). We include mensural ranges for the six key characters of all currently-recognised species of *Lepidophyma* for comparative purposes in Table 1.

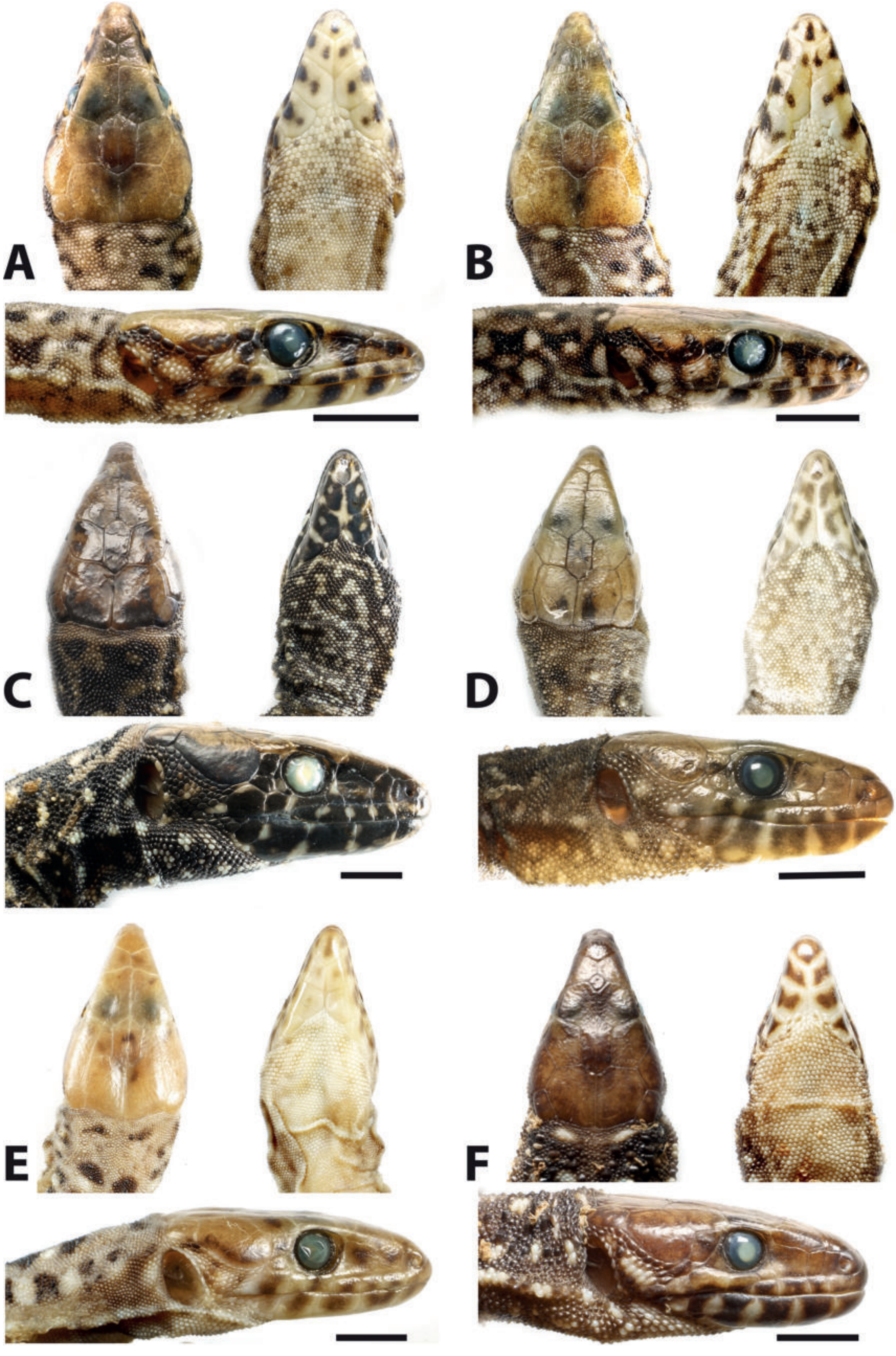

Figure 5. Comparison of head scalation of *Lepidophyma*. **A.** *Lepidophyma jasonjonesi* sp. nov. Holotype (INIRENA 2817); **B.** *Lepidophyma jasonjonesi* sp. nov. Paratype (INIRENA 2818); **C.** *Lepidophyma sylvaticum* (CIG 01550) Valle de Trinidad, Municipio de Xilitla, San Luis Potosí; **D.** *Lepidophyma sylvaticum* (CIG 01391) Loma Santa Rosita, Municipio de Yecuautla, Veracruz; **E.** *Lepidophyma micropholis* (CIG 00829) Grutas de Quintero, Municipio de El Mante, Tamaulipas; **F.** *Lepidophyma tarascae* (JRV 0239) Grutas San Gabriel, Municipio de Ixtlahuacán, Colima. The black line below each lateral profile represents 0.5 cm.

Table 1. Comparison of characteristic variation of *Lepidophyma jasonjonesi* sp. nov. to other species of *Lepidophyma*. Species are arranged by species group (*sensu* Bezy and Camarillo 2002), and *L. sylvaticum* data provided are only for humid environ populations (*sensu* Bezy 1984). This detail is noteworthy because what Bezy (1984) considered "*L. sylvaticum* Arid" we now know is composed of several taxa (see Discussion), and does not represent *L. sylvaticum* sensu stricto. Abbreviations used in the table are total femoral pores (FPT), lateral tubercle rows (LTR), gulars (GUL), dorsal scales along the mid-dorsal line between the post-parietals and a point above the vent (DOR), fourth toe lamellae (FTL) and paravertebral row (PVR).

Species	FPT	LTR	GUL	DOR	FTL	PVR
L .jasonjonesi sp. nov.	37–40	16	52–54	180–182	27–30	68–71
L. micropholis	28–36	27–35	55–72	230–251	24–29	52–85
L. occulor	17–22	22–25	58–71	213–242	22–25	49–69
L. sylvaticum	24–35	24–38	41–56	166–207	23–31	40–73
L. chicoasense	33–38	31–32	53–56	177–192	35–37	57–70
L. lipetzi	35–38	32–33	46–52	177–179	27–28	59–60
L. flavimaculatum	25–43	23–33	40–55	170–225	23–31	41–74
L. mayae	29–35	33–46	38–44	162–188	23–28	41–57
L. pajapanense	30–36	36–43	35–43	152–175	26–30	37–49
L. ramirezi	35–42	25–30	47–54	177–226	25–31	–
L. reticulatum	27–33	28–33	44–54	193–229	26–31	52–72
L. tuxtlae	18–29	30–40	37–52	150–190	22–29	37–55
L. zongolica	33–43	21–28	40–49	174–184	27–30	61–66
L. cuicateca	26	33–37	36–39	150–168	18–19	54–62
L. dontomasi	17–21	47–60	28–35	128–149	18–22	43–61
L. gaigeae	28–29	39–73	28–44	121–151	22–30	30–74
L. lowei	22–31	28–31	32–37	158–170	19–23	45–60
L. lusca	18–26	21–34	27–38	118–132	24–29	55–64
L. radula	20	24	32	135	20	46
L. inagoi	20–24	25–27	58–63	190–215	20–26	71–91
L. lineri	16–17	23–24	51–52	179–184	23–25	43–53
L. smithii	15–30	15–22	44–59	162–224	20–29	35–69
L. tarascae	14–18	16–25	40–43	145–159	22–23	42–49

Description of the holotype (Fig. 2). An adult male, with a rostral broader than high (2.2 mm broad, 1.15 mm high), followed by nasals which are in contact with median frontonasal; two prefrontals; two frontoparietals; no frontal; interparietal scale without parietal spot, but with parietal organ slightly visible and two parietals on the sides. Interparietal scale in contact with supraoculars, parietals and occipitals. Naris bordered by frontonasal, postnasal and first supralabial. Followed by two loreal scales, anterior loreal scale taller than postnasal and posterior loreal scale largest. Eight supralabials on both sides, fifth touching eye, sixth sub-square on left (1.3 tall long, 1.1 mm tall) and rectangular on right (1.6 mm long, 1.1 mm tall) and largest supralabial on both sides is seventh, rectangular in shape and on left 1.6 mm long, 1.1 mm tall and on right 1.8 mm long, 1.1 mm tall. Lower elongated postocular in contact with sixth supralabial on both sides.

Three supratemporal scales, first supratemporal scale in contact with parietal, second supratemporal scale is largest and in contact with parietal and occipital and third supratemporal in contact with occipital. The second supratemporal scale (3.8 mm long, 2.7 mm tall) is larger than the parietal (3.0 mm long, 2.8 mm wide). One large pretympanic scale between postoculars and seventh supralabial on both sides, with a reduced number of scales in temporal region, on left side, one large pretympanic scale and two small scales between anterior temporal and sixth and seventh supralabial, on right side only one large pretympanic scale between anterior temporal and sixth and seventh supralabials. The enlarged pretympanic scale (1.1 mm long, 1.0 mm tall) is subequal to the anterior temporal scale (1.2 mm long, 1.1 mm tall). Seven enlarged auricular scales bordering anterior portion of auricular opening, upper one dark and lower seven pale. Mental broader than long (2.7 mm broad, 2.3 mm long), follow by five pairs infralabials on both sides, the second and third are largest and the fifth is very reduced. Orbit in contact with one elongate preocular, fifth supralabial, two elongated postoculars and supraocular. No gulars contacting first pair of infralabials; 52 gulars along the ventral mid-line between second pair of infralabials and posterior gular fold.

The dorsal and lateral surfaces of the body covered by small granular scales of varying sizes, interspaced with enlarged tubercles, some of which are weakly keeled and some smooth (approximately three times the size of adjacent dorsal scales). Eighteen enlarged paravertebral tubercles from above axilla to above groin in the paravertebral row. One hundred and eighty dorsal scales along the vertebral line from the posterior edge of the occipitals to above the vent.

Square ventral scales are smooth and flat, in 10 longitudinal rows at mid-body; the lateral rows slightly smaller and keeled. Thirty-five transverse rows of ventral scales between gular fold and vent, including the anterior and posterior pre-anal scales. Scales on ventral surface of limbs heterogeneous in size, 37 total femoral pores (20 / 17). Twenty-nine sub-digital lamellae on fourth toe of left foot, 27 on fourth toe of right foot.

Regenerated tail approximately 80 mm long, regenerated at 25 mm from base. The unregenerated part with complete enlarged whorls, each separated by three rows of interwhorls; on ventral portion of tail these rows reduce to two.

Colouration in life (Figs 2, 3). Dorsal colouration of head chocolate brown; with a greenish tinge on the frontonasals, prefrontals and frontoparietals; interparietal, occipitals and supratemporals chocolate brown. Upper labials yellow, with a dark brown irregular loreal stripe which runs from the tip of the snout to the lower posterior edge of the occipitals. Dark loreal stripe chocolate brown and extends down on to third supralabial. Dorsal ground colouration of dorsum yellowish-cream, with unmarked pale yellowish-cream stripe down mid-dorsal region. Black reticulation on lateral portions of dorsum and flanks, with pale yellow highlights forming indistinct occelli. Tail pale grey with black blotches. Ventral colouration of head yellowish-cream, with brown spots. Venter white, with grey spots on throat and tail. Iris brass-copper coloured.

Colouration in preservative. After several years in preservative, the dorsal colouration of the head light tan, with green tinge faded away. Dark melanophores spread evenly on all large head scales. Labial region yellowish-cream ground colouration with one dark brown blotch on each supralabial, centred on the anterior five supralabials, concentrated on the posterior portion of the sixth supralabials and covering the most of the seventh and eight supralabials. Dark brown loreal lateral stripe on head begins anterior to naris, proceeds posteriorly through loreal region, where it extends down on to third supralabial and then posterior through upper orbit and posteriorly through the supratemporal scales to above the tympanum. The dorsal colouration of the body is pale tan, with two broken rows of dark brown blotches dorso-laterally and then dark brown spotting and reticulation. The mid-dorsal area, between the two paravertebral tubercle rows, is pale tan and complete unmarked. Two rows of pale cream ocelli are present between the rows of dark brown blotches, with 10–11 pale ocelli present per row. Dorsal colouration of tail pale cream with remnants of dark brown blotches turning into two alternating rows of dark brown spots. Tail is regenerated after proximal third and the regenerated portion lacks dark brown spots. Ventral colouration yellowish-cream. Ventral surface of head and throat yellowish-cream, with dark spotting. Three dark brown blotches on postmental, which are composed of dense clusters of little dark brown spots. These dark brown blotches continue on to the infralabials, where they are arranged in subequal pairs, the larger one nearest to the mouth. The throat has approximately 50 brown spots which consist of one gular each and extend back to the thirtieth row of gulars. The latter portion of the throat unmarked, cream. Venter unmarked, cream, lateral two rows of ventral scales haves some dark black stippling along the scale edges, most concentrated towards the anterior portion of the scale. Ventral portions of arms, hands, thighs, legs and feet are cream, unmarked. Ventral surface of tail white, unmarked. Eye black.

Measurements (mm). SVL 61.0 mm; TL 79.8 mm; TotL; 140.8 HL 15.1 mm; HW 9.65 mm; HH 4.7 mm; ED 2.4 mm; 4TL 7.7 mm.

Variation. Meristic variation of the two available specimens is given in Table 2. The paratype (INIRENA 2818) possesses a pale spot on the interparietal scale, towards the posterior portion of the scale and roughly encompassing the parietal organ. The paratype has 17 enlarged paravertebral tubercles in the paravertebral row between the axilla and the groin, as well as a total of 40 femoral pores. We have depicted variation of colour pattern of other individuals which were photographed (but not collected) at the type locality (Fig. 6). The dorsal colouration and pattern of all the individuals observed is the same; however, each individual varies slightly by amount and shape of the dark dorsal blotches and pale yellow dorsal ocelli.

Distribution and habitat. This species is known from semi-arid tropical deciduous forest on the lower leeward slopes of the Sierra Madre Oriental between Ciudad Victoria and Jaumave, Tamaulipas. The vegetation of the tropical deciduous forest in the Jaumave Valley grows to a low height and was described as "thorn desert" (= thornscrub) by Martin (1958). Martin reported 568 mm of mean annual precipitation and a mean annual temperature of 21.2 °C for Jaumave. The Koppen classification for the valley is BSh (hot semi-arid climate). The Jaumave Valley is unique for its Cactacea biodiversity and endemism (Martin 1958). *Lepidophyma jasonjonesi* sp. nov. is known with certainty only from 1005 m elev. at the type locality (Fig. 3), where it has been observed on several occasions. Sight records of small saxicolous *Lepidophyma* at other points along this stretch of highway and near it are likely to be of this species and we have included one of these on the distribution map (Fig. 7). A specimen from Gomez Farías, Tamaulipas (CIG 2050) appears to represent this species; however, we have decided not to include it as a paratype pending further molecular work. A population of *Lepidophyma* from San Roque (Nuevo León) is closely related, but whether it represent this species remains unresolved (see below, Fig. 8).

Table 2. Meristic variation of *Lepidophyma jasonjonesi* sp. nov. All measurements in mm.

Species	*L. jasonjonesi* sp. nov.	*L. jasonjonesi* sp. nov.
Specimen	**INIRENA 2817**	**INIRENA 2818**
Sex	Male	Male
Snout-vent Length	61.0	62.0
Tail Length	80.0	91.0
Total Length	141.0	153.0
Head Length (HL)	15.1	15.5
Head Width (HW)	9.7	9.5
Head Height (HH)	4.7	5.0
Fourth Toe Length (4TL)	7.7	8.6
Eye Diameter (ED)	2.4	2.4
HH/HL	0.31	0.32
HH/HW	0.49	0.53
Pretympanics	1 / 1	1 / 1
Gulars	52	54
Gulars contacting 1st Infralabials	0	1
Dorsals	180	182
Dorsals between Paravertebral Rows	4	5
Large Paravertebral Tubercles	18	17
Paravertebral Row	68	71
Lateral Tubercle Rows	16	16
Dorsal Interwhorls	3	3
Ventral Interwhorls	2	2
Ventrals, Longitudinal	35	38
Ventrals, Across	10	10
Femoral Pores	20 /17	20 / 20
Fourth Toe Lamellae	29 / 27	30 / 29
Divided Fourth Toe Lamellae	12 / 10	15 / 14
Parietal Spot	Absent	Present
Left 6th Supralabial Measurement (L / H)	1.3 x 1.1	0.9 x 1.0
Left 7th Supralabial Measurement (L / H)	1.6 x 1.1	2.1 x 1.1
Right 6th Supralabial Measurement (L / H)	1.6 x 1.1	1.3 x 1.0
Right 7th Supralabial Measurement (L / H)	1.8 x 1.1	1.5 x 1.2

Etymology. Named in honour of Jason Michael Jones, American-Mexican herpetologist and savvy field collector, who has always shared a profound interest for members of the family Xantusiidae and who collected the type series of the new species.

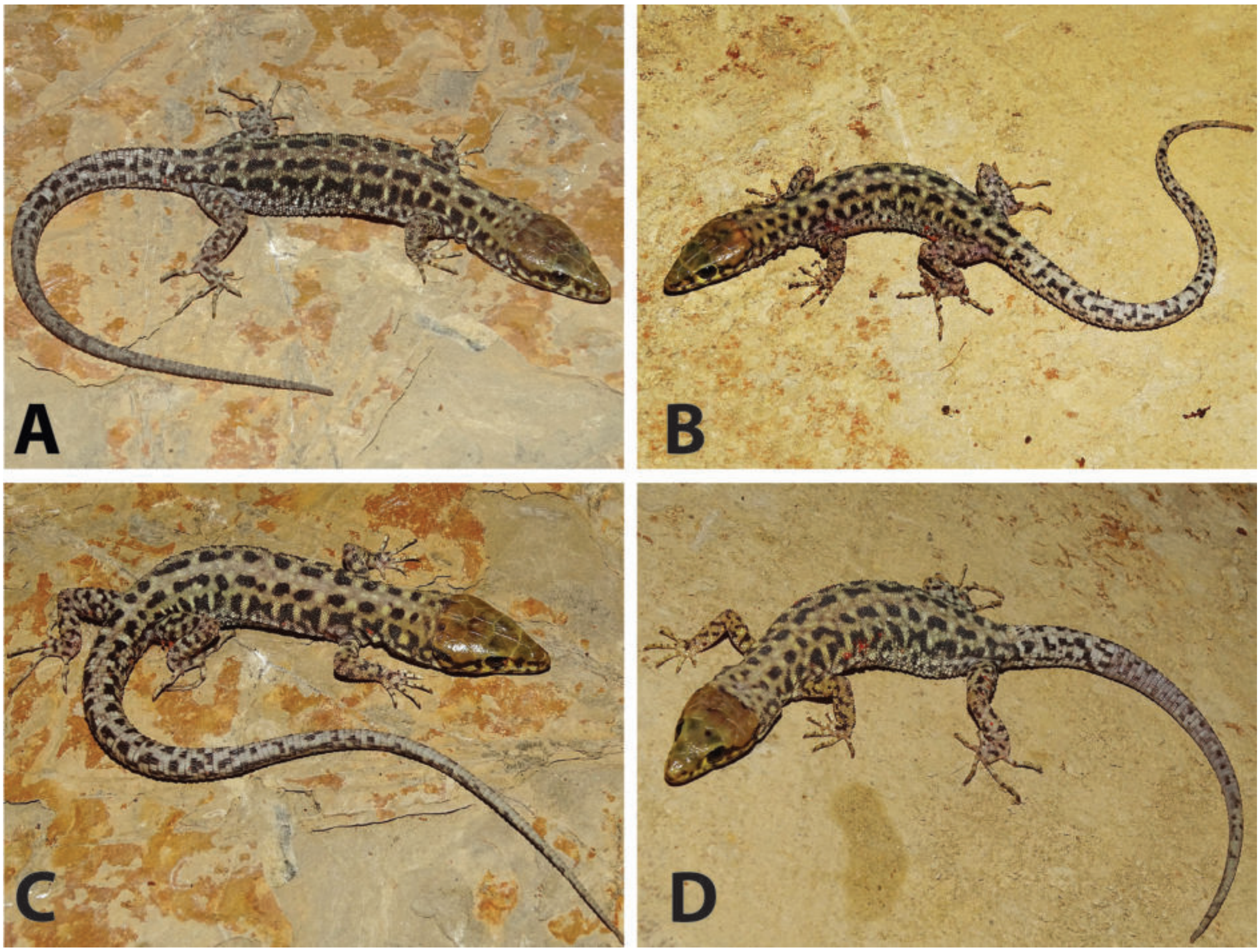

Figure 6. (**A–D**) Variation of *Lepidophyma jasonjonesi* sp. nov. individuals seen at the type locality. All photographs by Jason M. Jones, taken in October, 2016.

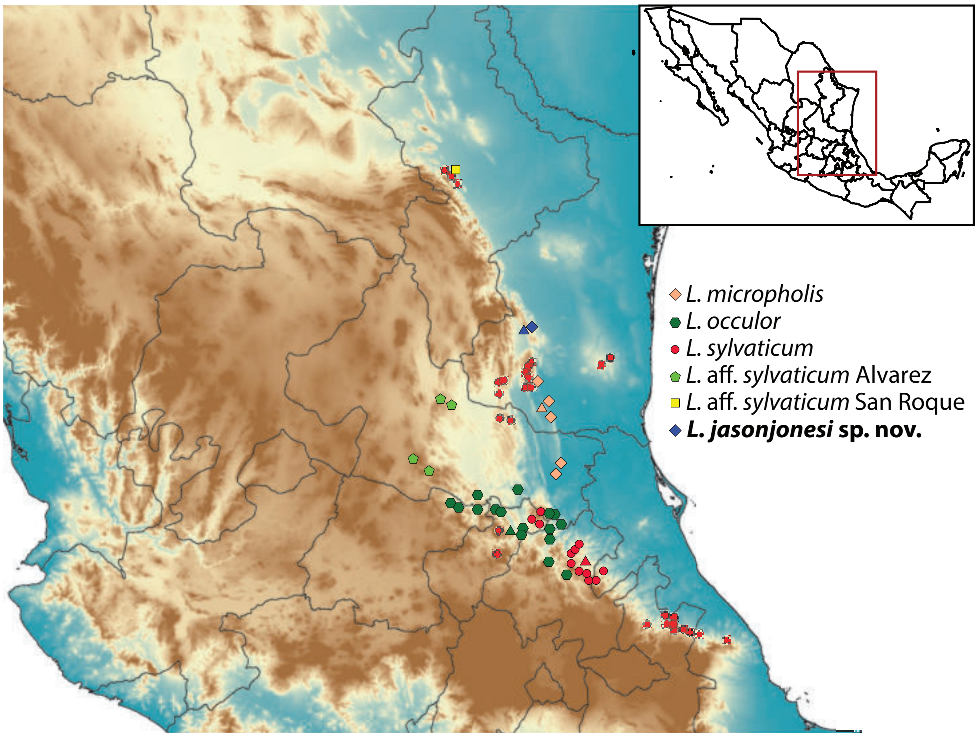

Figure 7. Distribution map of species in the *Lepidophyma sylvaticum* species group in north-eastern Mexico. Triangles represent type localities. Inset represents the paratype *L. jasonjonesi* sp. nov. (INIRENA 2818). See Materials and Methods for source of records.

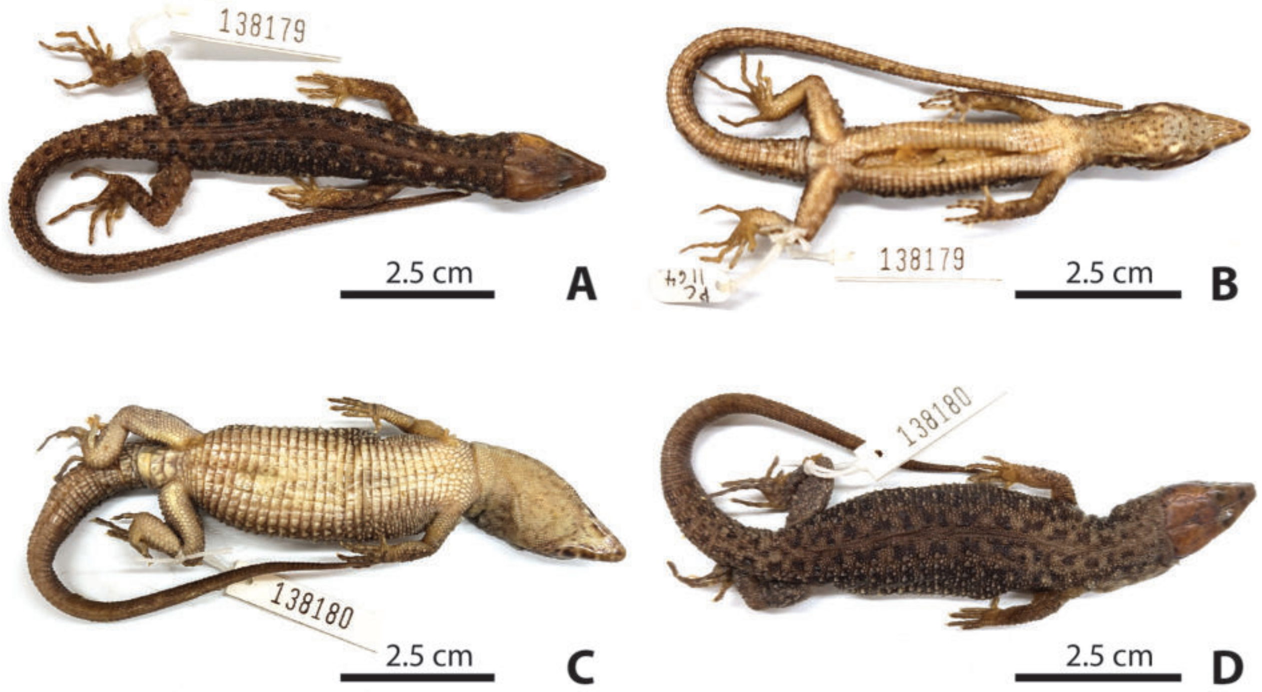

Figure 8. Photos of preserved specimens of a closely related species of *Lepidophyma* from San Roque, Municipio de Juárez, Nuevo León, Mexico. LACM 138179 (**A, B**); LACM 138180 (**C, D**).

Discussion

Additional taxa of the genus *Lepidophyma* continue to be discovered in Mexico. The majority of these new taxa have been described from the Sierra Madre Oriental and Sierra Madre del Sur and appear to be micro endemic. Our new species also appears to be rescripted to a small portion of the Sierra Madre Oriental; however, this apparent small range might be the result of poor sampling in the region and its distribution might be much larger than currently known (Fig. 7).

Our phylogenetic analysis shows that *L. jasonjonesi* sp. nov. is part of the *L. sylvaticum* group, which is restricted to north-eastern Mexico. Our results, alongside those of Noonan et al. (2013), show that the taxonomy of this group is in need of revision. The *L. sylvaticum* group appears to consist of at least five different taxa (Fig. 1), of which at least one is undescribed. These are *L. occulor*, *L. jasonjonesi* sp. nov., *L.* aff. *sylvaticum* Alvarez, *L. sylvaticum* and *L. micropholis*.

Individuals of *Lepidophyma* from central San Luis Potosí (*L.* aff. *sylvaticum* Alvarez) are the sister taxa to the remaining *L. sylvaticum* + *L. micropholis* (Fig. 1) and might require species recognition. The remaining populations of *L. sylvaticum* are represented by two distinct populations, one composed by individuals from eastern San Luis Potosí and Tamaulipas, while individuals from Querétaro and Hidalgo are sister to *L. micropholis*. Based on our results, we suggest additional studies are required to establish if the multiple populations of *L. sylvaticum* should be elevated to species and whether *L. micropholis* should be synonymised with *L. sylvaticum*.

The population of *Lepidophyma* from San Roque, Nuevo León (Fig. 8) requires additional study. Our molecular analysis shows that it is sister to *L. jasonjonesi* sp. nov. and it is likely that it represents a second population of this species. However, this population could also represent a second new species in the group, closely related to *L. jasonjonesi* sp. nov. As we do not have specimens of this population available for morphological study, we exclude them from our species description. Further specimens must be collected to properly resolve the status of this population. Two individuals of this population (LACM 138179, 138180; Fig. 8) show that they share a general resemblance to *L. jasonjonesi* sp. nov. and might share a similar saxicolous lifestyle. According to Noonan et al. (2013), a rock-crevice dwelling ecomorphotype (such as flattened body shape and smooth dorsal body scales lacking enlarged tubercles) evolved independently in *Lepidophyma* at least two times. Our data suggest that this rock-crevice dwelling ecomorphotype evolved independently as well within the *L. sylvaticum* group.

As stated above, one of the most striking characteristics of *Lepidophyma jasonjonesi* sp. nov. is the flattened head and body shape. This characteristic is reminiscent of the African *Platysaurus*, as well as the relatively closely related saxicolous *Xantusia* (*X. bezyi*, *X. bolsonae*, *X. henshawi*), but not seen to this degree in *Lepidophyma*. Upon first seeing the *L. jasonjonesi* sp. nov. specimens after preservation, two of authors (CIG, JRV) thought the flattened nature of the specimens was product of poor preservation or too much pressure being applied during preservation. However, live individuals are equally flat.

Conservation

Lepidophyma jasonjonesi sp. nov. is known only from south-western Tamaulipas, around the vicinity of the type locality. Due to its small distribution, we recommend that this species should be rewarded the highest level of protection possible from the Mexican government. It currently classifies as DD (Data Deficient) category as designated by the IUCN criterion.

Acknowledgements

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Appendix 1

Specimens examined:

Lepidophyma cuicateca: MEXICO: Oaxaca: Texcaltitlán (MZFC 16421).

Lepidophyma dontomasi: MEXICO: Oaxaca: Lachiguiri (ENEPI 3011, 3013).

Lepidophyma aff. flavimaculatum: MEXICO: Chiapas: La Venta (ENEPI 3794, 5793).

Lepiodphyma flavimaculatum: BELIZE: Blue Creek (CM 117260); COSTA RICA: Suerte (LACM 131068); Tortuguero (UF 65448); MEXICO: Chiapas: Montes Azules (RCMX 212–13, 232); HONDURAS: Gracias a Dios (USNM 563289–90); NICARAGUA: Diamante (OMNH 38246–47); PANAMA: Escobal (LACM 128560–61).

Lepidophyma gaigeae: MEXICO: Hidalgo: Durango (ENEPI 4090, 4095); Querétaro: El Madroño (ENEPI 4055); Lagunitas (LACM 127420). Tilaco (LACM 127346).

Lepidophyma jasonjonesi sp. nov.: MEXICO: Tamaulipas: 28 km NNE of Jaumave Federal Highway 101 to Ciudad Victoria, Municipio de Victoria (INIRENA 2817–18).

Lepidophyma lineri: MEXICO: Oaxaca: Portillo del Rayo, Candelaria Loxicha (JAC 242260); San Pedro Mixtepec (CIG 1903).

Lepidophyma lowei: MEXICO: Oaxaca: Zoogocho, 4 km SE of San Bartolome Zoogocho (CNAR 7498–99).

Lepidophyma lusca: MEXICO: San Luis Potosí: Tamul. (CNAR 32563–64).

Lepidophyma micropholis: MEXICO: San Luis Potosí: west of Ciudad Maíz (LACM 131141); Tamaulipas: Pachón Cave (JAC 24541); Gruta Quintero (CIG 0829).

Lepidophyma occulor: MEXICO: Querétaro: ex-Hacienda La Conca (TCWC 48499, CIG 1763).

Lepidophyma pajapanense: MEXICO: Veracruz: San Martín Tuxtla (LACM 135510); Catemaco, abandoned hotel above Playa Escondida (CIG 2084–85).

Lepidophyma aff. *radula*: MEXICO: Oaxaca: Mitla-Ayutla Highway (UTA 52402).

Lepidophyma reticulatum: COSTA RICA: Las Cruces (RLB 6317–18); Rincón (RLB 6319–20).

Lepidophyma aff. *smithii*: MEXICO: Guerrero: Puerto Márquez (LACM 130027–28).

Lepidophyma smithii: MEXICO: Chiapas: Acacoyagua (LACM 136363–64); Tuzantan (JAC 23062); Oaxaca: Juchitán (LACM 134468–69); Tehuantepec (LACM 128589); Chimalapas (JAC 23150, 23166), Tehuantepec (ENEPI O_4).

Lepidophyma sp.: MEXICO: Nuevo León: San Roque (LACM 138179–80).

Lepidophyma aff. *sylvaticum*: MEXICO: San Luis Potosí: Álvarez (ENEPI 4009–10).

Lepidophyma sylvaticum: MEXICO: Hidalgo: El Madroño (ENEPI 4029); Tepeoco (LACM 136365–66); Querétaro: Pinal de Amoles (CIG 1764); San Luis Potosí: El Naranjo (LACM 131147–48); west of Ciudad Maíz (ENEPI 4011–12), Valle de Trinidad (CIG 1550–51); Tamaulipas: Encino (TCWC 65549); Julilo (La Julila?) ENEPI 4076); Veracruz: Yecuautla (CIG 1391).

Lepidophyma tarascae: MEXICO: Colima: Grutas de San Gabriel (JRV 0219);

Lepidophyma tuxtlae: MEXICO: Oaxaca: Mirador (JAC 22720); VERACRUZ: San Andrés Tuxtla, above Estación Biologica UNAM (CIG 2087–88); Los Tuxtlas (ENS 10352); San Andrés Tuxtla (JAC 22619, 22626); Sontecomapan (LACM 136352, 136354).

Lepidophyma zongolica: MEXICO: Puebla: Tepeyac, Eloxochitlán (MZFC 22183–86).

Supplementary material 1

High resolution PDF file of comparative head shots

Authors: Jacobo Reyes-Velasco

Data type: PDF / image

Explanation note: (A) *Lepidophyma jasonjonesi* sp. nov. Holotype (INIRENA 2817); (B) *Lepidophyma jasonjonesi* sp. nov. Paratype (INIRENA 2818). (C) *Lepidophyma sylvaticum* (CIG 01550) Valle de Trinidad, Municipio de Xilitla, San Luis Potosí. (D) *Lepidophyma sylvaticum* (CIG 01391) Loma Santa Rosita, Municipio de Yecuautla, Veracruz. (E) *Lepidophyma micropholis* (CIG 00829) Grutas de Quintero, Municipio de El Mante, Tamaulipas. (F) *Lepidophyma tarascae* (JRV 0239) Grutas San Gabriel, Municipio de Ixtlahuacán, Colima.

Link: https://doi.org/10.3897/herpetozoa.36.e96184.suppl1

Supplementary material 2

Genbank sequences

Authors: Jacobo Reyes-Velasco

Data type: Spreadsheet

Explanation note: GenBank Numbers of sequences used in this study. New sequences are indicated in bold.

Link: https://doi.org/10.3897/herpetozoa.36.e96184.suppl2

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Trophic ecology of the Atlantic Forest endemic tree frog *Boana bischoffi* (Boulenger, 1887) (Anura, Hylidae)

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Abstract

Studies of natural history are important to accumulate knowledge about aspects of diet, reproduction, and habitat use, which can assist the conservation biology for endangered groups, such as amphibians. Here we evaluated the trophic ecology and sexual size dimorphism of *Boana bischoffi*, a widely distributed and endemic tree frog species of the Brazilian Atlantic Forest. We analysed 80 individuals, covering the distribution of the species and combined our data-set with data from the literature. Gastrointestinal items were separated, accounted, and identified to the highest possible taxonomic resolution. Subsequently, the size and mass of prey items were measured. Afterwards, we calculated rates of relative importance for each prey category. The items of greatest relative importance were beetles (Coleoptera), termites (Isoptera), crickets (Orthoptera) but also harvestmen (Opiliones). We did not find a relation between female snout-vent length, mouth width and length with prey length. In males, the mouth length and width are related to prey length. We found a sexual dimorphism in size typical for hylid frogs, with females being larger than males. Our data expand the knowledge about the alimentary biology of *B. bischoffi*, but further research focusing on other aspects of the natural history such as possible intersexual dietary divergence and food niche overlapping, environmental prey availability and selection is still needed.

Key Words

amphibians, diet, sexual dimorphism, trophic ecology

Introduction

Data about alimentary biology are essential to understand several aspects of an animal's life history and functional interaction between organisms, including energy flow and food webs as well as the development of conservation strategies (Anderson and Mathis 1999; Anderson 2002). Anurans mostly feed on invertebrates, e.g., arthropods (insects and spiders) but also molluscs and annelids, and occasionally small vertebrates (e.g., fish, birds, and even other frogs). Thus, they have an important role as regulators of the density of prey species (e.g., Duellman and Trueb 1986; Pizzatto and Shine 2008; Solé and Rödder 2009; Paunović et al. 2010; Luza et al. 2018).

Anurans play a fundamental role in the ecosystem since they are a source of food for several other animals, such as spiders (Foerster et al. 2017), snakes (Pombal 2007), fishes, insects (Haddad and Bastos 1997), and birds (Roulin and Dubey 2013), being an important component in trophic network (Toledo et al. 2007). Abundant species are good candidates for studies on the trophic biology, because of their important contribution to the matter and energy transfer between different trophic levels (Moser et al. 2017). *Boana bischoffi* (Boulenger, 1887) is a tree frog endemic to Atlantic Forest, that occurs from north of the state of São Paulo to the Rio Grande do Sul state, Brazil, and inhabits forests, open areas, and even degraded environments (e.g., Haddad et al. 2008; Garcia and Kwet 2010). Its body size varies from 40 to 65 mm (Ribeiro et al. 2005) with females being visually larger than males (personal communication).

Even though *B. bischoffi* is a widely distributed species, aspects related to its natural history have been poorly studied, with only a single study describing the diet of a population in the Rio Grande do Sul, Brazil (Moser et al. 2019). Furthermore, there are no data on sexual size dimorphism and trophic biology from other populations of this species. Thus, in this study, we evaluate the alimentary biology of the tree frog *Boana bischoffi*, a widely distributed and endemic species of the Atlantic Forest, to answer the following questions: (1) What is the diet composition of *B. bischoffi*? (2) Which are the most important component preys? (3) Is there sexual dimorphism in size in *B. bischoffi*?

Methods

We examined 80 adults of *Boana bischoffi* from 26 localities in total (Fig. 1, Appendix 1) deposited at the Célio F. B. Haddad Amphibian Collection (**CFBH**), Universidade Estadual Paulista, Rio Claro, São Paulo, Brazil. We selected specimens to have a balance that was representative of the four seasons (spring, summer, fall and winter) and both sexes.

We determined the sexes through direct observation of the gonads and secondary sexual characteristics of males such as the presence of a vocal sac and/or vocal slits. We measured the snout-vent length (**SVL**), mouth width (**MW**), and mouth length (**ML**) following Napoli (2005), with a digital calliper (to the nearest 0.1 mm). We examined the sexual size dimorphism (**SSD**) of SVL, HL and HW carrying out Student's t-testes. We tested for possible deviation from a normal distribution of the data using the Shapiro-Wilk test (Zar 2010). We also calculated the Sexual Size Dimorphism index following (Lovich and Gibbons 1992).

We analysed the composition of the diet examining both stomachs and intestines of each specimen to increase the efficiency to find a respective gut content (following Silva and Britto-Pereira 2006). First, we carefully separated the prey using scissors and forceps, under a stereomicroscope Nikon SMZ 745, and identified the order and family level when possible. Second, we measured the length and width of each prey with a digital calliper (to the nearest 0.1 mm). Third, we measured the mass of each prey after drying the material under 60 °C for about 5 minutes, using a digital scale Toledo XS205 (to the near-

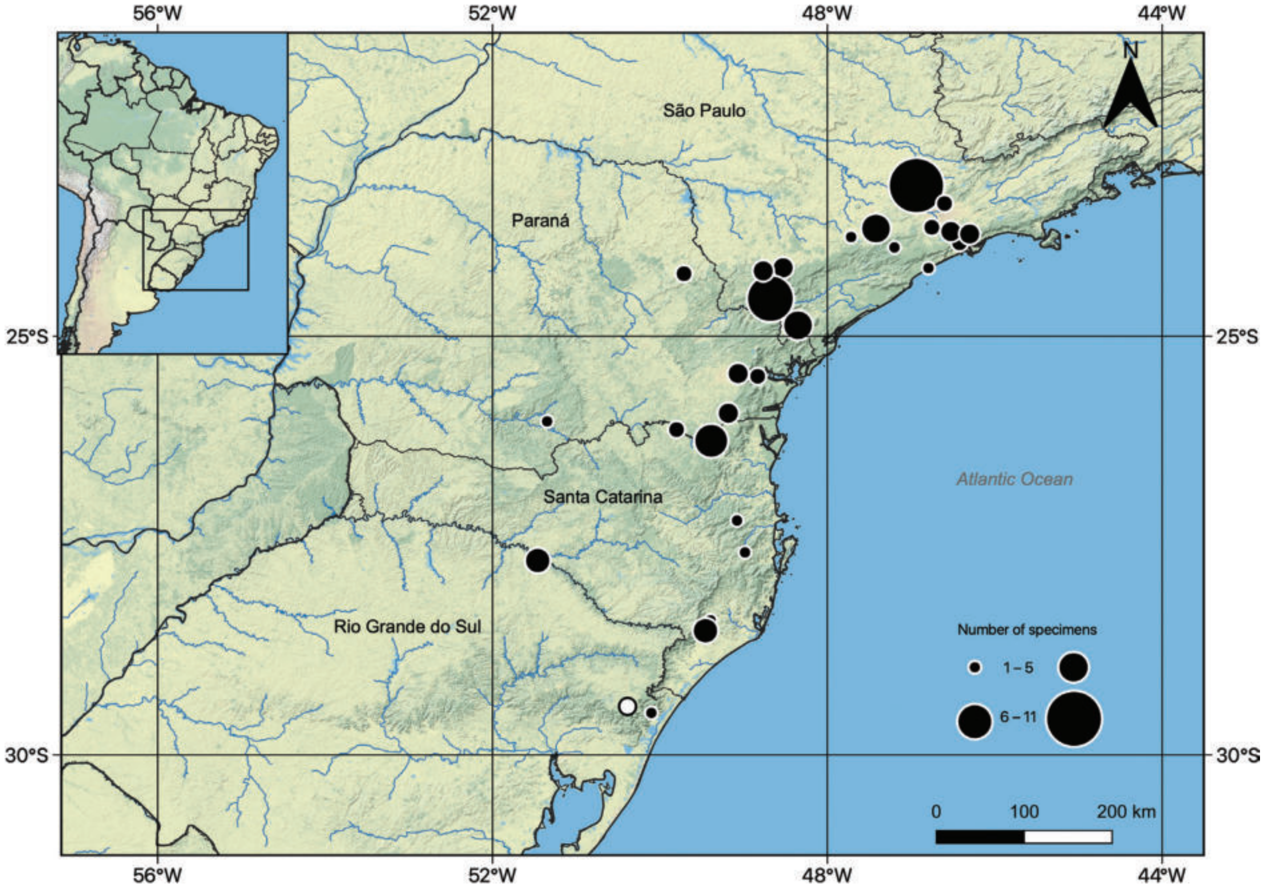

Figure 1. Geographic distribution of *Boana bischoffi* populations used for our trophic biology study. Black circles = examined specimens; white circle = literature data (from Moser et al. 2019).

est 0.00001 g). Then, we identified each prey using Thyssen (2010), Brusca et al. (2016), and Gullan and Cranston (2017), considering mature and immature as different categories. In order to estimate the importance of each prey category in the diet of *B. bischoffi*, we calculated the index of relative importance (IRI) proposed by Pinkas et al. (1971), substituting volume for mass in this formula following Martin et al. (1996): IRI = %O × (%N +%M), where %O is the relative occurrence, %N is the relative abundance, and %M is the relative mass.

To evaluate whether the sampling used was sufficiently representative, we performed two rarefaction curves with prey categoric frequency, one using only our data and a second compiling data set from Moser et al. (2019), both using species diversity estimates in the R package iNEXT (Chao et al. 2014; Hsieh et al. 2020) under the incidence-frequency data option.

We also analysed the possible influence of frog snout-vent length, mouth width, and length in relation to the length of the prey ingested, represented by a simple regression between frog measurements, by sex, and the length of the largest prey item found in each gastrointestinal tract. For this analysis, we only considered intact prey to avoid bias.

All analyses were performed using the R program (R CORE TEAM, 2018).

Results

For the analysis of the sexual dimorphism relative to the SVL, we sampled 80 adult specimens (38 females and 42 males) of *Boana bischoffi*. Females were larger (SVL ranged from 43.9 to 61.1 mm; mean 54.9 ± 3.7 mm) than males (SVL range 34.4–46.2 mm, mean 39.9 ± 2.6 m; $t = 20.639$, DF = 66.641, $p = 0.0001$) and had wider (females: 16.2–22.3 mm, mean 19.0 ± 1.4 mm; males: 11.1–16.4 mm, mean 14.1±1.2 mm; $t = 16.493$, DF = 73.739, $p < 0.0001$) and longer mouth (females: 14.5–20.1 mm, mean 17.6 ± 1.4 mm; males: 10.7–15.5 mm, mean 12.7±1.1 mm; $t = 17.745$, DF = 771.752, $p < 0.0001$). The size dimorphism index also shows that females were larger than males (SDI = 0.376).

We analysed the gastrointestinal contents of 38 females and 42 males of *B. bischoffi*. Of these, 43 (six only with fragments of plants) had their digestive tracts with prey (53.8%), of which 18 were females and 25 males. The percentages of gastrointestinal tracts with some contents were relatively higher in the summer (54%) and spring (64%) than in the fall (45%) and winter (42%). We identified 112 alimentary items in total distributed into 13 prey categories (Table 1). *B. bischoffi* consumed exclusively arthropods, totalling the 13 prey categories, with possible accidental ingestion of plants only. The most abundant prey items in the diet of *B. bischoffi* were termites (Isoptera) (56.3%), beetles (Coleoptera) (12.5%), and crickets (Orthoptera) (7.1%). beetles (26.1%), crickets (17.4%), and spiders (Araneae) (13.0%) were the most frequent prey in the gastrointestinal tracts. The relatively most massive prey items were harvestmen (Opiliones) (24.1%), crickets (16.2), and beetles (14.1%). Considering the index of relative importance, beetles (36%), termites (18.9%), crickets (17.2%), and harvestmen (13.4%) were the most important prey items consumed by female and male *B. bischoffi*.

Table 1. Diet composition of the frog *Boana bischoffi* (n = 43). O = total number of each prey category in the gastrointestinal tract; N = frequency of gastrointestinal tracts containing a given category; M = total mass (g) of each prey category; IRI = Index of Relative Importance; % = percentage value over total.

Prey Categories	O	O%	N	N%	M	M%	IRI%
Araneae	6	5.4	6	13	29.63	2.1	5.8
Opiliones	6	5.4	5	10.9	339.39	24.1	13.4
Blattaria	1	0.9	1	2.2	2	0.1	0.1
Coleoptera	14	12.5	12	26.1	199.32	14.1	36
Diptera	1	0.9	1	2.2	0.81	0.1	0.1
Hymenoptera (ants)	3	2.7	3	6.5	0.69	0	1.3
Hemiptera	1	0.9	1	2.2	58.21	4.1	0.4
Isoptera	63	56.3	2	4.3	4.93	0.3	18.9
Lepidoptera	1	0.9	1	2.2	516.2	36.6	2.5
Larvae of Coleoptera	1	0.9	1	2.2	1.3	0.1	0.1
Larvae of Lepidoptera	6	5.4	4	8.7	25.84	1.8	4
Neuroptera	1	0.9	1	2.2	4.42	0.3	0.2
Orthoptera	8	7.1	8	17.4	228.42	16.2	17.2
Plant matter	27	–	16	–	37.87	–	–

In the gastrointestinal tracts of *B. bischoffi*, we observed 13 prey categories increasing to 20 when we included data from references. In the richness of prey estimative, we found 30 categories for our data and 21 compiling data from Moser et al. (Fig. 2). Female and male snout-vent length (females: $r = -0.1195$; $F = 0.03963$; $p = 0.8472$; $n = 10$; males: $r = 0.01984$; $F = 1.304$; $p = 0.2727$; $n = 16$) were not related to prey length. The female mouth length ($r = -0.0372$; $F = 0.6772$; $p = 0.4344$; $n = 10$) and width ($r = -0.0262$; $F = 0.77702$; $p = 0.4057$; $n = 10$) did not have a relation to prey length. However, the male mouth length ($r = 0.3124$; $F = 7.816$; $p = 0.0143$; $n = 16$) and width ($r = 0.2388$; $F = 5.705$; $p = 0.03155$; $n = 16$) were related to prey length.

Discussion

Female *Boana bischoffi* were larger than males in agreement with the findings of other congeneric species such as *B. albopunctata*, *B. cordobae*, and *B. curupi* (Guimarães et al. 2011; Bastiani et al. 2016; Otero et al. 2017). Shine (1979) observed that, in most anuran species he analysed, females were the larger-sized sex. This pattern is probably related to fecundity because larger females can produce and store a larger number of eggs (Kupfer 2007; Nali et al. 2014; Maerker et al. 2016). Another hypothesis about this pronounced sexual dimorphism in size can

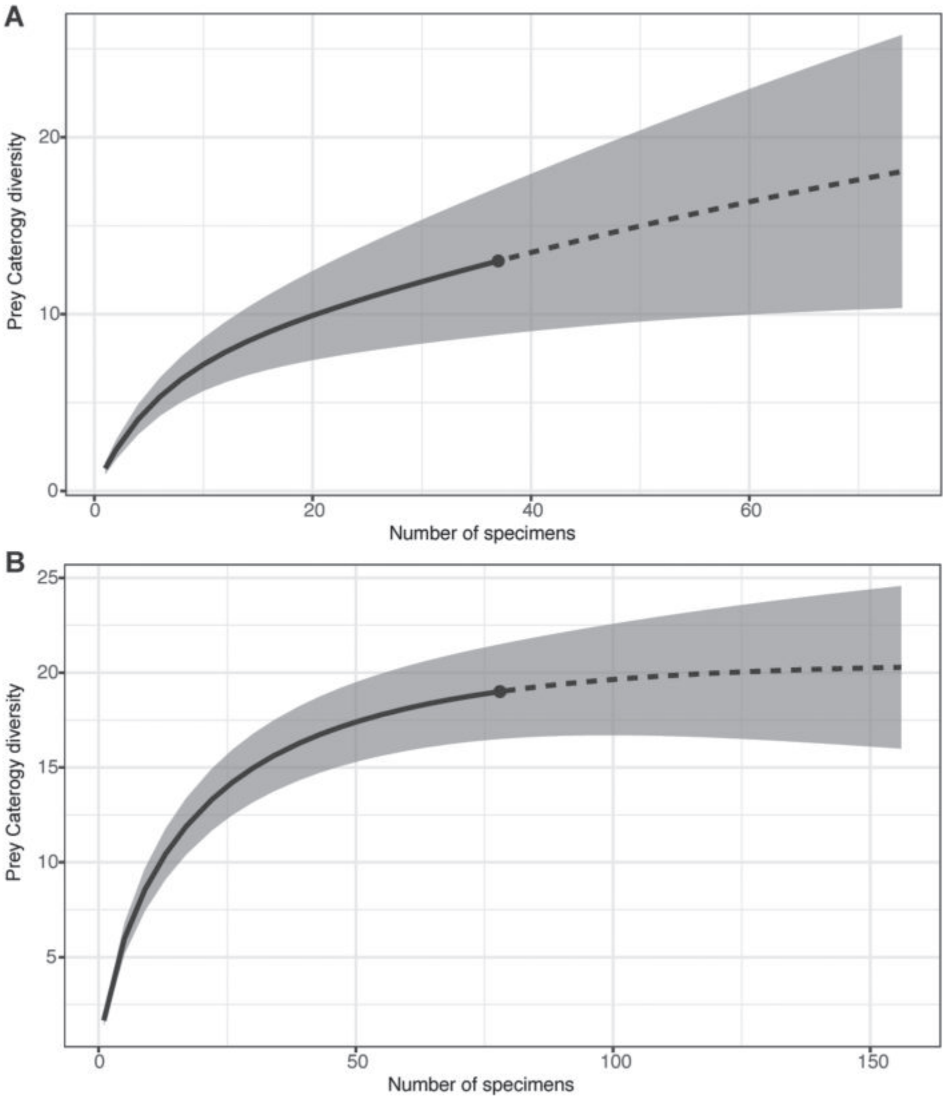

Figure 2. Rarefaction curve of prey categoric frequency in gastrointestinal tracts of *Boana bischoffi* using the data from (**A**) this study (**A**) and (**B**) compiling data of Moser et al. (2019). Continuous line = interpolated; Dashed line = extrapolated.

be related to the arboreal habitat, which is the case with the *B. bischoffi* females that must carry males during the amplexus through the vegetation, leading to larger female body sizes (Silva et al. 2020). More detailed analyses focusing on the sexual dimorphism of arboreal *Boana* species are important to evaluate not only the difference in size but also other body characters and shapes using a geometric morphometric approach.

The absence of prey in about half of the gastrointestinal tracts may be due to the use of specimens deposited in collections of natural history museums, which were collected for many other purposes but were not specifically collected for diet biology studies. Specimens to be used in alimentary biology studies must be euthanized and fixed immediately after being collected (following e.g., Solé et al. 2005; Magalhães et al. 2016; Machado et al. 2019), which is not necessarily the case with specimens found in natural history collections. Also, most of the frogs may have been collected during the reproductive season, when individuals are focused on reproductive success and are not feeding (Duellman and Trueb 1986; Solé and Pelz 2007). After Ceron et al. (2020) *B. bischoffi* is continuously breeding and males keep calling during a prolonged period of nine months, which also may influence its foraging activity. On the contrary, our study specimens with digestive contents were captured during the reproductive period in summer or spring. Therefore, complementary studies comparing the feeding and reproductive activities are necessary to further elucidate the high proportion of empty gastrointestinal tracts of *B. bischoffi*.

We found 13 prey categories in the diet of *B. bischoffi*, two less than that reported earlier by Moser et al. (2019). However, four invertebrate prey groups were newly recorded i.e., Opiliones, Isoptera, Neuroptera, and Formicidae in the diet, thus increasing the prey richness up to 20 groups, almost matching the prediction of 21 prey groups. Usually, alimentary biology studies have used only their own sampling to estimate the richness of prey items, but they did not reach the plateau, as an indicative of the need to increase sampling efforts (Telles et al.

2013; Magalhães et al. 2016). Including literature data in our analyses proofed an important tool to infer the diet of the Neotropical anurans, which are still poorly studied (Anjos et al. 2020).

Coleoptera, Orthoptera, Isoptera and Opiliones were the most important prey categories in the diet of *B. bischoffi* in accordance with congeners, where Araneae and Diptera also have high representativity (López et al. 2009; Sabagh et al. 2010; Rosa et al. 2011; Barbosa et al. 2014; Moser et al. 2019, 2022; Tupy et al. 2021). In the previous study on the trophic ecology of *B. bischoffi*, Moser et al. (2019) reported Araneae and Coleoptera as the most relative important items. However, we found Coleoptera, Orthoptera, Isoptera, and Opiliones as the main prey items. The difference might be explained by the prey availability in the environment because we examined specimens from several localities (see Fig. 1) but Moser et al. (2019) from only one locality (Floresta Nacional São Francisco de Paula, Rio Grande do Sul, Brazil). Nevertheless, future works focusing on prey availability and food selectivity are necessary to further elucidate this finding.

We found plant material (seed and fragments of leaves) in the gastrointestinal tract of *B. bischoffi*, which was previously reported for other species of *Boana* (López et al. 2009; Sabagh et al. 2010; Barbosa et al. 2014; Moser et al. 2019; Tupy et al. 2021). This ingestion is likely accidental when frogs are capturing prey, as plants are not considered an important part of the anuran diet (e.g., Korschgen and Moyle 1955; Solé and Pelz 2007; Solé and Rödder 2009). However, other authors (e.g., Das 1996; Anderson and Mathis 1999; Silva and Britto-Pereira 2006) have argued that plant ingestion could be intentional, serving as a source of water and nutrients but also constitute a method to eliminate parasites. Future field ethological studies are still necessary to better clear up the controversy about an herbivorous diet of anurans.

We did not find a relation between snout-vent length, mouth width and length to prey length in females of *B. bischoffi*. However, in the males, the mouth length and width are related to prey length, which was also reported earlier for the congener *B. albomarginata* (Tupy et al. 2021). However, Guimarães et al. (2011) and Tupy et al. (2021) showed that frog and prey size were correlated in *B. albopunctata* and *B. pombali*, indicating that predator size also determines prey size (see also Caldwell and Vitt 1999).

Conclusions

Boana bischoffi is a generalist feeder; i.e., its diet is mainly based on several major arthropod groups such as Coleoptera, Isoptera and Orthoptera but also Opiliones. It has an accentuated sexual size dimorphism with females being larger than males.

Future studies comparing alimentary biology are necessary to elucidate possible intersexual dietary divergence or food niche overlapping. Furthermore, environmental prey availability and selection are other important aspects to be evaluated in the future. Our study contributes to understanding the trophic ecology of anurans in the Atlantic Forest and reinforces the importance of including literature data in the analysis.

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Appendix 1

Adults of the frog *Boana bischoffi* in this study

BRAZIL: **Paraná:** Cruz Machado: Estrada para Usina Hidrelétrica Salto do Vau (♂, CFBH 18261); Jaguariaíva: Fazenda do Edilson (Dono do Hotel Conde Alemão) (2♂♂: CFBH 24725, CFBH 24727); Morretes: Parque Estadual do Pico do Marumbi (♀, CFBH 21926; ♂, CFBH 21927); Piraquara: Brejo na Estrada da casa do Senhor Airton (♀,CFBH 31048), Mananciais da Serra, região Barragem Piraguara (♀,CFBH 11047), Poça na casa do Senhor Airton, estrada para a Pousada Tempero da Serra (♀,CFBH 31044); Tijucas do Sul: Estrada entre Tijucas do Sul e BR 376 (♂, CFBH 8432; 2♀♀,CFBH 8436, CFBH 8438). **Rio Grande do Sul:** Barracão: Parque Estadual do Espigão Alto (4 ♂♂, CFBH 21913 to 21914, CFBH 21916, CFBH 21922); Itati: Reserva Biológica Estadual da Paludosa (♂, CFBH 14592). **Santa Catarina:** Angelina (♂, CFBH 8479); Botuverá: Comunidade Lajeada Baixa; riacho Vicinity (♀, CFBH 10959); Lauro Muller: Brejo próximo a estrada da Serra do Rio do Rastro (♀, CFBH 30324); Mafra: Ponto I (♂, CFBH 8585; ♀, CFBH 8590); São Bento do Sul: Estrada e brejo na “Fazenda 26”, estrada velha pra Rio Vermelho (2 ♀♀, CFBH 10975 to10976; 2 ♂♂, CFBH 10986 to 10987), Estrada Saraiva (2 ♀♀, CFBH 3009 to 3010); Treviso: Mina do Carvão, TRV4 (♂, CFBH 12401), próximo a Criciuma (♀, CFBH 8489; ♂ CFBH 8490), Treviso (♂, CFBH 10329). **São Paulo:** Apiaí: Parque Estadual Turístico do Alto Ribeira (♀, CFBH 38662), Base Bulha d’água e capinzal (2 ♀♀, CFBH 26768 to 26769), Núcleo Caboclos (2 ♀♀, CFBH 26805, CFBH 26807; ♂ CFBH 26806), Núcleo Ouro Grosso (3 ♂♂, CFBH 6303 to 6304, CFBH 6324); Barra do Turvo: Parque Jacupiranga (5 ♂♂, CFBH 6339 to 6340, CFBH 6344 to 6345, CFBH 6347); Cubatão: COPEBRAS (♂, CFBH 9243; ♀ CFBH 25828); Guapiara (3 ♀♀, CFBH 14691 to 14692; CFBH 14721); Iporanga (♀, CFBH 14582); Itanhaém, Parque Estadual da Serra do Mar, Núcleo Curucutu, Trilha Mambu (♂, CFBH 12210); Jundiaí, Serra do Japi (2 ♀♀, CFBH 718, CFBH 802; 9 ♂♂, CFBH 8374 to 8376, CFBH 8618 to 8621, CFBH 14433, CFBH 14440), Juquitiba: Parque Estadual de Jurupará (♀, CFBH 38600), Piedade: Parque Estadual do Jurupará (♀, CFBH 23266), Vila Elvio (♀, CFBH 15989; 3 ♀♀, CFBH 15991 to 15992, CFBH 22260); Pilar do Sul (♀, CFBH 8346); Ribeirão Branco, Fazenda São Luiz (Mathedi) (2 ♀♀, CFBH 255 to 256); Ribeirão Branco (♂, CFBH 11290); Santo André: Parque do Pedroso (♂, CFBH 11627), Parque Estadual Municipal Nascentes de Paranapiacaba, próximo a Cachoeira do Pedro Lisa (2 ♀♀, CFBH 28966–28967); São Paulo, Parque Ecológico da Guarapiranga (♂, CFBH 26677), Sítio Roda D’Água (♀, CFBH 31086); Serra da Cantareira (♀, CFBH 5689; ♂, CFBH 5690).

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First record of *Hoplobatrachus litoralis* Hasan, Kuramoto, Islam, Alam, Khan & Sumida, 2012 (Anura, Dicroglossidae) from China

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Abstract

We report the first record of *Hoplobatrachus litoralis* Hasan, Kuramoto, Islam, Alam, Khan & Sumida, 2012 from China based on seven specimens from Baoshan City, western Yunnan. Morphologically, the specimens from China mostly agree with the original description of *H. litoralis* and phylogenetically show a small genetic distance (1.7%) in the 16S rRNA gene with the specimens (including one paratype) of *H. litoralis* from its type locality in Bangladesh. Our work increased the species number of the genus *Hoplobatrachus* Peters, 1863 in China to two. This record is the easternmost distribution of this species at present. Furthermore, we found that the species *H. salween* Thongproh, Chunskul, Sringurngam, Waiprom, Makchai, Cota, Duengkae, Duangjai, Hasan, Chuaynkern & Chuaynkern, 2022 recently described from north-western Thailand is morphologically and genetically very similar to *H. litoralis* and, therefore, we discuss the validity of *H. salween* as a separate species.

Key Words

16S rRNA, Baoshan, distribution, Longling, western Yunnan

Introduction

For a long time, it was considered that only one species of the genus *Hoplobatrachus* Peters, 1863 was distributed in China, namely, *H. chinensis* (Osbeck, 1765) and it was thought to be widely distributed in southern China (Fei et al. 2009, 2012; AmphibiaChina 2022; Frost 2022). Although this species has a wide distribution range, it is regarded as a legally protected species in China due to the rapid decline of its population size (Jiang and Xie 2021).

Hoplobatrachus litoralis Hasan, Kuramoto, Islam, Alam, Khan & Sumida, 2012 is a species originally described from Bangladesh (Hasan et al. 2012). Subsequently, Mulcahy et al. (2018) and Zug (2022) extended the range of this species into Myanmar, while Purkayastha and Besak (2018), Mondal et al. (2018), Bohra et al. (2019), Kundu et al. (2020) and Lalremsanga et al. (2022) recorded this species in India and Wangyal et al. (2020) reported this species from Bhutan. *Hoplobatrachus litoralis* inhabits vegetated, marshy ditches or ponds. It is characterised by large body size, a broad black band from anterior corner of eye through the nostrils to anterior edge of upper jaw, a band along the lateral margin of upper jaw, a distinct black margin in the inner side of the upper arm, a black inner metatarsal tubercle and inter-orbital distance that is much narrower than eyelid width and inter-nostril distance (Hasan et al. 2012).

PENSOFT.

While studying the *Hoplobatrachus* specimen collection of Kunming Natural History Museum of Zoology, Kunming Institute of Zoology, Chinese Academy of Sciences (KIZ), we discovered a jar with seven specimens determined as *H. chinensis* and collected from Longling, Baoshan, Yunnan. After detailed examination of these specimens, we found that they more closely resemble *H. litoralis* rather than *H. chinensis*. Since molecular analysis conducted in this study also indicated that these specimens belong to *H. litoralis*, we report this new record for China in detail.

Materials and methods

Genomic DNA was extracted from liver tissues using the standard phenol-chloroform extraction protocol (Sambrook et al. 1989). A partial fragment of the mitochondrial 16S rRNA was amplified for all samples via the polymerase chain reaction (PCR) using the primers L2188 (Matsui et al. 2006): 5’–AAAGTGGGCCTAAAAGCAGCCA–3’ and 16H1 (Hedges 1994): 5’–CTCCGGTCTGAACTCAGATCACGTAGG–3’. The products were purified and sequenced by Tsingke Biotechnology (Beijing) Co., Ltd. All new sequences were deposited in GenBank. *Euphlyctis cyanophlyctis* (Schneider, 1799) and *E. hexadactyla* (Lesson, 1834) were chosen as outgroups according to Hasan et al. (2012). Homologous and outgroup sequences were obtained from GenBank (Table 1). The technical computation methods for the best substitution model selection, Bayesian Inference and Maximum Likelihood phylogenetic analyses were the same as those in Liu et al. (2020).

Measurements were taken with a digital caliper to the nearest 0.1 mm. Morphological terminology follows Hasan et al. (2012). Abbreviations of the morphometric traits are as follows: snout-vent length (**SVL**), head length (**HL**), head width (**HW**), snout to nostril distance (**S-N**), inter-nostril distance (**N-N**), nostril to eye distance (**N-E**), horizontal eye diameter (**ED**), inter-orbital distance between inner borders of upper eyelids (**E-E**), eyelid width (**ELW**), horizontal tympanum diameter (**TD**), forelimb length (**FLL**), forearm and hand length (**FHL**), forearm width (**FAW**), hand length (**HAL**), length of 1st to 4th finger (**F1-F4**), hind-limb length (**HLL**), femur length (**FEL**), tibia length (**TIL**), tarsus and foot length (**TFL**), foot length (**FOL**), length of 1st to 5th toe (**T1-T5**) and inner metatarsal tubercle length (**IMT**).

Results

Bayesian Inference and Maximum Likelihood analyses showed consistent topology. The sequences of the specimens from Baoshan, western Yunnan, China, clustered with the sequences of the specimens from Mae Hong Son, Thailand and Bago and Yangon, Myanmar and all together clustered with the sequences of the specimens of *Hoplobatrachus litoralis* from Bangladesh (including one paratype IABHU 3974) and India (Fig. 1). The average genetic distance (uncorrected p-distance) between the specimens from China and the specimens of *H. litoralis* from its type locality in Bangladesh (including one paratype IABHU 3974) is 1.7% and the average genetic distance (uncorrected p-distance) between the specimens from China and the specimen of *H. litoralis* from India is 1.4% (Table 2). Morphological measurements of the specimens from China are presented in Table 3. As the specimens from China are not adults, we only compared the body ratios of these specimens (Table 4) with the relevant data from the original description of *H. litoralis* by Hasan et al. (2012). The specimens from China have relatively greater snout to nostril distances (S-N/SVL 0.080–0.094 vs. 0.044–0.076), relatively greater eye diameters (ED/SVL 0.118–0.133 vs. 0.063–0.107) and relatively greater lengths of fourth toe (T4/SVL 0.397–0.438 vs. 0.277–0.368); apart from these, all other ratios of the specimens from China overlap with the relevant data from the original description of *H. litoralis*. In addition, the colour patterns on these specimens from China agree well with the original description of *H. litoralis*.

Taxonomic account

Hoplobatrachus litoralis Hasan, Kuramoto, Islam, Alam, Khan & Sumida, 2012

Figs 2, 3, 4A, B

Specimens examined. KIZ 034006–KIZ 034012, seven subadults, all collected on 21 August 2013 from Manguanhe Village, Mengnuo Town, Longling County, Baoshan City, Yunnan Province, China (24°20'41"N, 99°1'11"E; elevation 750 m).

Morphological description. Head longer than wide (HL/HW 1.13–1.23), obtusely pointed. Canthus rostralis blunt. Loreal region concave. Nostril slightly nearer to tip of snout than to eye (S-N/N-E 0.78–0.97). Tympanum smaller than eye (TD/ED 0.51–0.59). Inter-orbital space much narrower than eyelid width and inter-nostril space (E-E/ELW 0.48–0.70, E-E/N-N 0.50–0.78).

Fingertips blunt without disc, no webbing on fingers. Finger length F3 > F1 > F2 > F4 or F3 > F1 > F4 > F2. Subarticular, thenar and palmar tubercles distinct.

Hind-limb moderately long (HLL/SVL 1.53–1.71). Femur length almost equal to tibia length (FEL/TIL 0.92–1.01). Toe tips blunt, slightly rounded. Toe length T4 > T5 > T3 > T2 > T1 or T4 > T3 > T5 > T2 > T1. Webbing reaching the base of toe tip, incurved between toe tips. Subarticular tubercles moderate, inner metatarsal tubercle distinct, no outer metatarsal tubercle.

Many distinct thin longitudinal ridges on dorsum. Indistinct small round warts over dorsal and lateral body. Dorsal limbs smooth. Supratympanic fold from behind eye to posterior margin of tympanum. Weak tarsal ridge extending from proximal end of inner metatarsal tubercle to heel.

Table 1. Sequences used for phylogenetic analysis in this study.

Taxon	Voucher no.	Locality	GenBank no.
Hoplobatrachus chinensis	SYS a006157	Guangdong, China	ON615100
Hoplobatrachus chinensis	SCUM0437941	Yunnan, China	DQ458251
Hoplobatrachus chinensis	MVZ 224079	Tam Dao, Vinh Phuc, Vietnam	EU979844
Hoplobatrachus chinensis	ZFMK TZ55	Ky Thuong, Ha Tinh, Vietnam	AF285208
Hoplobatrachus chinensis	2007.6265	Huu Lien, Lang Son, Vietnam	KR827770
Hoplobatrachus chinensis	ZFMK TZ301	Ky Thuong, Ha Tinh, Vietnam	AY014372
Hoplobatrachus chinensis	VUB 0684	Vietnam	AY322289
Hoplobatrachus chinensis	2003.8636	Phongsaly, Laos	KR827767
Hoplobatrachus chinensis	0033Y	Uttaradit, Thailand	KR827766
Hoplobatrachus crassus	BAUFBG 20865	Khulna, Bangladesh	AB272595
Hoplobatrachus crassus	IABHU 3859	Chittagong, Bangladesh	AB543601
Hoplobatrachus crassus	IABHU 3973	Cox’s Bazar, Bangladesh	AB671184
Hoplobatrachus crassus	MNHNF 20698	Assam, India	AB290413
Hoplobatrachus crassus	CDZMTU282	Nepal	MT983032
Hoplobatrachus crassus	CDZMTU283	Nepal	MT983033
Hoplobatrachus litoralis	IABHU 3975	Cox’s Bazar, Bangladesh	AB671173
Hoplobatrachus litoralis	IABHU 3974	Cox’s Bazar, Bangladesh	AB671174
Hoplobatrachus litoralis	IABHU 3985	Cox’s Bazar, Bangladesh	AB671175
Hoplobatrachus litoralis	IABHU 3982	Cox’s Bazar, Bangladesh	AB671176
Hoplobatrachus litoralis	IABHU 3977	Cox’s Bazar, Bangladesh	AB671177
Hoplobatrachus litoralis	IABHU 3983	Cox’s Bazar, Bangladesh	AB671178
Hoplobatrachus litoralis	IABHU 3976	Cox’s Bazar, Bangladesh	AB671179
Hoplobatrachus litoralis	IABHU 3988	Cox’s Bazar, Bangladesh	AB671180
Hoplobatrachus litoralis	IABHU 3978	Cox’s Bazar, Bangladesh	AB671181
Hoplobatrachus litoralis	MZMU-1777	Mizoram, India	OM501581
Hoplobatrachus litoralis	USNM 587325	Yangon, Myanmar	MG935819
Hoplobatrachus litoralis	USNM 587404	Yangon, Myanmar	MG935820
Hoplobatrachus litoralis	MBM-USNMFS 35607	Bago, Myanmar	MG935818
Hoplobatrachus litoralis	KKUC 01173/THNHM 26829	Mae Hong Son, Thailand	MW244089
Hoplobatrachus litoralis	THNHM 26827	Mae Hong Son, Thailand	MW244088
Hoplobatrachus litoralis	THNHM 26828	Mae Hong Son, Thailand	MW244087
Hoplobatrachus litoralis	KIZ 034006	Baoshan, Yunnan, China	OQ535767
Hoplobatrachus litoralis	KIZ 034007	Baoshan, Yunnan, China	OQ535768
Hoplobatrachus litoralis	KIZ 034008	Baoshan, Yunnan, China	OQ535769
Hoplobatrachus litoralis	KIZ 034009	Baoshan, Yunnan, China	OQ535770
Hoplobatrachus litoralis	KIZ 034010	Baoshan, Yunnan, China	OQ535771
Hoplobatrachus litoralis	KIZ 034011	Baoshan, Yunnan, China	OQ535772
Hoplobatrachus litoralis	KIZ 034012	Baoshan, Yunnan, China	OQ535773
Hoplobatrachus occipitalis	MVZ235754	Nouakchott, Mauritania	EU979845
Hoplobatrachus occipitalis	MVZ234146	Mwanza, Tanzania	EU979846
Hoplobatrachus occipitalis	ZFMK 65186	Uganda	AY014374
Hoplobatrachus occipitalis	ZFMK 23WB01	Mauritania	AY014373
Hoplobatrachus occipitalis	FMNH 257224	Ivory Coast	AF261263
Hoplobatrachus occipitalis	IABHU 20699	Africa	LC640619
Hoplobatrachus tigerinus	IABHU 3902	Mymensingh, Bangladesh	AB530500
Hoplobatrachus tigerinus	IABHU 4000	Mymensingh, Bangladesh	AB671182
Hoplobatrachus tigerinus	IABHU 4001	Mymensingh, Bangladesh	AB671183
Hoplobatrachus tigerinus	CDZMTU285	Nepal	MT983035
Hoplobatrachus tigerinus	CDZMTU286	Nepal	MT983036
Euphlyctis cyanophlyctis	MNHN 2000.650	Cochin, India	AY014366
Euphlyctis hexadactyla	EH98	India	GU136102

Colouration. In preservative, dorsum light grey to dark grey, a whitish mid-dorsal stripe from tip of snout to vent. Many large black spots on dorsal and lateral body. Large oval black spots on dorsal surface of limbs. A distinct black stripe from tip of snout through nostril to anterior corner of eye on each side. A short black stripe below eye each side. A gradually widening black stripe along supratympanic fold on each side. Upper lips brown, lower lips white with some black spots. A light thick stripe from posterior corner of eye to groin on each side. Rear side of thigh heavily mottled. Ventral side white, except for some black spots along the edge of lower jaw to the base of forelimb.

Recommended Chinese name. 孟加拉虎纹蛙 (Pinyin: mèng jiā lā hǔ wén wā).

Comparison between *Hoplobatrachus litoralis* and *H. chinensis*. *Hoplobatrachus litoralis* differs from *H. chinensis* by snout to anterior eye stripe present in *H. litoralis* vs. absent in *H. chinensis*, supralabial stripe present in *H. litoralis* vs. absent in *H. chinensis*, mid-dorsal stripe present in *H. litoralis* vs. absent in *H. chinensis*, distinct large round black spots on dorsum of *H. litoralis* vs. indistinct irregular small black spots on dorsum of *H. chinensis*, light stripe from posterior corner of eye to groin present in *H. litoralis* vs. absent

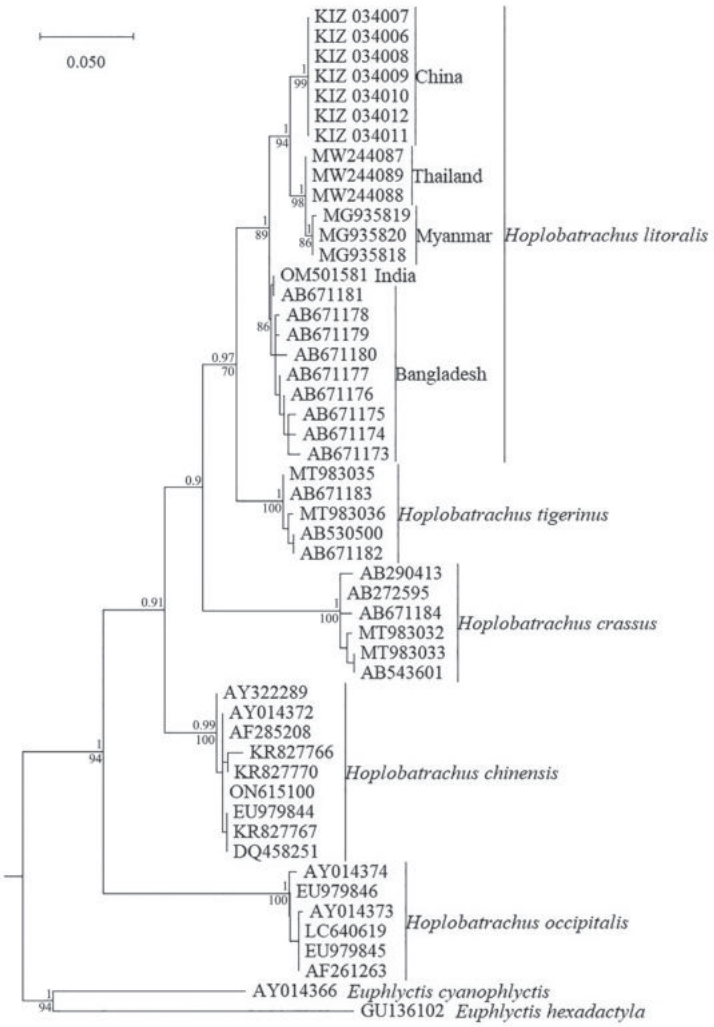

Figure 1. Maximum Likelihood tree based on 16S rRNA sequences. Numbers above branches indicate Bayesian posterior probabilities (values below 0.9 are not shown) and numbers below branches indicate bootstrap support for Maximum Likelihood analysis (values below 70 are not shown).

Table 2. Genetic uncorrected p-distances (%) based on partial 16S rRNA sequences.

	1	2	3	4	5	6	7	8	9	10
1 *Hoplobatrachus litoralis* (China)										
2 *Hoplobatrachus litoralis* (Thailand)	1.3									
3 *Hoplobatrachus litoralis* (Myanmar)	1.6	0.4								
4 *Hoplobatrachus litoralis* (India)	1.4	1.4	1.8							
5 *Hoplobatrachus litoralis* (Bangladesh)	1.7	2.2	2.5	0.6						
6 *Hoplobatrachus chinensis*	4.9	4.2	4.5	3.7	4.2					
7 *Hoplobatrachus crassus*	6.5	6.4	6.0	5.6	5.9	6.3				
8 *Hoplobatrachus occipitalis*	11.4	10.5	11.0	10.9	11.2	9.7	11.6			
9 *Hoplobatrachus tigerinus*	4.3	4.0	4.2	2.8	3.3	4.6	5.4	10.7		
10 *Euphlyctis cyanophlyctis*	12.6	11.9	12.0	11.7	12.5	12.9	11.7	13.4	12.0	
11 *Euphlyctis hexadactyla*	13.6	12.6	12.7	11.9	12.8	13.5	13.9	15.2	11.9	12.8

or indistinct in *H. chinensis*, ventral side of head with no spot except for some spots along the edge of lower jaw in *H. litoralis* vs. ventral side of head scattered with black spots in some individuals of *H. chinensis* and dorsal skin relatively smoother in *H. litoralis* vs. dorsal skin relatively rougher in *H. chinensis* (see Fig. 4).

Discussion

This study is the first record of *Hoplobatrachus litoralis* from China. The new locality in China is approximately 770 km away from the type locality of this species and is also the easternmost part of its distribution range to date (Fig. 5). Our work brings the species number of the genus *Hoplobatrachus* in China to two. Species of the genus *Hoplobatrachus* have been regarded as legally protected in China. Finding out how many species of this genus live in China contributes to the better protection of these endangered amphibians.

The specific name of this species is derived from the Latin *litoralis* meaning coastal, as this species was origi-

Figure 2. The specimens of *Hoplobatrachus litoralis* from China in preservative. **A.** Dorsal view; **B.** Ventral view.

Table 3. Measurements (in mm) of the specimens of *Hoplobatrachus litoralis* from China. For abbreviations, see Materials and methods.

	KIZ 034006	KIZ 034007	KIZ 034008	KIZ 034009	KIZ 034010	KIZ 034011	KIZ 034012
SVL	48.0	44.1	35.3	55.3	53.3	39.3	34.8
HL	20.3	17.5	15.2	22.4	20.9	16.2	14.3
HW	16.5	14.8	13.0	19.8	18.7	13.5	12.5
S-N	4.5	3.7	3.1	5.1	4.8	3.7	2.8
N-N	3.2	2.7	2.4	4.0	3.8	3.0	2.5
N-E	5.1	4.5	3.4	5.8	5.2	3.8	3.6
ED	6.0	5.3	4.7	6.5	6.4	4.9	4.6
E-E	2.3	2.1	1.8	2.7	2.4	1.5	1.7
ELW	3.3	3.2	2.7	4.0	3.9	3.1	2.7
TD	3.5	3.0	2.4	3.7	3.8	2.8	2.6
FLL	24.2	21.0	17.4	27.5	27.3	19.9	17.9
FHL	18.7	16.5	13.4	22.0	21.3	15.2	13.5
FAW	4.2	3.4	2.6	4.7	4.4	2.6	2.4
HAL	9.8	9.6	7.3	12.7	12.0	8.2	7.4
F1	5.1	5.0	3.8	6.5	6.1	3.7	3.9
F2	4.0	3.7	2.7	4.7	4.1	3.2	2.8
F3	5.8	5.5	4.0	7.6	6.6	5.0	4.6
F4	4.1	3.4	2.6	5.1	4.3	3.4	3.2
HLL	82.1	67.5	55.5	90.9	88.6	62.6	54.6
FEL	23.6	20.0	16.7	26.9	25.8	18.9	15.5
TIL	25.7	20.8	16.9	28.9	27.4	18.8	16.9
TFL	37.8	30.9	25.6	42.3	40.6	28.2	24.9
FOL	25.8	21.7	16.7	28.7	27.0	19.6	16.4
T1	5.9	5.3	4.2	7.2	5.8	4.4	4.2
T2	9.1	7.9	6.6	10.6	9.2	7.8	6.8
T3	13.6	12.1	10.0	16.4	15.1	12.0	9.5
T4	20.3	17.5	15.0	22.8	22.2	17.2	14.0
T5	13.9	11.0	9.2	15.5	14.3	11.1	8.3
IMT	2.6	2.1	2.1	2.9	3.0	1.9	1.7

Table 4. Comparison of body ratios between the specimens of *Hoplobatrachus litoralis* from its type locality and from China. Data for the specimens from its type locality were obtained from Hasan et al. (2012).

	***Hoplobatrachus litoralis* (Bangladesh, n = 27)**		***Hoplobatrachus litoralis* (China, n = 7)**	
	Mean	**Min–Max**	**Mean**	**Min–Max**
HL/SVL	0.378	0.349–0.436	0.410	0.392–0.431
HW/SVL	0.359	0.316–0.400	0.351	0.336–0.368
S-N/SVL	0.062	0.044–0.076	0.089	0.080–0.094
N-N/SVL	0.063	0.051–0.073	0.070	0.061–0.076
N-E/SVL	0.090	0.065–0.108	0.101	0.096–0.106
ED/SVL	0.082	0.063–0.107	0.125	0.118–0.133
E-E/SVL	0.041	0.029–0.054	0.047	0.038–0.051
ELW/SVL	0.075	0.060–0.094	0.074	0.069–0.079
TD/SVL	0.069	0.051–0.083	0.070	0.067–0.075
FLL/SVL	0.541	0.484–0.626	0.500	0.476–0.514
FHL/SVL	0.384	0.331–0.418	0.388	0.374–0.400
FAW/SVL	0.086	0.059–0.121	0.077	0.066–0.088
HAL/SVL	0.193	0.175–0.206	0.215	0.204–0.230
F1/SVL	0.104	0.081–0.131	0.109	0.094–0.118
F2/SVL	0.077	0.060–0.112	0.081	0.076–0.085
F3/SVL	0.111	0.091–0.132	0.126	0.113–0.137
F4/SVL	0.074	0.053–0.093	0.084	0.074–0.092
HLL/SVL	1.586	1.400–1.703	1.612	1.531–1.710
FEL/SVL	0.498	0.438–0.569	0.474	0.445–0.492
TIL/SVL	0.504	0.469–0.531	0.498	0.472–0.535
TFL/SVL	0.726	0.625–0.782	0.739	0.701–0.788
FOL/SVL	0.479	0.436–0.539	0.500	0.471–0.538
T1/SVL	0.100	0.075–0.117	0.119	0.109–0.130
T2/SVL	0.179	0.149–0.218	0.188	0.173–0.198
T3/SVL	0.244	0.193–0.280	0.286	0.273–0.305
T4/SVL	0.323	0.277–0.368	0.416	0.397–0.438
T5/SVL	0.228	0.177–0.280	0.267	0.239–0.290
IMT/SVL	0.053	0.043–0.069	0.052	0.048–0.059
HL/HW	0.055	0.961–1.222	1.168	1.118–1.230
S-N/N-E	0.693	0.479–0.909	0.881	0.778–0.974
ED/E-E	2.054	1.333–2.808	2.684	2.407–3.267
TD/ED	0.846	0.600–1.152	0.566	0.511–0.594
N-N/E-E	1.575	1.150–2.192	1.507	1.286–2.000
ELW/E-E	1.875	1.234–2.909	1.603	1.435–2.067
F1/F2	1.375	1.011–1.632	1.351	1.156–1.488
TIL/FEL	1.015	0.871–1.137	1.052	0.995–1.090
FOL/FEL	0.966	0.966–1.169	1.055	1.000–1.093
TIL/FOL	1.054	0.973–1.148	0.997	0.959–1.030

nally considered to be distributed only in the coastal belt of Bangladesh (Hasan et al. 2012). However, *Hoplobatrachus litoralis* was subsequently found frequently in inland areas far from the coastal belt (Mulcahy et al. 2018; Purkayastha and Besak 2018; Bohra et al. 2019; Kundu et al. 2020; Wangyal et al. 2020; Lalremsanga et al. 2022). This shows that this species is also widespread beyond coastal areas. Therefore, we did not directly translate the specific name plus the generic name as the Chinese name, but chose the translation of Bangladesh plus the generic name as the Chinese name of this species.

Recently, Thongproh et al. (2022) described the specimens from north-western Thailand as a new species of *Hoplobatrachus*, namely *Hoplobatrachus salween* Thongproh, Chunskul, Sringurngam, Waiprom, Makchai, Cota, Duengkae, Duangjai, Hasan, Chuaynkern & Chuaynkern, 2022 and they considered the previous record of *H. litoralis* from northern Tanintharyi, Myanmar, also to belong to *H. salween* (Thongproh et al. 2022). However, through our phylogenetic analysis based on 16S rRNA, the specimens from China, the specimens of *H. salween* from Thailand and Myanmar and the specimens of *H. litoralis* from Bangladesh and India formed a monophyletic clade (Fig. 1). Although the average genetic distance between the specimens of *H. salween* and the specimens of *H. litoralis* from Bangladesh reached 2.2%–2.5% in this study, the genetic distance between the specimens of *H. salween* and the specimen of *H. litoralis* from India is only 1.4%–1.8% (Table 2). In addition, the average genetic distance between the specimens of *H. salween* and the specimens from China is only 1.3%–1.6% (Table 2). Therefore, the molecular data do not support *H. salween* and *H. litoralis* being two separate species. Morphologically, according to Thongproh et al. (2022), the snout to anterior eye stripe is indistinct in *H. salween*, whereas distinct in *H. litoralis*, the supralabial stripe is indistinct in *H. salween*, whereas distinct in *H. litoralis*, the dark supratympanic stripe is present in *H. salween*, whereas absent in *H. litoralis*, the hand pattern is mottled in *H. salween*, whereas uniform in *H. litoralis*, the web pattern is mottled with an irregular pattern in *H. salween*, whereas uniform grey in *H. litoralis*, the tubercles on the dorsal tibia of males are densely in *H. salween*, whereas few in *H. litoralis*, sexual dichromatism is present in *H. salween*, whereas absent in *H. litoralis* during the breeding season, the relative finger

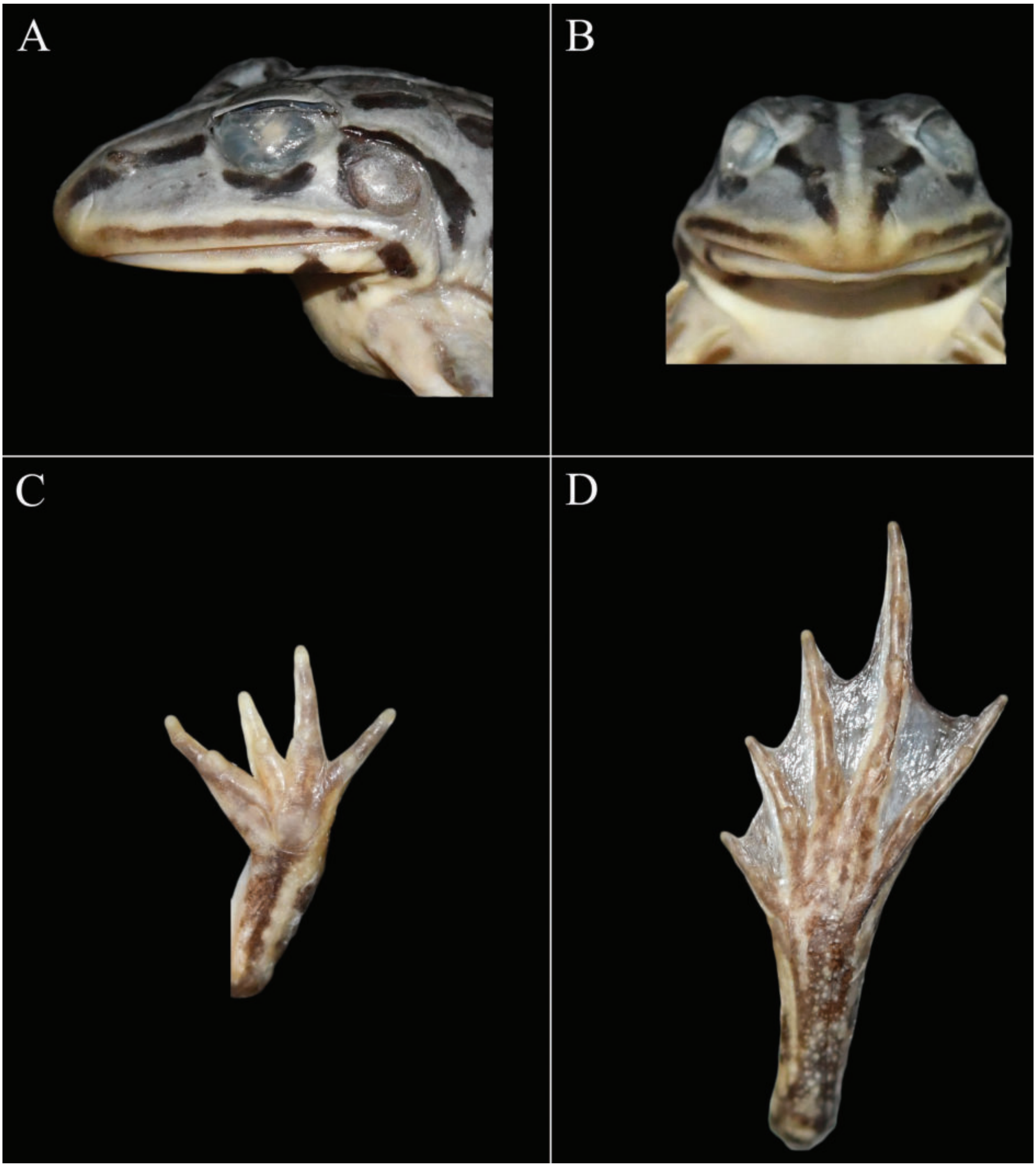

Figure 3. Close-up views of the specimen (KIZ 034010) of *Hoplobatrachus litoralis* from China in preservative. **A.** Lateral view of the head; **B.** Front view of the head; **C.** Volar view of left hand; **D.** Plantar view of left foot.

length is F3 > F1 > F4 > F2 in *H. salween*, whereas F3 > F1 > F2 > F4 in *H. litoralis* and the relative toe length is T4 > T3 > T5 > T2 > T1 in *H. salween*, whereas T4 > T5 > T3 > T2 > T1 in *H. litoralis*. However, the snout to anterior eye stripe is distinct in some individuals of *H. salween* (see fig. 6A in Thongproh et al. (2022)), the supralabial stripe is distinct in some individuals of *H. salween* (see fig. 6B in Thongproh et al. (2022)), the dark supratympanic stripe is present in some individuals of *H. litoralis* (see fig. 1A in Hasan et al. (2012) and fig. 2 in Lalremsanga et al. (2022)) and, in the specimens from China, the hand pattern is not uniform in the specimens from China, the relative finger length is either F3 > F1 > F2 > F4 or F3 > F1 > F4 > F2 in the specimens from China and the relative toe length is either T4 > T5 > T3 > T2 > T1 or T4 > T3 > T5 > T2 > T1 in the specimens from China. In addition, we found that the adult males from western Yunnan in life also present almost completely yellow colouration during the breeding season. We are not sure whether the remaining two differences in the web colouration and the tubercles

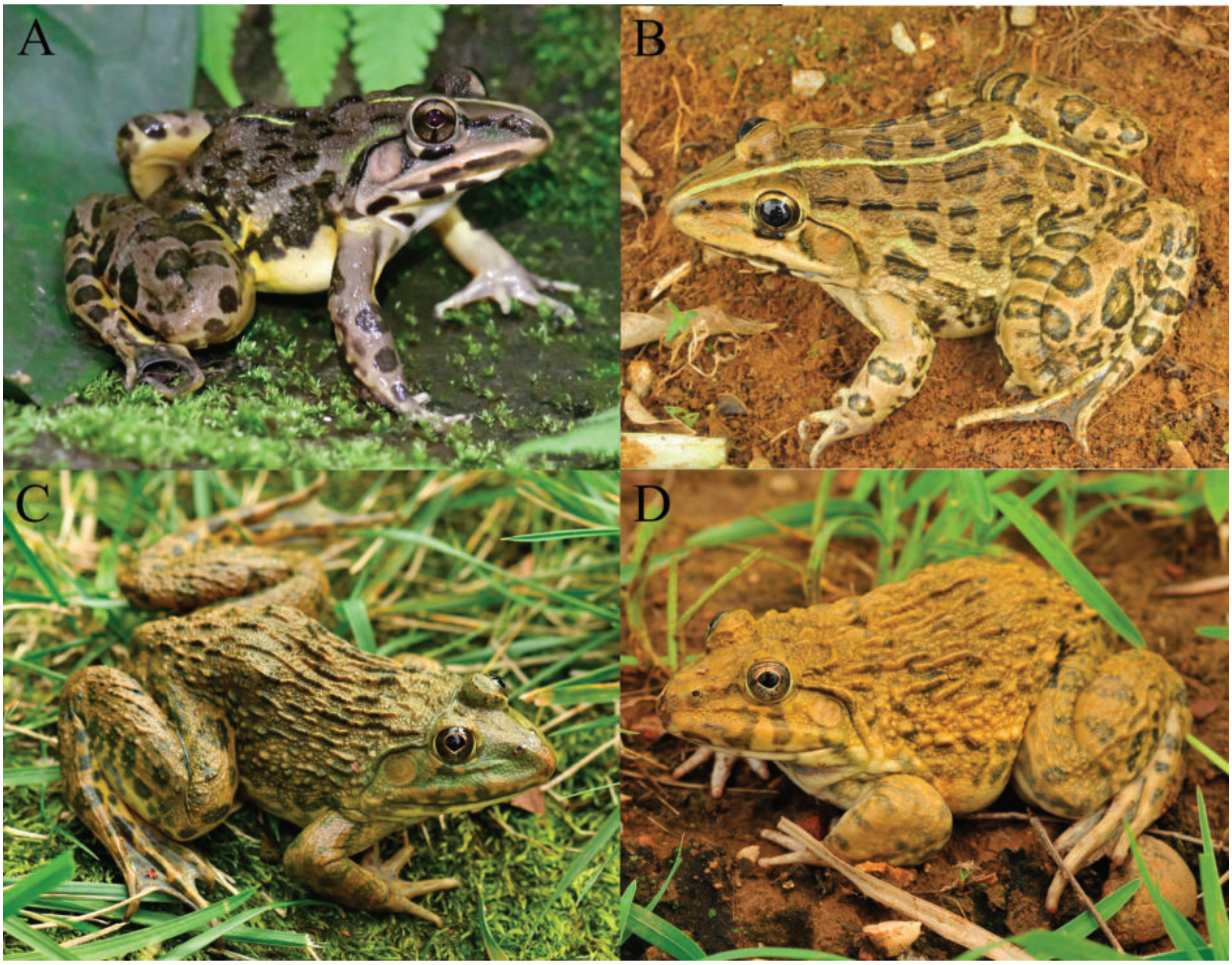

Figure 4. Comparison of *Hoplobatrachus litoralis* and *H. chinensis*. **A**, **B.** *H. litoralis* in life from western Yunnan, China; **C**, **D.** *H. chinensis* in life from southern Yunnan, China.

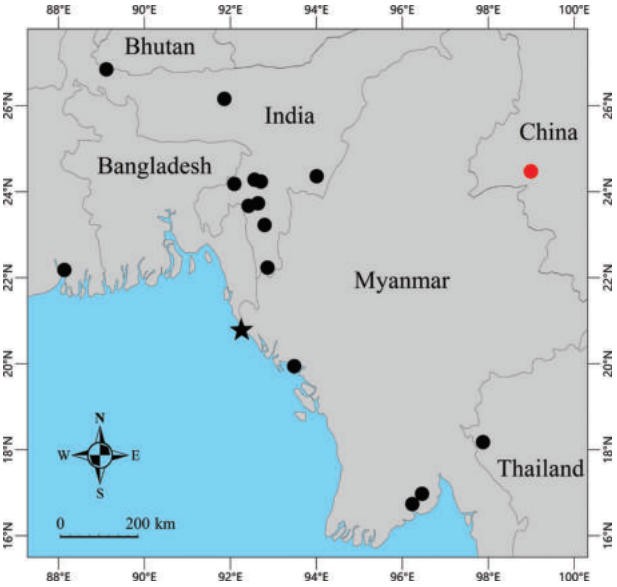

Figure 5. Map showing the currently known distribution of *Hoplobatrachus litoralis*. The black star indicates the type locality, black dots indicate previously published records and the red dot indicates the new record in China.

on the dorsal tibia are stable and these differences seem to be insufficient to distinguish different species, they may be treated as intraspecific variation. To sum up, *H. litoralis* and *H. salween* cannot be completely separated in both morphological and molecular markers and the specimens from China seem to represent the transitional type between *H. litoralis* and *H. salween*. Therefore, we consider that *H. salween* should be a junior synonym of *H. litoralis*.

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A contribution to taxonomy and biology of *Spalerosophis diadema diadema* (Schlegel, 1837) along with a new record of *Spalerosophis atriceps* (Fischer, 1885) from the Poonch District of Jammu and Kashmir, India (Reptilia, Squamata, Colubridae)

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Abstract

The present study is aimed to validate the occurrence of two species of Diadem or Royal snakes of Genus *Spalerosophis* Jan, 1865 from the Poonch District of Jammu and Kashmir, India along with the presentation of eight different colour morphs and diagnostic characteristics. The eight different colour morphs include four colour morphs of adults of *S. d. diadema* (Schlegel, 1837), two colour morphs of adults of *S. atriceps* (Fischer, 1885), one colour morph of subadults of unknown parentage and one colour morph of the adult of *S. diadema* (Schlegel, 1837) represented by a single individual whose identification at subspecies level (ssp. *cliffordii/diadema*) is the subject for further investigations for want of more specimens. This solitary specimen shares many similarities with *S. d. diadema*, but it has less number of sub-caudal scales which point towards the likelihood of *S. d. cliffordii* (Schlegel, 1837). The distribution, activity, habitat and behaviour have also been reported along with the morphological, morphometric and meristic characters. Between two identified species *S. atriceps* is a new report from the Poonch District. Reported specimens are mapped across the study area and are depicted here in the distribution map.

Key Words

Colour morphs, taxonomy, *Spalerosophis atriceps*, *S. d. diadema*, Pir Panjal, Poonch, Western Himalayas

Introduction

Genus *Spalerosophis* Jan, 1865 of the family Colubridae has a very large range of distribution in arid and semi-arid regions from North Africa in the west through Arabia, Iran and Pakistan to central India in the east (Marx 1959; Minton 1966; Mertens 1969; Gasperetti 1988; Whitaker and Captain 2004, 2008, 2015; Sharma 2007; Schätti et al. 2010; Sindaco et al. 2013; Uetz 2015; Yadollahvandmiandoab et al. 2018). The systematics and taxonomy of *S. diadema* (Schlegel, 1837) from the Euphrates and the Caspian Sea to the Indian subcontinent need clarification (Schätti et al. 2010). The probable reasons behind the confusion in the identification of *Spalerosophis* spp. are: the different counting of scales of the head, printing lapses and different descriptions of supranumeral scales in the pileus region, particularly the prefrontals (Dumeril et al. 1854; Gunther 1864; Zugmayer

1905; Schmidt 1930; Marx 1959; Minton 1966; Baig and Masroor 2008; Schätti et al. 2010). In present study, labelled sketches of scales of the pileus region (Fig. 1) and different arrangements of prefrontals (Fig. 2) have been given to avoid confusion and misinterpretation.

Minton (1966) reported that the Indo-Pakistan segment is represented by *Spalerosophis arenarius* (Boulenger, 1890), *S. diadema diadema* (Schlegel, 1837), *S. diadema schirazianus* (Jan, 1863) and *S. atriceps* (Fischer, 1885). *Spalerosophis atriceps* was considered a species of its own by Minton (1966), Baig and Masroor (2008), Schätti et al. (2009, 2010), Whitaker and Captain (2015) and colour morph of adult *S. diadema* by Marx (1959), Mertens (1969) and Khan (2006). In the present study, we too have considered *S. atriceps* as a valid species. Within the Indo-Pak region, *S. d. schirazianus* was shown distributed along Iran, Afghanistan and western India, *S. arenarius* from Gujrat and only *S. d. diadema* was shown with a little wider range of distribution from the central, north and north-west part of India (Marx 1959). Baig and Masroor (2008) considered *S. schirazianus* as junior synonyms of *S. diadema.* Following Marx (1959), Schätti et al. (2009) reported *S. diadema* as a polytypic species represented by *S. d. diadema* and *S. d. cliffordii*, the latter from Iran. Marx (1959) separated the *S. d. diadema* and *S. d. cliffordii* based on the number of subcaudals, i.e. 80 or more in *S. d. diadema* versus less than 80 in *S. d. cliffordii*. In our study, we followed the determination key given by Schätti et al. (2009) for the genus *Spalerosophis* Jan, 1865. Working on the herpetofauna of Jammu and Kashmir, Sahi and Duda (1985) have shown the presence of *S. d. diadema* from districts Kathua, Jammu, Dumel, Udhampur, Reasi, Poonch and Ramban; *S. atriceps* from districts Kathua, Udhampur and Kishtawar and *S. arenarius* from districts Jammu and Bhaderwah. In the literature, we have not found any mention of *S. d. cliffordii* from India. It is, for this reason, we kept a specimen representing a subcaudal count of less than 80 (resembling *S. d. cliffordii*) for further investigations. Schätti et al. (2009) reported that the occurrence of *S. diadema* from north-west India (Kashmir) requires serious investigations. The lack of knowledge of the reptilian fauna of Jammu and Kashmir in general and the current study area (i.e. Pooch District which is located in the Pir Panjal range of Western Himalayas) in particular is supposed to be mainly caused by the remoteness of the area and by the area's instability since the 1990s owing to its location near the border of Pakistan. The presence of *S. diadema* in India was put in question for investigations and confirmation by Schätti et al. (2010) due to the absence of its mapping by Baig and Masroor (2008) and due to reporting based on subadult specimens. In the current study, the occurrence of *Spalerosophis* spp. from different localities of the study area has been mapped (Fig. 3) besides reporting on adult individuals along with their diagnostic characteristics (Table 1). In addition, we are presenting a new report on the occurrence of *S. atriceps* along with revalidation of the occurrence of *S. d. diadema* from Poonch District of Jammu and Kashmir, India. *Spalerosophis* spp. are represented by different colour morphs both within the species and between its different species which has led to great confusion in identification (Baig and Masroor 2008). Here, we are reporting eight colour morphs of the genus *Spalerosophis* Jan, 1865 spread across three species under discussion. These findings will confirm the distribution of these species from north India, in general and Poonch District of Jammu and Kashmir, in particular, besides supplementing the diagnostic characteristics for identification.

Materials and acronyms

On sighting the specimens in the field, the activity of the snake, time, date, climate, coordinates, photographs and videos have been taken. The threatening behaviour of *S. d. diadema* has been recorded in captivity. Specimens which were found dead on the field were preserved in the Mendhar College Museum of Zoology (MCMZ) for reference. Different localities of Poonch District from where 56 specimens belonging to genus *Spalerosophis* have been reported during the years 2019–2021 (Fig. 3) exhibit 8 different colour morphs (Figs 4–7). The meristic and morphometric characters of 12 collection-vouchered non-types, including two photo vouchers and ten vouchered specimens are shown in Table 1. Out of 12 preserved specimens, MCMZ0619 is a subadult of unknown parentage (*diadema/atriceps*, Fig. 5A), eight specimens belong to *S. d. diadema* (Figs 5B, C, 6A, B), one male specimen (MCMZ0119) of *S. diadema* remains unidentified at the level of subspecies viz. *cliffordii* or *diadema* (Fig. 6C) and two specimens belong to *S. atriceps* (includes one male, MCMZ0920 and one female, MCMZ1020) (Fig. 7).

Terminology used for describing meristic and morphometric characters (Figs 1, 2, Table 1)

To avoid terminological confusion while interpreting the number of head scales, we give labelled head sketches in Figs 1, 2. While counting the number of scales even a small granule has also been taken into account. 'Anterior scale rows -asr' denote anterior dorsal scale rows counted one head-length behind the head excluding the ventrals. 'Mid-body scale rows -msr' are the dorsal scale rows counted at the level of the mid-ventral scale, excluding the latter. 'Posterior scale rows -psr' refers to the dorsal scale rows one head length anterior to the anal plate, excluding the ventrals. 'Anterior temporal -at' is a vertical row of scales immediately behind the postocular touching below the supralabials and above the parietals. 'Circumocular -co' is a ring of scales in contact with the eye (i.e. 'preocular-pro' + 'subocular-so'+ 'postocular-po' + 'supraocular-spo'). 'Dorsal blotches - db' are mid-dorsal large dark spots running behind the head down the tail. 'Frontal-fr'

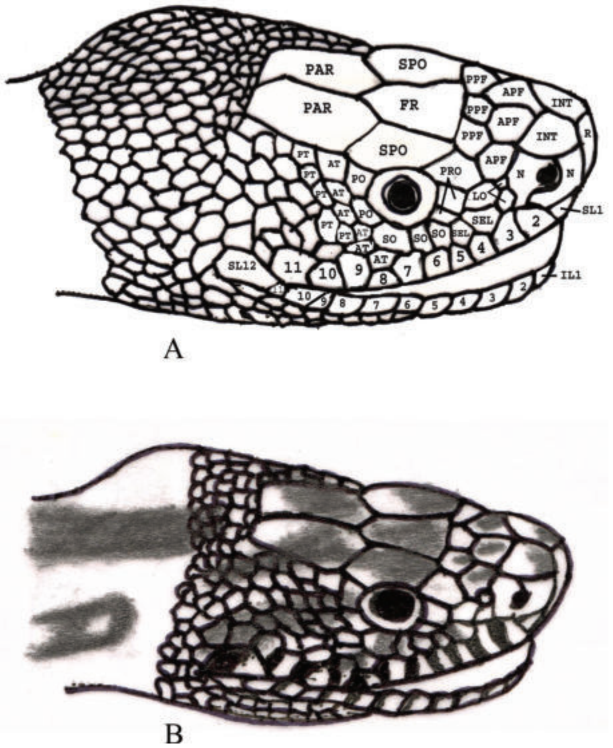

Figure 1. A, B. Dorsolateral sketch of head of *Spalerosophis diadema diadema* (MCMZ0314). **A.** The characteristics head scales pattern and nomenclatures used for *Spalerosophis* spp. under report; **B.** The characteristic black markings on supralabials scales found in *Spalerosophis* spp. under report.

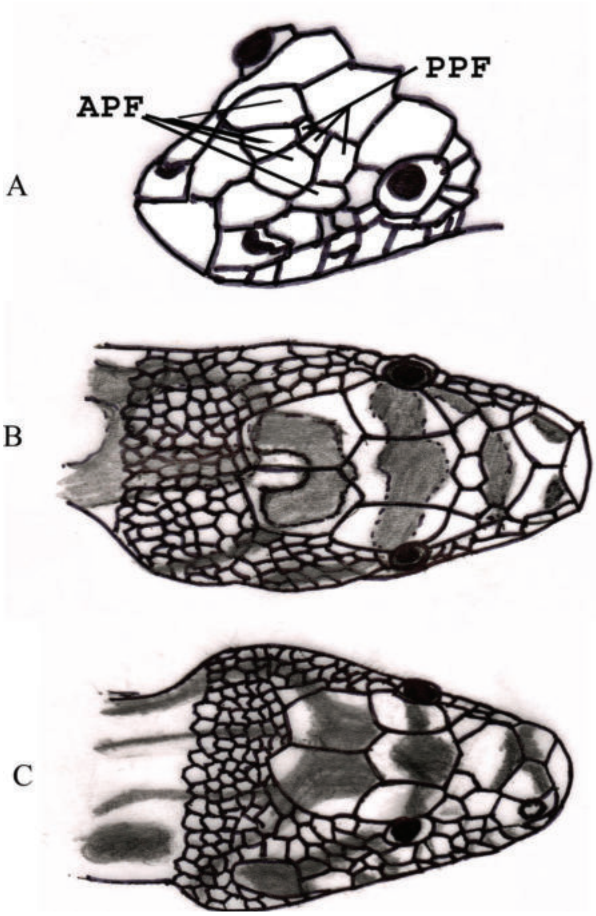

Figure 2. A–C. Showing the variations in the arrangement of the head scale of *Spalerosophis* spp (*diadema diadema/ atriceps*) with a special focus on prefrontals. **A.** MCMZ0119; **B.** MCMZ0819; **C.** MCMZ0514.

is the large scale present on the dorsal side of the head, between the eyes and adjacent to the supraocular. 'Infra labials -il' and 'supralabials -sl' are scales of the lower lip and upper lip, respectively. 'Internasals-int' are the scales along the dorsal side of the snout connecting the nasals on both sides of the head. 'Loreals -lo' are the scales situated on or above a straight line parallel to the mouth from the lower posterior tip of the nasal to the circumocular ring and below the prefrontals. 'Prefrontals -pf' are the scales on the dorsal side of the head, often arranged in two rows, between 'internasal-int' at their anterior and 'frontal-fr' at their posterior, bordering laterally with loreal and preocular. 'Anterior prefrontals-apf' is the horizontal row of prefrontal scales touching internasal anteriorly, while 'posterior prefrontals- ppf' is the horizontal row of prefrontal scales touching frontal and supraocular posteriorly. 'Secondary labials - sel' are the scales below the loreals, anterior to scales of the circumocular ring and in contact with the supralabials. 'Rostral-r' is the single scale present at the tip of the snout. 'Ventrals - vent' are the scales counted from the first transverse scale on the ventral side of the head just posterior to the gular up to the anal plate. 'Anal - an' is the last ventral scale covering the anal opening. 'Sub-caudal -scd' are the scales on the ventral side of the tail. 'Temporals' are the scale rows present on the sides of the head immediately behind the postocular, between the parietal above and supralabials below. A vertical row of temporals present adjacent to the postocular is 'anterior temporal- at' row and a vertical row of temporals present posterior and adjacent to anterior temporals is 'posterior temporal- pt'. 'Parietal- par' are the two large scales present on the head connected to the posterior margin of the supraocular and frontals. If the left and right counts are different, they are separated by a slash.

Results

The *Spalerosophis* spp. in the study area are represented by a moderately large population. A total of 56 individuals have been reported from different locations of study area during the years 2019–21, out of which three individuals represent *S. atriceps*, 47 individuals represent *S. d. diadema*, five individuals represent subadults of unknown parentage (*S. d. diadema/S. atriceps*) and one specimen represent *S. diadema* whose identification at subspecies level is subjected for further investigations. Observed specimens have shown distinctive colourations and markings on the body. Common characteristics features shown by all three species under report include: (1) an elongated and oval head well-demarcated from the neck; (2) long and moderately obtuse snout; (3) eye with a round pupil and golden iris;

(4) rostral broader than high; (5) orbit surrounded from all sides by a ring of ocular scales (Figs 1A, B); (6) prefrontals and loreals broken into small scales (Figs 1A, B, 2A–C); (7) a high number of temporal scales (4–6 in the first row) (Fig. 1A, B); (8) one undivided sub-pentagonal frontal; (9) parietals two, not in contact with postoculars; (10) divided nasals; (11) two inter-nasals; (12) posterior dorsal scale rows 19–21; (13) each supralabials scale with a vertical dark marking /band on the posterior margin (Figs 1B, 4A–J); (14) white underside of the head and chin (Figs 5–7 ventral); (15) an entire anal plate and (16) round tail which is shorter in males as compared to females.

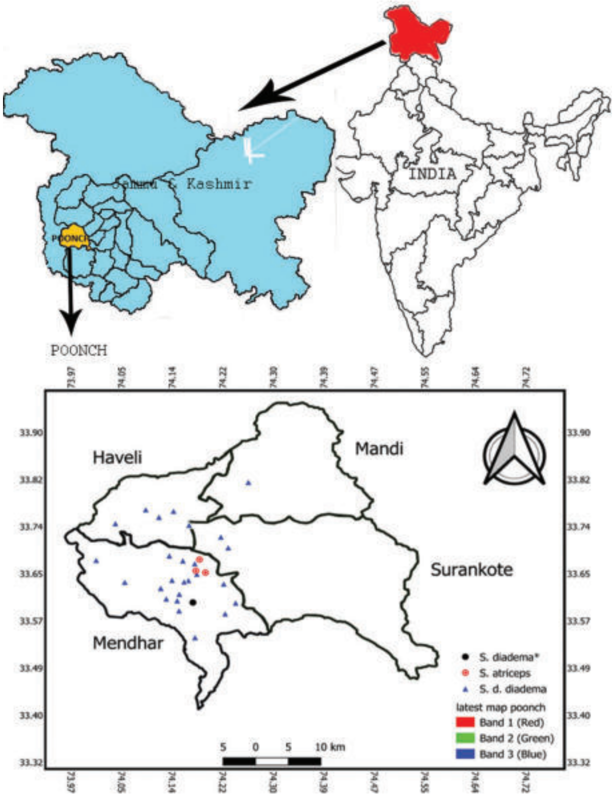

Figure 3. Map showing the distribution of *Spalerosophis* spp. in the study area (Poonch District). * Identification at the subspecies level (*cliffordii/ diadema*) is yet to be ascertained.

Morphology (Figs 4–7)

Body colour, markings and blotches. The genus *Spalerosophis* shows a great variant of inter- and intra-specific colouration, markings and blotches.

Colours and markings of subadults of unknown parentage are more or less similar with that of adults of *S. d. diadema* but not with the adults of *S. atriceps*. Out of 56 individuals observed in the present study, five were subadults with a grey background colour of the dorsal body (Fig. 5A). A total of 47 adults of *S. d. diadema* have a background colour of the dorsal body from dark brown (Fig. 5B, C) to ruddy brown (Fig. 6A, B dorsals) with five rows of large darkish spots (blotches) quincuncially arranged passing down the back from the nape well on to the tail. Spots on the median row are large, rounded or rhomboid in outline alternating with the two rows of smaller spots on each lateral side (Figs 5, 6). *Spalerosophis diadema cliffordii/ diadema* (MCMZ 0119) also has ruddy brown body colour with a similar pattern of spots as depicted by *S. d. diadema*, except that the spots are restricted to a group of 4–6 scales and it seems that the spots are being created by grouping of dark scales (Fig. 6C).

Table 1. Morphometric and meristic characters of *Spalerosophis diadema diadema*, *S. d. cliffordii/diadema* and *S. atriceps*. Abbreviations: **asr** - anterior scale rows, **alt** - altitude, **at** - anterior temporal, **br** - broken, **co** - circumocular (**pro** - preocular + **so** - subocular + **po** – postocular + **spo** -supraocular), **db** -dorsal blotches, **ds** - dorsal scales, **f** – female, **il** - infralabials, **k -** keeled, **lo** - loreal (when 2 - one behind the other, when 3- two anterior and one posterior, when 4- two anterior and two posterior), **m** – male, **MCMZ** - Mendhar College Museum of Zoology (unique specimen identifier), **msr** - midbody scale rows, **pv** – photo voucher, **psr** - posterior scale rows, **pf** - prefrontals (**apf** - anterior prefrontal + **ppf** - posterior prefrontals), **sa** – subadult, **scd** -subcaudal, **sel** - secondary labials, **sl** - supralabials, **SVL** - snout-vent length, **TL** - tail length, **vent** - ventrals, **wk** - weakly keeled. If the left and right counts are different, they are separated by a slash.

							Meristic characters								Morphometric characters	
MCMZ	sex	asr	msr	psr	ds	vent	scd	pf	lo	co	sel	at	sl	il	db	SVL (mm)	TL (mm)
0619 *diadema/ atriceps*	sa	27	29	19	wk	248	106	7 (4+3)	2	8 (2+3+2 +1)	1	4/5	10/12	12	64	309	87
0719 *diadema diadema*	f	27	29	21	wk	244	106	8 (4+4)	2	9 (3+3+2 +1)	2	5	11/12	14	63	640	200
0819 *diadema diadema*	f	25	27	19	wk	249	96	7 (3+4)	2	9 (2+4+ 2 +1)	1	5	11	12/13	88	860	241
0514 *diadema diadema*	f	br	29	19	wk	241	102	5 (3+2)	2	9/8 (3+3+2+1/ 2+3+2+1)	2	4	11	13/14	54[†]	1130	340
PV0220 *diadema diadema*	m	26	29	19	wk	246	86	8 (4+4)	2	9 (2+3+3 +1)	2	5	11	12	60[†]	1280	230
0314 *diadema diadema*	f	25	29	19	wk	242	110	7 (4+3)	2/3	9/10 (3+3+2+1/ 3+3+3+1)	2	6	11/12	12	61	1203	384
0219 *diadema diadema*	m	26	29	19	wk	248	52 br	8 (4+4)	2	8 (2+3+2 +1)	1	4/5	11	13	62	1545	210
0413 *diadema diadema*	f	29	29	19	wk	234	111	7 (3+4)	3	9 (3+3+2 +1)	1	4	11	13	60	1340	450
PV0120 *diadema diadema*	m	26	29	19	wk	254	82	7 (4+3)	2	8 (2+3+2 +1)	2	5	11	12	64	1440	325
0119 *diadema cliffordii/ diadema*	m	26	29	19	wk	240	78	7 (4+3)	2	8 (2+3+2 +1)	1	4	11	11/12	85	1415	321
0920 *atriceps*	m	27	29	19	k	238	105	8 (4+4)	2	7/8 (2+2+2+1/ 2+3+2+1)	1/2	4/5	11	13	57[‡]	1110	285
1020 *atriceps*	f	28	31	21	k	250	109	7 (4+3)	3/4	9/11 (3+3+2+1/ 3+3+4+1)	2/2	4/6	12/13	13	nil	1230	333

† faded on the tail, not seen clearly; ‡ dull shades of blotches only.

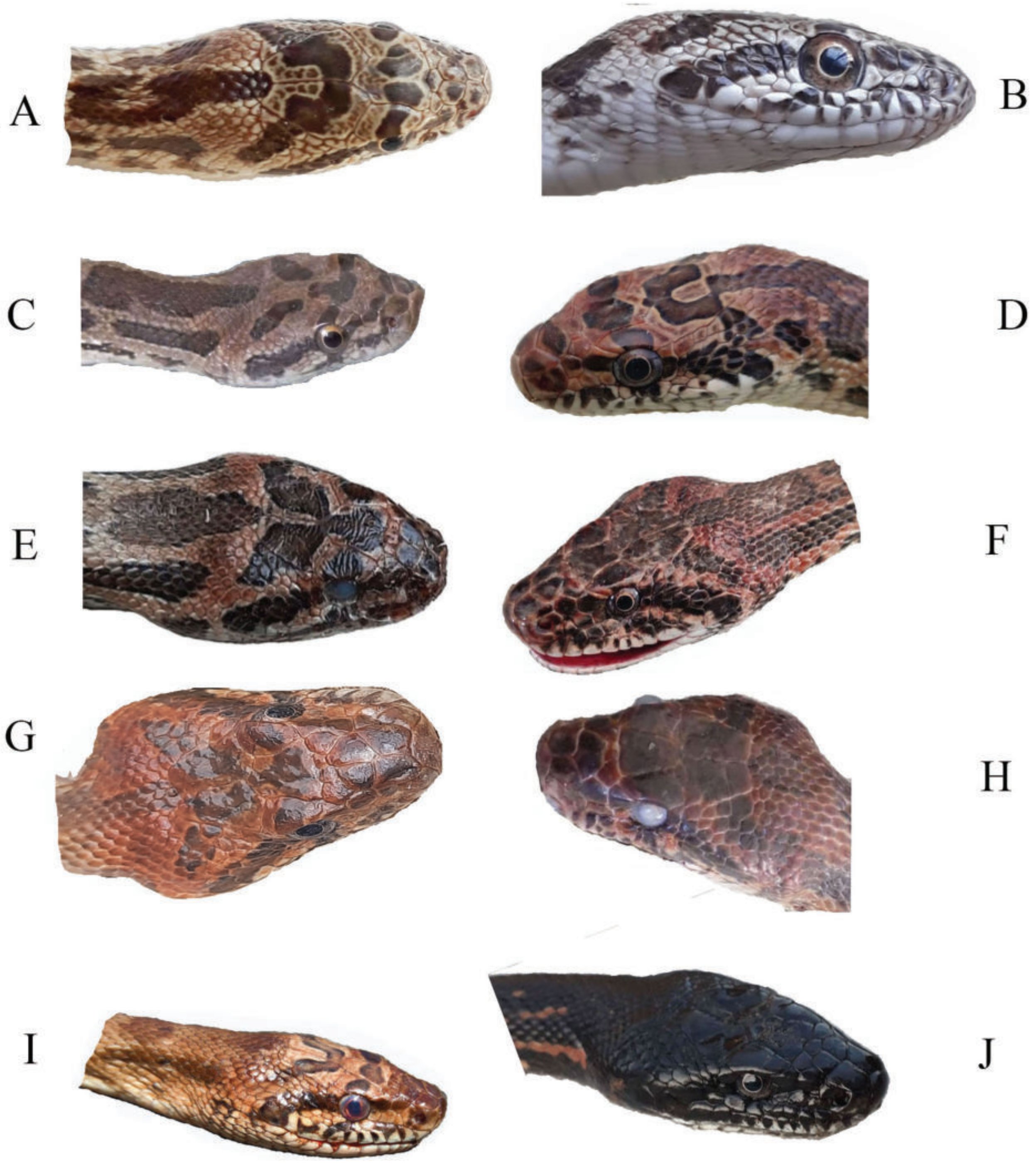

Figure 4. **A-J.** Colouration and dark markings of heads of *Spalerosophis* spp: subadult (**A, B.** MCMZ0619), *S. d. diadema* (**C.** MCMZ0719; **D.** MCMZ0819; **E.** Photo Voucher; **F.** MCMZ0219; **G.** Photo Voucher), *S. d. cliffordii/ diadema* (**H.** MCMZ0119) and *S. atriceps* (**I.** MCMZ0920; **J.** MCMZ1020).

Only three specimens of *S. atriceps* were encountered during the study period. One was alive and two were found killed. The live one was showing an exactly similar colour pattern as that of MCMZ1020 (Fig. 7B). The pattern of marking is quite variable in differently-aged individuals of *S. atriceps.* In the smaller-sized individual, the body is straw yellow with irregular black flecks restricted to few scales and faded dorsal blotches as if the snake had been spattered with tar, the head giving a pink hue and mottled with black marking similar to forma typica (Fig. 7A). These spots of *S. atriceps* differ from *S. diadema cliffordii/diadema* (Fig. 6C) in the manner that they are not present in rows and do not show any uniform pattern. A more grown individual of *S. atriceps* has a highly melanistic dark bluish to black body with few ruddy brown scales (Fig. 7B).

In *S. d. diadema*, the belly is whitish in all the half-grown specimens (Fig. 5A, B ventrals), but, in grown-up individuals, it is suffused with pink, especially in the middle line (Figs 5C, 6A ventrals) or there are frequently greyish spots or mottling at the lateral edges of the ventrals (Fig. 6B ventral). The belly of *S. d. cliffordii/diadema* (Fig. 6C ventral) resembles that of *S. d. diadema* (Fig. 6B ventral), whereas, *S. atriceps* has a ruddy white or uniform rosy pink belly in younger (Fig. 7A ventral) and light black in fully grown individuals (Fig. 7B ventral).

The number of blotches vary a great degree from individual to individual. In the present study, the number of blotches remain countable (54–88) in all sizes of *S. d. diadema* and *S. d. cliffordii/diadema*, but remain observable only in the younger and moderately melanistic form of *S. atriceps* (Tables 1, 2). With little deviation from the two lateral rows of spots on each side of the body of adult forms, younger individuals have three rows of lateral spots on each side of the fore-body which are reduced to one or two rows on the tail (Fig. 5A).

Head markings. The head is light brown or copper colour or ruddy brown, variously spotted or mottled with dark spots in almost all the sizes of *S. d. diadema* (Figs 1B, 4 A-J) and *S. d. cliffordii/diadema* (Fig. 4H). The dark markings of the head are well distinct in younger forms, but become less distinct as age advances and this is true for both the species viz. *S. d. diadema* and *S. atriceps*. Markings of the head are often broken up, but the most constant is a band between the eyes, an oblique strip from behind the eye to the angle of the mouth and a quoit-like mark on the parietals (the diadem mark). The diadem mark is often connected with the band between the eyes by a median stripe (Fig. 4E–H) or remains quite detached (Fig. 4A–D, I) or throws back one to three short stripes posteriorly (Fig. 4A, D, F–I). Many departures from this arrangement may be seen either towards a confluence or a disintegration of these marks and, in many specimens, the interorbital and diadem marks are barely suggested (Fig. 4F, G, J). Smaller-sized individuals of *S. atriceps* have a light scarlet colour on the head and neck (Fig. 4I). Fully grown *S. atriceps* has a completely black coloured head (Fig. 4J). A very constant feature observed in all specimens of both the species is the presence of one light and one dark vertical band on each supralabial scale (Fig. 1B). This feature is so common that it is even retained by the intensely melanistic form of *S. atriceps* (Fig. 7B) where all the rest of the markings of the head are not visible.

Variation in mid-dorsal body blotches. In younger forms of *S. d. diadema*, the vertebral line of spots seems broken down into three spots: a median rhomboid large dark spot with a lateral slightly narrow band on each side. On the tail, a single narrow long mid-dorsal dark streak is present.

In older forms, the three small spots of vertebral line may become completely fused to form a single large rhomboidal dark spot (Figs 5B, 6A, C) or the median rhomboid remains distinct, but connected at its middle with two lateral smaller bands (Fig. 6B). In addition to this, the dorsal spots may fade away and may look quite dull, rendering them almost invisible (Fig. 5C). Similar to the pattern of younger individuals, the mid-dorsal spots on the tail of adult individuals may sometime fuse to form a single dark line at mid-dorsal position (Fig. 6B).

Table 2. The altitudinal range of occurrence along with a range of characteristics of *Spalerosophis diadema diadema*, *S. diadema cliffordii/diadema* and *S. atriceps*. Number in parenthesis are the number of specimens; means are in brackets; M=Male; F=Female. Different left and right counts are separated by a slash.

S.No	Characters	*S. d. diadema*	*S. diadema cliffordii/ diadema*	*S. atriceps*
1	Altitudes in metres	780–1920	990	1120–1630
2	Number of Individuals for meristic characters	8	1	2
3	Anterior Dorsal Scale Rows	25–29 (7)	26	27–28
4	Midbody Dorsal Scale Rows	27–29	29	29–31
5	Posterior Dorsal Scale Rows	19–21	19	19–21
6	Ventrals	234–249 F (5) 246–254 M (3)	240 M (1)	250F (1) 238M (1)
7	Subcaudals	96–111F (5) 82–86M (2)	78 M (1)	109 F (1) 105 M (1)
8	Prefrontals	5–8	7	7–8
9	Loreals	2–3	2	2–4
10	Circumocular	8–10	8	7–11
11	Secondary Labials	1–2	1	1–2
12	Anterior Temporals	4–6	4	4–6
13	Supralabials	10–12	11	11–13
14	Infralabials	11–14	11/12	13
15	Dorsal Blotches	54–88	85	57-Nil
16	Total length	840–1790 F (5)[1357.6] 1510–1765 M (3)[1752]	1736 M (1)	1563 F (1) 1395 M (1)
17	Tail Body Ratio	0.31 F 0.17 M	0.22 M	0.27 F 0.25 M

Variation in lateral body blotches. Lateral spots of the body in *S. d. diadema* form a complete dark blotch in the majority of the cases (Figs 5A, B, 6A, B), while sometimes, the lateral blotches may become faded from the inside, thus forming a dark ring on their margin (Fig. 5C). These lateral blotches are seen restricted to few scales only in *S. d. cliffordii/diadema* (Fig. 6C).

Morphometric and meristic characters

Scalation patterns and body sizes of *S. d. diadema*, *S. atriceps* and *S. d. cliffordii/diadema* are given in Tables 1, 2.

Loreals (Fig. 1). *Spalerosophis diadema diadema* and *S. d. cliffordii/diadema* have the same range of loreal count (i.e. 2–3), whereas *S. atriceps* differs in having a loreal scale range of 2–4. As far as the arrangement of loreals is concerned, when there are two, then they are positioned anterio-posteriorly in a single row; when three, they are partially arranged in two rows i.e. two are present anteriorly, positioned one above the other and one posteriorly and, when 4, two are present anteriorly and two posteriorly.

Prefrontals (Figs 1, 2). Scales in the prefrontal, range between 5–8 in *S. d. diadema* and *S. d. cliffordii/diadema* and 7–8 in *S. atriceps*. The lowest number of prefrontals are presented by MCMZ514 and the highest by MCMZ0719, MCMZ0219 and MCMZ0920. They are always found ar-

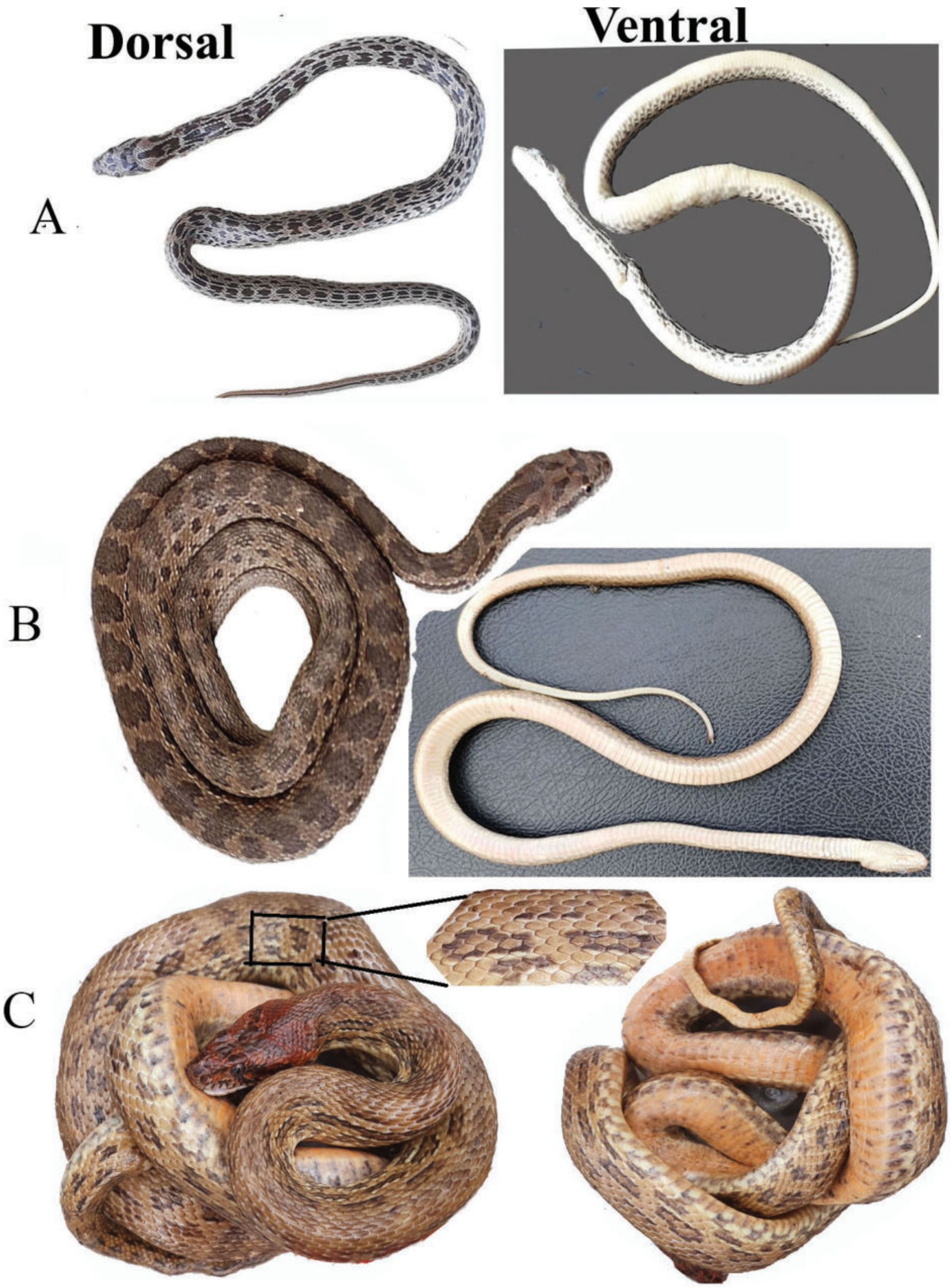

Figure 5. **A–C.** Dorsal and ventral view of *Spalerosophis* spp. **A.** Subadult (MCMZ0619) and **B.** Adult of *S. diadema diadema* (MCMZ0719). Both have uniformly white ventrals **C.** Adult *S. diadema diadema*. Photo Voucher showing fading of dark spots on the body.

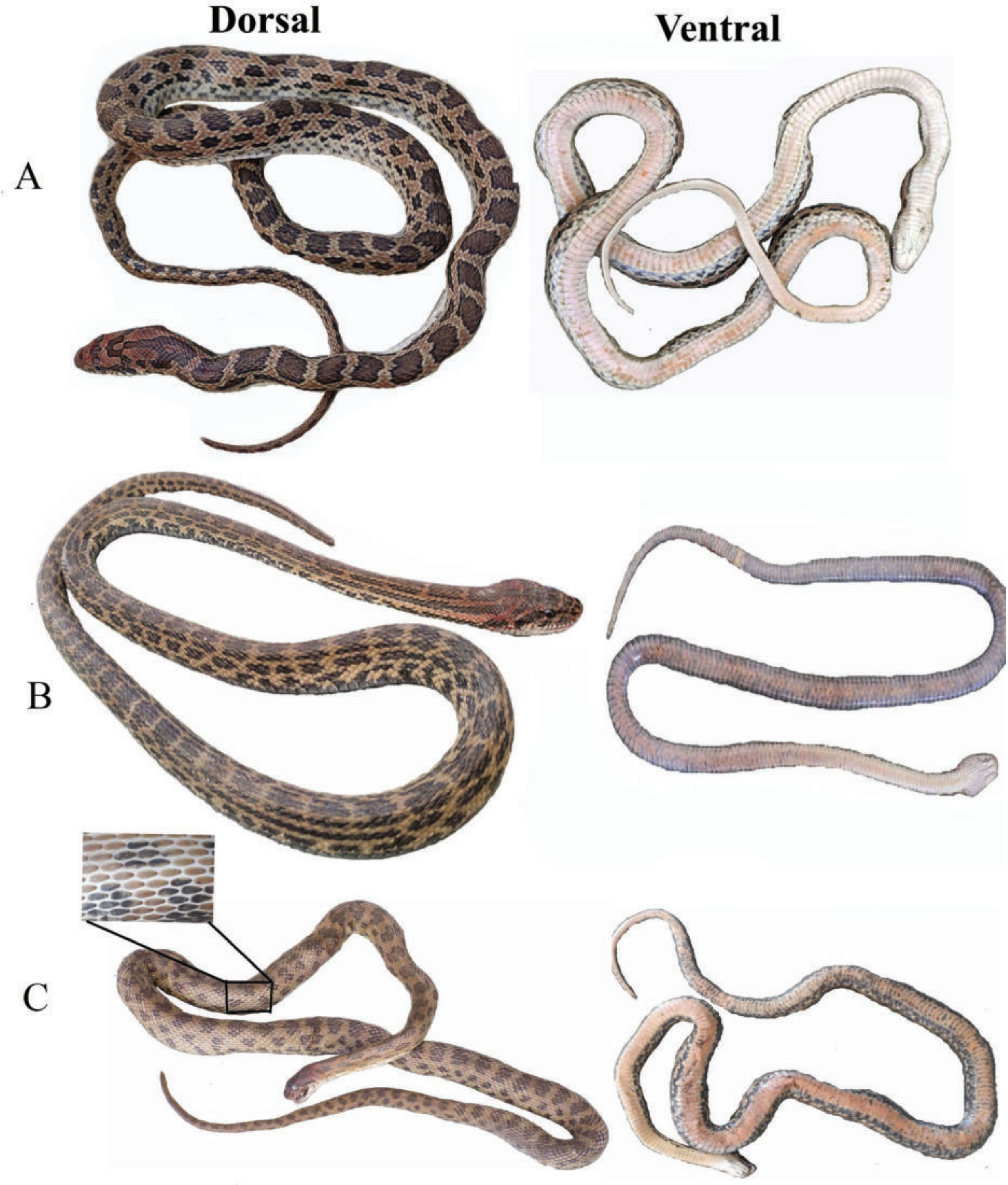

Figure 6. A-C. Dorsal and ventral view of adults of *Spalerosophis diadema diadema.* (**A.** MCMZ0819; **B.** MCMZ0219) and *Spalerosophis diadema cliffordii/ diadema* (**C.** MCMZ0119). Ventral scales of all are mottled with dark markings.

ranged in two rows, the anterior prefrontals and posterior prefrontals (Fig. 1A). Scales of both the rows show a variable size, shape and configuration. As shown in Table 1, the number of scales in the anterior prefrontal and posterior prefrontal rows may be 3–4 and 2–4, respectively. Scales of two rows may be restricted in their respective rows as depicted in specimen MCMZ0314 (Fig. 1) or one scale of the anterior prefrontal row may be so large that it touches the frontal scale directly as in MCMZ0119 and MCMZ0514 (Fig. 2A, C) or simply wide enough to restrict the number of anterior prefrontals to two as in MCMZ0819 (Fig. 2B).

Circumocular. The number of scales in the circumocular ring has a slightly lower range in *S. d. diadema* and *S. d. cliffordii/diadema* i.e. 8–10 scales than that of *S. atriceps* where this range is 7–11. This count includes 2–3 preocular, 2–4 postocular, 3–4 subocular and one large supraocular. The subocular scales completely separate the supralabials from the eye.

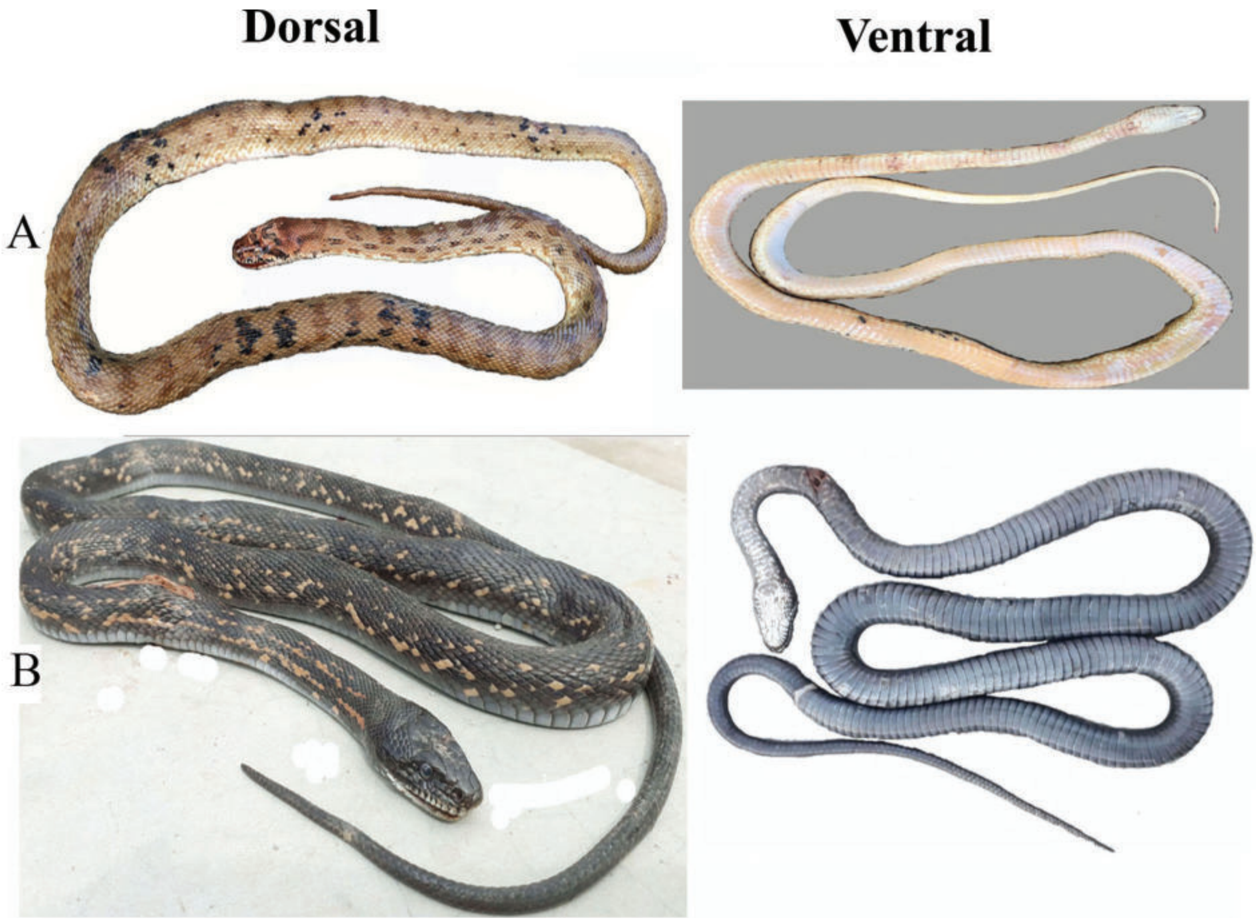

Figure 7. **A, B.** Dorsal and ventral view of *Spalerosophis atriceps.* **A.** Moderately melanistic, MCMZ0920 (Male); **B.** Intensely melanistic, MCMZ1020 (Female).

Labials. The count of supralabials and infralabials in *S. d. diadema* and *S. d. cliffordii/diadema* is in the range of 10–12 and 11–14, respectively. In *S. atriceps*, the supralabials count ranges between 11–13, whereas the infralabials count remains 13 for both individuals. The number of secondary labials ranges between 1 and 2 in *S. d. diadema* and *S. d. cliffordii/diadema* and between 0 and 2 in *S. atriceps*.

Temporals. Temporals range between 4 and 6 in all individuals.

Ventrals. Ventrals in males of *S. d. diadema* range between 246 and 254 and in females 234 and 249. The only male specimen of *S. d. cliffordii/diadema* reported in present study has 240 ventrals which are within the range of *S. d. diadema*. In the case of *S. atriceps*, ventral count is 238 in male and 250 in female. Anal is entire in all species.

Subcaudals. The number of subcaudals ranges between 96 and111 and 82 and 86 in females and males of *S. d. diadema*, respectively. The only male specimen of *S. d. cliffordii/diadema* reported in present study has 78 subcaudals which is lower than the lowest number of subcaudals count of *S. d. diadema*. In the *S. atriceps*, the subcaudal count of the female is within the range of *S. d. diadema* i.e. 109, whereas the male has a much higher number of subcaudals i.e. 105 as compared to the subcaudals count of males of *S. d. diadema*.

Dorsal scale rows. Dorsal scales are weakly keeled in *S. d. diadema* and *S. d. cliffordii/diadema*. In both of them, the count of rows of dorsal scales at the anterior (one head length behind the head), mid-body (at mid-ventral scale) and posterior (one head length ahead of anal) ranges between 25 and 29, 27 and 29 and 19 and 21, respectively. On the other hand, *S. atriceps* have keeled dorsal scales. The count of anterior and posterior dorsal scale rows of *S. atriceps* ranges between 27 and 28 and 19 and 21, respectively which is very much in the range of anterior and posterior dorsal scale rows of *S. d. diadema*, but the number of mid-body dorsal scales rows is again on the higher side i.e. 29–31 when compared with *S. d. diadema*.

Size. Males are smaller in size in both species. The maximum total length as reported in the present study is 1790 mm (tail 450 mm) for females and 1765 mm (tail 325 mm) for males of *S. d. diadema*. The total body length of the single male specimen of *S. d. cliffordii/diadema* is 1736 mm which lies within the highest range of size of the male of *S. d. diadema*. Though only one individual each of male and female has been reported for *S. atriceps*, the size of the male is again on the lower side (total length 1395 mm, tail length 285 mm) compared with that of the female (total length 1563 mm, tail length 333 mm). The tail/body-length ratio is 0.31 for females and 0.17 for males of *S. diadema diadema* and 0.27 and 0.25 for females and males of *S. atriceps*, respectively. The tail/body-length ratio of *S. diadema cliffordii/diadema* is 0.22.

Distribution (Fig. 3). Both the species of *Spalerosophis* viz. *S. d. diadema* and *S. atriceps* have shown sympatric distribution in the study area. *Spalerosophis diadema diadema* has shown a wider range of distribution as it has been reported from both temperate (tehsils (administrative regions) Surankote and Mandi) and sub-tropical regions (tehsils Haveli and Mendhar) of the study area within an altitudinal range of 780–1920 m, whereas *S. atriceps* has been reported from sub-tropical region (tehsil Mendhar) only within the altitudinal range of 1121–1633 m. The temperate zone of the study area has recorded less number of individuals of *S. d. diadema* as compared to the sub-tropical regions. Only a single specimen of *S. d. cliffordii/diadema* has been reported from a quite low altitude of tehsil Mendhar i.e. 990 m.

Habitat. *Spalerosophis diadema diadema* has been found in the crop field, crevices, bare area rocks, grass fields, mosaic vegetation and inside more often on the roof of a kaccha house (made of wood and soil) and walls. *Spalerosophis atriceps* has been reported from crop fields and bare rocky area. *Spalerosophis diadema cliffordii/diadema* has also been reported from a house. Vegetation of the areas under the report includes dispersed shrubs, annual grasses, maize and wheat crop fields and woody trees.

Activity and behaviour. The activity period of *Spalerosophis* spp. ranges between spring and autumn (May-October). Inside houses, *S. d. diadema* is found more active during the night-time. On two occasions, it was found coiled around a common house rat as if the latter were being killed by constriction. On being cornered, it suddenly raised its fore-body and glided sidewise in search of the escape route. On capturing, it expanded and contracted its body, produced a hissing sound and struck quickly. Younger ones are more active than adults. Out of the three specimens of *S. atriceps*, two were found dead in the field and the alive one was found basking in the sun after rain in the month of August and escaped before being captured. Similarly, the only single specimen of *S. diadema cliffordii/diadema* was found dead. Thus, we remain unable to gather details about the activity and behaviour of both *S. atriceps* and *S. d. cliffordii/diadema*.

Discussion

The study and identification of species is one of the first and most important steps to be taken before formulating a species-specific policy for the conservation of biological diversity. *Spalerosophis* spp. are adapted to a wide range of habitats (Rastegar-pouyani et al. 2008). As these species feed on some rodents, their role may be considered important in the biological control of the rodent population (Yadollahvandmiandoab et al. 2018). Present findings of reporting of *Spalerosophis* spp. from crop fields, kaccha houses and, on some occasions, wrapped around the common rat suggest the role of this species in rodent control.

Despite the observation on the distribution of *S. d. diadema* from the north-western parts of India i.e. Jammu and Kashmir, Punjab, Uttar Pradesh, Haryana, Rajasthan and Gujrat (Smith 1943; Sahi and Duda 1985; Sharma 2007), Baig and Masroor (2008) have restricted the distribution of *S. d. diadema* in the Indo-Pakistan region along the Pakistan border with Iran and Afghanistan only. In addition to this, Whitaker and Captain (2008, 2015), while describing the snakes of India, have not depicted *S. d. diadema* in the coloured plates and discussions. Sahi and Duda (1985) have mentioned the presence of *S. atriceps* from other districts of Jammu and Kashmir, but not from the Poonch District. Thus, the present study, besides re-validating the presence of *S. d. diadema* from the Poonch District, is also presenting a new record of *S. atriceps* from the study area.

Following Marx (1959), Schätti et al. (2009) considered *Spalerosophis diadema* as a polytypic species including *S. d. cliffordii* and *S. d. diadema*. Schmidt (1939) and Marx (1959) separated the *S. d. diadema* and *S. d. cliffordii*, based on the number of subcaudals, i.e. 80 or more in the former versus less than 80 in the latter. The present finding of one specimen MCMZ 0119 having a subcaudal count of 78 points to the likelihood of this specimen as the candidate of *S. d. cliffordii*. However, we refrain from this decision of establishing this species from the current study area and subject it to further investigations, based on the fact that none of the authors has ever reported *S. d. cliffordii* from India. Schätti et al. (2009) have shown the presence of *S. d. cliffordii* from the western Sahara to southwest Iran only. Moreover, Khan (2002, 2006) and Baig and Masroor (2008) have considered the subcaudals range of 78–114 for *S. d. diadema*. Thus, the establishment of *S. d. cliffordii* from this area needs more profound analysis, larger sample size and additional diagnostic characteristics.

Smith (1943), Marx (1959), Sharma (2007), Baig and Masroor (2008) and Whitaker and Captain (2008, 2015) reported that adults of *S. diadema* and *S. atriceps* show strikingly different head and dorsal colour patterns, but this does not hold true for subadults and juveniles, thus making their identification difficult. This also holds true in the present finding as a less grown individual of *S. atriceps* MCMZ 0920 is showing faded blotches and head markings as if the individual is losing its typical subadult markings of the head and body. Specimens MCMZ 920 having a SVL of 1110 mm showing a faded blotched pattern, as well as a moderately melanistic body and specimen MCMZ 1020 having SVL of 1230 mm exhibiting an intensely melanistic body, are identified as *S. atriceps* on the bases of the findings of Baig and Masroor (2008) who reported that *Spalerosophis atriceps*, on exceeding snout-vent length of 1000 mm, gradually loses the blotched pattern and changes into straw yellow colour with irregular flecks and blotches (Fig. 7A) and on attaining further growth changes to another melanistic form (Fig. 7B). In addition to this, the scalation pattern of these two specimens of *S. atriceps* as per our data are clearly in line with the earlier findings of Minton (1966) and Baig and Masroor (2008), mentioned hereinafter in brackets viz. mid-dorsals 29–31 [27–31 and 29–30]; ventrals 238–250 [232–254 and 230–252]; subcaudals 105–109 [96–114 and 100–112]. Thus, these two specimens are clear candidates for belonging to *S. atriceps*.

Specimens in the present study show little variation in the range of scales when compared with the findings of Wall (1914), Smith (1943), Marx (1959); Sahi and Duda (1985), Sharma (2007), Baig and Masroor (2008) and Whitaker and Captain (2008, 2015) and these variations may be ascribed to the different habitat of the present study area not explored earlier. Thus, the diagnostic key of *Spalerosophis* needs more inputs from its wide range of distribution areas as rightly pointed out by Schätti et al. (2009) that the systematic position of *Spalerosophis* has been subject to modifications over the past 140 years.

Body colour patterns as reported in smaller individuals of *Spalerosophis atriceps* (MCMZ 0920) having a yellowish-brown background with irregularly scattered dark brown or black spots either confined to individual scales or much more thickly distributed, forming large rhomboidal dorsal spots, similar in position to the dorsal larger-sized spots of *Spalerosophis diadema diadema* along with uniform rose pink belly (Fig. 7A) and colour pattern shown in the present study by the fully grown individual (MCMZ 1020) having an entirely black head with a deep red hue, which becomes deep red on nape and temples (Fig. 7B) are clearly in line with the findings of Smith (1943) and Sharma (2007) who have reported the occurrence of *S. atriceps* from Gilgit, Agra, Jeypore, Allahabad, Delhi and Harrand.

Though *Spalerosophis diadema diadema* and *S. atriceps* are sympatric species (Marx 1959), the latter is nocturnal in habit (Sharma 2007) and is uncommon (Whitaker and Captain 2015). Thus, the smaller number of individuals of *S. atriceps* found during the present investigations as compared to *S. diadema diadema* is probably because of the nocturnal habit and uncommon distribution of the former. In addition, low densities, elusiveness and long periods of inactivity are often the causes behind the low detection of the snake species (Seigel 1993) leading to underestimation of their distribution range compared with the other reptiles (Santos et al. 2006; Bombi et al. 2009)

Our findings of 54–88 dorsal blotches in *S. d. diadema* are very close to those of Baig and Masroor (2008) who have reported 56–84 dorsal botches from the Pakistan population of *S. diadema*. In our study, *S. d. cliffordii/diadema* has shown 85 dorsal blotches, whereas the blotches were absent in the fully melanistic form of *S. atriceps.* The number of blotches in the moderately melanistic form of *S. atriceps* has been counted up to 57 which is, again, in the range of the number of blotches as reported by Baig and Masroor (2008) i.e. 55–78 from Pakistan.

Arrangement of scales as observed in the present study, such as: (1) a complete ring of oculars in which suboculars are excluding the orbit from supralabials, (2) prefrontals and loreals broken up into small scales, (3) a high number of temporal scales and (4) an entire anal plate, are the diagnostic characteristics of *Spalerosophis* spp. (Marx 1959).

The number of ventrals showing sexual dimorphism in both species as observed in the present study (Table 2) is a diagnostic characteristic feature of *S. diadema* (Marx 1959; Schätti et al. 2010).

Keeled dorsals in *S. atriceps* and weakly-keeled dorsals in *S. d. diadema* as observed in the present findings have also been reported by Minton (1966) from Pakistan. Subadults have also shown weak keeling in the present study.

In our study, *S. atriceps* is showing a higher range of number of scales vis à vis *S. diadema diadema* [count of scales of *S. d. diadema* in brackets] in mid-body dorsal scales rows 29–31 [27–29], subcaudals 105–109 [82–111], loreals 2–4 [2–3], circumocular ring 7–11 [8–10] and supralabials 11–13 [10–12]. The count of scales of *S. atriceps* had also remained high in the findings of Baig and Masroor (2008) [count of *diadema* in bracket] for mid-body dorsal scales 29–30 [25–31], ventrals 230–252 [220–254] and subcaudals 100–112 [78–114]. Owing to a smaller number of individuals of *S. atriceps* in the present study, conclusions on the snout-vent length remain inconclusive and contrary to the findings of Baig and Masroor (2008) who have reported *S. atriceps* as larger-sized species as compared to *S. d. diadema.*

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Supplementary material 1

Threatening behaviour of *Spalerosophis diadema diadema*

Author: Sarshad Hussain

Data type: Video

Explanation note: It shows hissing and quick biting by *S. diadema diadema* in captivity.

Link: https://doi.org/10.3897/herpetozoa.36.e94456.suppl1

Supplementary material 2

Table of localities

Author: Sarshad Hussain

Data type: Occurence

Explanation note: Showing latitude, longitude, altitude and name of the locality from where species of the genus *Spalerosophis* have been reported.

Link: https://doi.org/10.3897/herpetozoa.36.e94456.suppl2

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Body size and body condition in the nose-horned viper (*Vipera ammodytes*): effects of sex and populations

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https://zoobank.org/59B15D27-0CB6-4945-9A19-0DC825852E30

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Abstract

Snakes are ecologically and morphologically plastic organisms that exhibit extensive variations in body size and body condition in response to environmental factors. Documenting inter-population variations is important to describe species comprehensively across their distribution range and to monitor trends over time (e.g. decreasing body condition due to alteration of habitat). Thus, we analyzed the influence of population and sex on body size and body condition in three populations of nose-horned vipers (*Vipera ammodytes*) in Serbia. In one population, males were larger than females ($F_{1,39}$=4.802, p=0.034), but not in the two other populations ($F_{1,36}$=0.075, p=0.786; $F_{1,21}$=0.018, p=0.893). Females exhibited higher body condition (residual values from the regression of log-body mass against log-body size) than males ($F_{1,90}$=10.444, p=0.002); this sex difference was not found in one population when analyzed separately ($F_{1,35}$=1.834, p=0.184). Moreover, we found strong inter-population differences in mean body size and mean body condition ($F_{2,96}$=8.822, p<0.001 and $F_{2,90}$=10.319, p=0.001, respectively). While inter-population difference in body size was driven by males, inter-population difference in body condition was driven by females. These results suggest that, in this species, body size might be an important determinant of mating success in males, while body condition may play a major role in female fecundity.

Key Words

inter-population difference, morphological traits, sexual dimorphism, Viperidae

Introduction

Body size is a central phenotypic trait for most organisms (LaBarbera 1989). In snakes, indeterminate growth means that body size after maturity can be influenced by environmental factors such as food availability; this trait is thus subjected to significant inter-population variation (Madsen and Shine 2000; Aubret 2012). Body condition (body mass scaled by body size) is another key phenotypic trait that affects reproductive success in snakes (Naulleau and Bonnet 1996; Reading 2004). Body condition also responds to environmental variables such as trace metal contamination or climate change (Brischoux et al. 2012; Lettoof et al. 2022). By definition, body size and body condition are uncorrelated (Bonnet et al. 2001). However, in terms of physiology these two traits are not independent (Stearns 1992). Indeed, trophic resources are limited, thus growth in stature trade-offs against body condition increases (Stearns 1992). Overall, complex interactions between environmental factors, sexual dimorphism, reproductive strategies and trophic ecology affect body size and body condition in snakes (Bonnet et al. 2001, 2002; Aubret et al. 2002; King 2002; Beaupre 2008; Zuffi et al. 2010).

We studied body size and body condition in free-ranging nose-horned vipers (*Vipera ammodytes)* from three

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widely separated populations in Serbia. Because environmental factors are likely to vary among sites (populations), differences in body size and body condition were expected. We also considered sex, because in this snake species, males tend to attain larger body size than females (Tomović et al. 2022).

Methods

Study species

Vipera ammodytes is a venomous viper distributed in Europe and Asia Minor (Agasyan et al. 2009). In Europe, it inhabits southern Austria, northern Italy, and the Balkan Peninsula (Crnobrnja-Isailović and Haxhiu 1997). Preferred habitats of this species are xerothermic rocky places with shrubby vegetation cover, but nose-horned vipers can also be found in sparse forests and man-made stone walls (Agasyan et al. 2009). *Vipera ammodytes* displays sexual dimorphism in tail length with males having longer tails, more subcaudal scales and larger body size, while females have wider ventral scales (Tomović et al. 2002, 2022).

Study sites and data sampling

The three study sites were located in Serbia (Fig. 1): 1) Natural Monument Lazarev Canyon (**LC**) is a protected area located in Eastern Serbia (about 440 m a.s.l.). The rocky canyon is covered by dry deciduous forest. 2) Svilajnac surroundings (**SV**) is situated in Central Serbia (about 100 m a.s.l.) near the Velika Morava River and near the town of Svilajnac; habitats include deciduous forest, shrubs, and arable land. In this locality nose-horned vipers have been harvested for venom extraction since 1990s (Čubrić and Crnobrnja-Isailović 2022). 3) The Ibar River Gorge (**IR**) is situated in the central part of Serbia (270 m a.s.l.). In this rocky gorge, sparse vegetation cover includes shrubs and patches of deciduous forest.

In each of the three locations, we (TČ for all three populations, TČ and JC-I (for 2 field trips) for SV and IR populations) visually searched vipers from 10:00h to 18:00h along two standard transects of approximately the same length (4000 m). Searching was performed over approximately five consecutive days (±2 d) per season (spring (April-May), summer (June-August), autumn (September-October)) per locality. IR and SV were monitored from 2016 to 2020 (roughly 70 days), LC in 2018 and 2019 (roughly 40 days).

We captured snakes by hand. We measured snout to vent length (**SVL**) and total length (**TL**) to the closest mm using plastic meter tape (precision 1 mm). Body mass (**BM**) was recorded using a spring scale (±5 g). Sex was determined in juveniles and adults by palpation of hemipenes (neonates with 17.8<SVL<25.0 cm were not sexed and were discarded from analyses). We calculated Body Condition as the residual values from the linear regression between Log-BM against Log-SVL; therefore, body condition was not correlated with body size, by definition (Bonnet et al. 2001). Vipers were marked individually by scale clipping and every animal was photographed. We did not include the recaptures in the analysis. We performed all measurements *in situ* and the vipers were released immediately after processing. To count for observer's bias, all measurements were done by the first author.

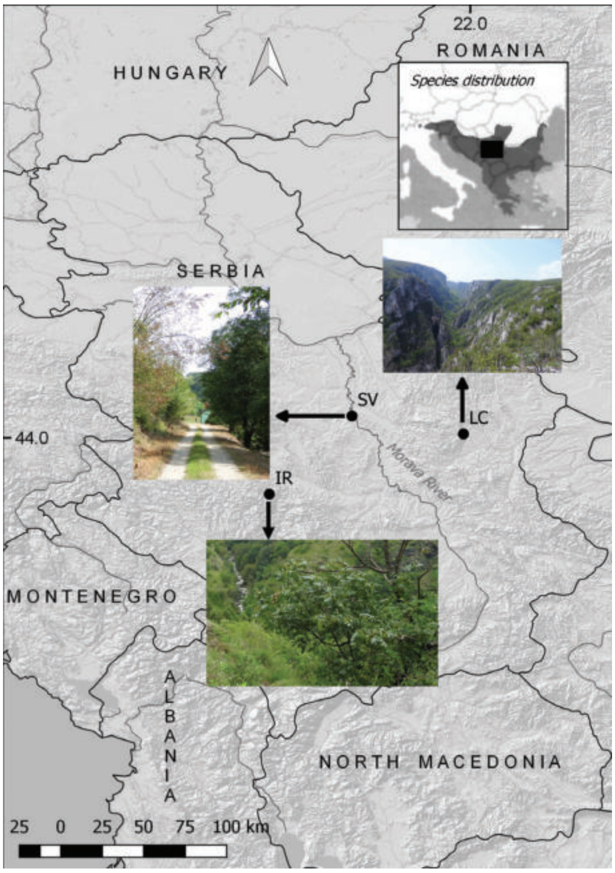

Figure 1. Indicative locations of the studied populations. Three sites were monitored: Lazarev Canyon (LC), Ibar River Gorge (IR) and Svilajnac surroundings (SV). The photographs inserted illustrate the type of habitat (photographs by T. Čubrić).

Vipers have been and still are, in some parts of Serbia, illegally killed and collected for venom extraction and possibly pet trade (Čubrić and Crnobrnja-Isailović 2022). Therefore, we do not provide the exact GPS coordinates of the populations (Console et al. 2020). Indicative locations of the studied populations are shown in Fig. 1.

Statistical analyses

Each individual was represented only once in the analyses and only data from the first capture were used. Data were Ln transformed prior to analyses. Body measurements were analyzed using ANOVA and General Linear

Model (**GLM**). Measuring body size and body condition is relatively straightforward in elongated and limbless animals such as snakes; SVL and BM provide major descriptors of individual's morphology. Nonetheless, body condition does not merely correlate with fat stores, thus its meaning should be interpreted with care (Weatherhead and Brown 1996). In this study, snakes were not fully palpated in order to limit disturbance; body condition calculation included vitellogenic and gravid females along with individuals with food in the gut. Thus, different elements (e.g., body fat reserves, follicles, prey remains) contributed to body condition; but all of them were positively associated with food intake, thereby reflecting local food availability. Analyses were also performed separately for each population to examine more finely possible sex effect (thus, the population effect on the total variance was diminished). Parturition occurs in late summer, provoking a sudden drop in female body mass. Restricting analyses to spring (or spring and summer) did not change the patterns, but several results became marginally significant due to the loss in statistical power. This suggests that post parturition drop of body mass did not alter the observed trends. Consequently, we retained the three seasons during analyses. Statistical tests were performed using STATISTICA 7.0.

Results

Table 1 provides an overview of the number of snakes sampled in each population. The initial total sample consisted of 128 individuals – 37 from LC, 53 from SV and 38 from IR). However, as we discarded snakes with SVL of less than 25cm for analyses, several BM were not recorded; the resulting sample size was of 102 vipers.

Body size and body mass

The smallest male was from IR (SVL 29.5 cm, TL 33.0 cm) and the largest was from SV (SVL 69.8 cm, TL 80.0 cm). BM ranged from 20 g in LC to 220 g in SV. Both the smallest and the largest female were from LC (SVL 26.5 cm, TL 30.7 cm and SVL 60.0 cm, TL 67 cm, respectively), while BM ranged from 20 g to 165 g, respectively (Table 1).

Table 1. Body size and body mass (mean±SE) in three populations of nose-horned vipers surveyed in Serbia. LC stands for Lazarev Canyon, SV for Svilajnac surroundings and IR for Ibar River Gorge.

Population	Sex	N	SVL	BM
LC	males	11	45.22±2.79	69.54±10.28
	females	12	45.35±3.40	87.91±14.45
SV	males	28	55.21±1.48	110.51±11.48
	females	13	50.66±1.41	114.00±6.60
IR	males	23	44.86±1.24	74.77±5.28
	females	15	43.44±1.48	72.69±3.86

We found a strong effect of population on SVL (ANOVA with Ln-SVL as the dependent variable and sex and population as the factors: $F_{2, 96}$=8.822, p<0.001), but no effect of sex ($F_{1, 96}$=1.614, p=0.207) and no interaction between population and sex ($F_{2, 96}$=0.914, p=0.404). SV vipers (both sexes pooled) were larger (52.9±8.2 [SD], N=41) than LC (45.3±10.4, N=23) and IR vipers (43.8±6.0, N=38). Body mass was highly correlated to SVL (r=0.870, $F_{1, 100}$=311.83, p<0.001); similar results were obtained using BM instead of SVL (strong population effect without effect of sex). SV males tended to be larger than the females, but this effect was detected only when SVL was analyzed specifically in this population ($F_{1, 39}$=4.802, p=0.034) (Table 2). However, BM did not differ between sexes in SV population ($F_{1, 39}$=0.016, p=0.900). Similarly, no sex differences were found in the two other populations when analyzed separately.

Body condition

Body condition (GLM with Ln-BM as the dependent variable, sex and population as the factors and Ln SVL as covariate) was influenced by sex and population without interaction between these factors (Table 3, Fig. 3). Mean body condition was higher in females (Fig. 2) and in SV population ($F_{1,.90}$=10.444, p=0.002; $F_{2,90}$=10.319, p=0.001 respectively). When conducted separately for each population (Table 2, Fig. 3), significant sex effects were found in SV and LC where females exhibited higher body condition than males ($F_{1, 38}$=11.528, p=0.002 and $F_{1, 20}$=6.203, p=0.023 respectively), but not in IR ($F_{1, 35}$=1.834, p=0.184) (Table 2, Fig. 3) .

Table 2. Effect of sex on body size and body condition analyzed separately for each of the three populations. Significant effects are in bold.

Population	SVL		Body condition	
	F	*p*	*F*	*p*
SV	4.80	**0.034**	11.52	**0.002**
IR	0.075	0.786	1.83	0.184
LC	0.018	0.893	6.20	**0.023**

Table 3. Results from a GLM with Ln-BM as the dependent variable and sex and population as the factors and Ln-SVL as covariate. Body condition was significantly influenced by sex and population without interaction between these factors. Significant effects are bolded.

Effect	SS	Df	*F*	*p*
Intercept	1.505	1	38.958	**>0.001**
Ln-SVL	9.243	1	239.322	**>0.001**
Sex	0.403	1	10.444	**0.002**
Population	0.797	2	10.319	**>0.001**
sex*ln SVL	0.355	1	9.180	**0.003**
population*ln SVL	0.757	2	9.800	**>0.001**
sex*population*ln SVL	0.211	2	2.732	0.070
sex*population	0.214	2	2.770	0.068
Error	3.476	90		

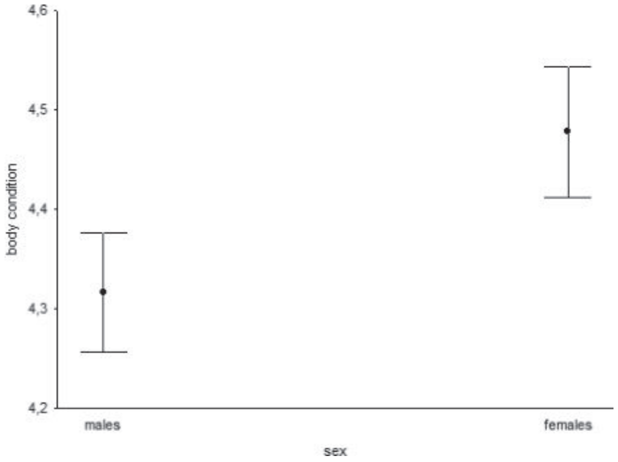

Figure 2. Comparison of body condition between sexes (all populations pooled).

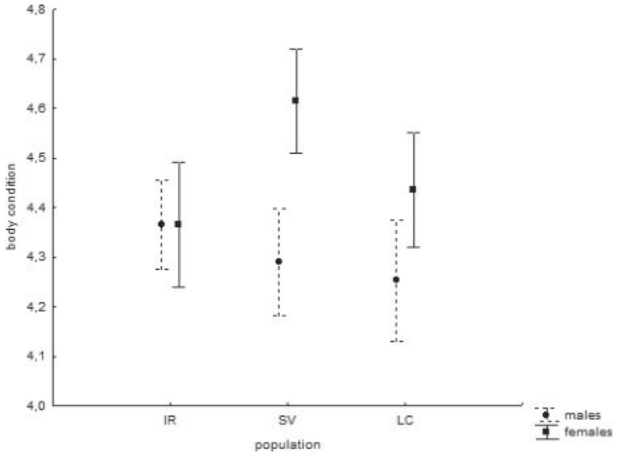

Figure 3. Comparison of body condition between sexes and among populations.

Discussion

We found both sex and population effects on mean body size and mean body condition of *V. ammodytes* studied in three different sites in Serbia, suggesting a role for local conditions such as food availability on growth and body reserve storage. Svilajnac (SV) population hosts the largest and relatively heaviest individuals. This population has been subjected to hunting for venom supply; snake collectors may have targeted a healthy or easily accessible population. Rodents, the main prey of nose horned vipers, are favored by traditional agriculture that creates semi-open habitats with many hedgerows and abundant grass and foraging resources. Such landscapes and fragmented woods persist in SV. Vipers can thrive in peri-urban context provided that favorable partly open bushy habitats are available (Bonnet et al. 2016). The two other populations (Lazarev Canyon, Ibar River Gorge) are situated in markedly less disturbed rocky habitats where lizards are abundant. Smaller body size and lower mean body condition indicate that food availability and prey quality (lizards are less beneficial food items for vipers than micromammals; Zuffi et al. 2010; Tomović et al. 2022) might be lower compared to SV traditional agriculture landscape.

Besides broad geographic patterns, sex differences revealed complex pattern. Both males and females from SV were larger compared to other populations; but this effect was essentially driven by the large SV males (restricting population comparison to males, $F_{2,\,58}$=8.675, p<0.001), rather than females (restricting population comparison to females, $F_{2,\,38}$=2.834, p=0.071). Consequently, sexual size dimorphism (SSD) was detected only in SV vipers. This outcome mirrors the results from previous studies documenting a lack of, or a significant SSD with larger males in the nose horned viper depending upon study sites (Tomović et al. 2002, 2022; Mebert et al. 2017). Body condition results did not fully match those obtained with SVL. Indeed, in this case females were the main driver of population differences. SV females were in better condition compared to the two other populations (restricting population comparison to females, $F_{2,\,35}$=14.381, p<0.0001) while no difference was found in males (restricting population comparison to males, $F_{2,\,55}$=1.926, p=0.155; in fact, IR males exhibited the highest body condition). Overall, in the population that may benefit from high food availability, males invest resources in stature growth and females in body reserve storage (plus vitellogenesis) resulting in marked sexual dimorphism in body size (larger males) and body condition (relatively heavier females). In areas characterized by lower food availability, both growth and reserve storage might be strongly constrained, resulting in attenuated sexual dimorphism.

The positive influence of body size on male reproductive success versus the positive influence of body condition on female fecundity has been documented in vipers. Larger male adders (*V. berus*) are more likely to defeat smaller rivals and to court successfully females (Madsen et al. 1993). In the asp viper (*V. aspis*) females need to reach a body condition threshold (meaning that they need to accumulate enough energy reserves) to be able to reproduce (Naulleau and Bonnet 1996). A similar role for body reserves was suggested in the nose horned viper (Luiselli and Zuffi 2002; Phelps 2007). Moreover, body condition positively correlates with fecundity in the aspic viper (Bonnet et al. 2001). The notion that males may benefit from the allocation of resources into growth while females' reproductive success should be enhanced by the allocation of resources into body reserves and vitellogenesis might be general in snakes (Shine 2003).

Although body size and body condition are highly plastic traits in snakes (Bonnet et al. 2021), we cannot exclude the possible role of local genetic adaptation (King and Lawson 2001). Čubrić et al. (2019) identified two overlapping genetic clades of *Vipera ammodytes* in SV region, while in IR and LC only one clade was found. Genetic investigations are required to clarify this issue.

Conservation issues

Habitat loss, climate change, contamination, over-collection for venom supply and illegal pet trade represent a cocktail of threats for the nose-horned viper, but the

IUCN Least Concern species status could make it rather unattractive for conservation funding agencies (Čubrić and Crnobrnja-Isailović 2022). Therefore, monitoring of the nose-horned viper populations is essential to prevent silent declines. Our data contribute to the establishment of a landmark for long-term population monitoring. In this endeavor, the complex interactions between habitat, local environmental conditions and sex on body size and body condition in this species must be considered.

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The advertisement calls of *Pristimantis galdi* Jiménez de la Espada, 1870 and *Pristimantis katoptroides* (Flores, 1988) (Anura, Strabomantidae)

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Abstract

In this study we describe for the first time the calls of *Pristimantis galdi* and *Pristimantis katoptroides*. Recordings were obtained in Sangay National Park, Ecuador. We highlight the importance of recording *P. galdi* since its call has been recorded after 153 years of having been described as a species. The call of *P. galdi* consists of 7 to 9 short notes, the sounds of which are similar to a hammer hitting a nail, with a mean dominant frequency of 2.39 kHz. In turn, the call of *P. katoptroides* consists of a single note, the sound of which is similar to a metallic "tic", with a mean dominant frequency of 1.74 kHz. We compared the advertisement calls of *P. galdi*, *P. katoptroides* and *P. roni* as these species share similar morphological characteristics and are grouped in the spiny green frog's ecotype. Despite these morphological similarities, their advertisement calls are different. Obtaining calls of *Pristimantis* species in Ecuador might prove difficult with short-term studies due to the great sampling efforts that may be needed to get these recordings. Therefore, implementing active and passive monitoring could help improve our knowledge of acoustic signals in Ecuador's rainfrogs.

Key Words

acoustic communication, calls, Sangay National Park, spiny green frogs

Acoustic communication is one of the Anurans' most important and conspicuous ethological traits, through which they transmit messages between conspecifics (Duellman and Trueb 1994; Angulo 2006; Wells 2007). Acoustic emissions respond to a specific social context and function (Toledo et al. 2015), where the advertisement call is the most commonly heard one in nature. This type of call announces sexual receptivity, position in a territory, and individual size (Gerhardt and Huber 2002; Wells 2007; Toledo et al. 2015; Köhler et al. 2017). In general, the advertisement call consists of a repetitive series of notes or pulses and, as a result of this stereotypical structure, tends to have a high degree of specificity among species (Gerhardt 2001; Wells and Schwartz 2007). These characteristics have allowed the use of the advertisement call as an identification feature among species (Angulo 2006). Moreover, analyzing and determining call variations has allowed for gaining a better understanding of the diversity of certain groups. Especially in those where morphological characteristics are insufficient to differentiate and diagnose species. (Angulo and Reichle 2008; Rivera-Correa et al. 2022).

PENSOFT.

Pristimantis Jiménez de la Espada, 1870 is a genus of anuran restricted to the New World, known as the most speciose vertebrate genus (595 species; Frost 2023). Nearly 20 species of *Pristimantis* have been described in the last two years in Ecuador, thereby making it the country with the highest description rate per year of this genus (Reyes-Puig and Mancero 2022). In fact, Ecuador counts 254 species, out of which 61.8% (157 species) are endemic (Ron et al. 2022). Despite this considerable rate of discoveries and publications, a large percentage of the vocalizations of the *Pristimantis* individuals of Ecuador remains unknown (Batallas and Brito 2016). In this study, we describe for the first time the advertisement calls of *Pristimantis galdi* Jiménez de la Espada, 1870 and *Pristimantis katoptroides* (Flores, 1988). Furthermore, we compare these results with the call of *Pristimantis roni* Yanez-Muñoz, Bejarano-Muñoz, Brito-M and Batallas-Revelo, 2014, since it is a species with some morphological resemblance.

Fieldwork was conducted in June and September of 2014 in the lower zone of Sangay National Park (administrative jurisdiction comprising the eastern foothills of the park within an altitudinal range of 600 to 1800 m), which corresponds to the Subtropical zoogeographic region (Albuja et al. 2012). The ecosystem comprises montane evergreen forest on the southern slope of the Eastern Andes (Ministerio Del Ambiente del Ecuador 2013). The habitat is characterized by trees whose gnarled trunks and branches support several species of epiphytes such as orchids and bromeliads. The emergent vegetation reaches up to 30 m in height, and there is a visual predominance of romerillo (*Prumnopitys montana*) and palma real (*Dictyocaryum lamarckianum*) trees. In the understory, there is visual predominance of herbaceous plants of the Araceae and Marantaceae families (Brito et al. 2017).

Two calls from a *Pristimantis galdi* individual collected at Sardinayacu lake complex (2°04'20.5"S, 78°12'52.4"W, 1,800 m) were recorded on June 24, 2014, between 14:00–16:00h. Meanwhile, 30 calls from three *Pristimantis katoptroides* individuals collected at Danu (2°04'45.3"S, 78°09'37.3"W, 1,360 m) were recorded on September 21, 2014, between 19:00–20:00h. The calls were obtained using a digital recorder (Olympus WS–802), coupled to a directional microphone (Sennheiser ME 66–K6), placed between 1–3m from the active individual. All recordings were made at a sampling rate of 44.1 kHz and 16 "bits" resolution, saving the audio files in the uncompressed WAV format. It is also worth noting that no fixed hours were set for recording during fieldwork, and it was rather the vocal activity of species that influenced the sampling effort. Collected specimens were sacrificed according to Chen and Combs (1999) protocols and preserved following Simmons (2002). They are deposited at the División de Herpetología del Instituto Nacional de Biodiversidad (DHMECN), Quito, Ecuador. The recordings are deposited at the Fonoteca Zoológica (www.fonozoo.com) del Museo Nacional de Ciencias Naturales (CSIC), Madrid, Spain (Appendix 1). Identification of specimens was based on morphological characteristics described in Lynch and Duellman, (1980), Flores (1988), and Duellman and Lehr (2009).

The spectral and temporal properties of the advertisement calls were analyzed with the software Raven 1.6 (K. Lisa Yang Center for Conservation Bioacoustics at the Cornell Lab of Ornithology 2022). The spectrograms were obtained using the Hann window at 90% overlap and 512 samples of DFT size. The parameters analyzed were: (1) Dominant frequency of the entire call and each of the elements emitted in it; (2) Harmonics; (3) Duration of calls; (4) Intervals between calls; (5) Calls/minute; (6) Notes/call; (7) Duration of notes; (8) Intervals between notes; (9) Notes/second. Definitions, terminology and measurements of acoustic parameters follow the terms of Köhler et al. (2017), and Sueur (2018). The figures were processed in R software (R Core Team 2022). For this, the audio files were imported with the tuneR package version 1.4.1 (Ligges et al. 2018). Subsequently, the oscillogram and spectrogram were created using the Seewave package version 2.2.0 (Sueur et al. 2008). With the values of the analyzed parameters, the measures of central tendency (means) and dispersion (maximum, minimum, and standard deviation) were calculated.

Pristimantis galdi Jiménez de la Espada, 1870 (Fig. 1A)

The recorded male was calling from shrub vegetation approximately 150 cm above the ground. The advertisement call (Fig. 2A, Table 1) consists of 7 to 9 notes. Call duration ranges from 1730 to 2017 ms. The mean note duration is 44.62 ± 12.50 ms, with a mean rate of 4 ± 0.96 notes/second. The mean interval between notes duration is 217.57 ± 54.16 ms. The mean dominant frequency is 2.39 ± 0.16 kHz, showing a partial harmonic whose mean frequency is 4.76 ± 0.36 kHz. Its calls are a constant metallic sound, similar to a hammer hitting a nail.

Pristimantis katoptroides (Flores 1988) (Fig. 1B)

Recorded males were calling perched on tree branches, about 2–4 m above the ground. This species vocalizes in choruses of about seven to ten males, often observed in smaller groups of up to four individuals in the same tree. The advertisement call consists of a single note with a mean duration of 54.73 ± 5.69 ms. The mean interval between notes is 44.84 ± 25.14 ms, and mean rate is 1.80 ± 0.99 calls/minute. The mean dominant frequency is 1.74 ± 0.11 kHz, showing four partial harmonics in the spectrogram (Fig. 2B, Table 1). The calls have a metallic sound, onomatopoeically resembling the "tic" of a bell.

We describe for the first time the spectral and temporal parameters of the advertisement call of *Pristimantis galdi* and *Pristimantis katoptroides*. The former is assigned to the *Pristimantis* (*Pristimantis*) *galdi* species group (Hedges et al. 2008; Padial et al. 2014), where other species' calls remain unknown. Both, *P. galdi* and *P. katoptroides* (not assigned to any group), are placed in the spiny green frog's ecotype (Yanez-Muñoz et al. 2014). Within this ecotype, *Pristimantis roni* is morphologically similar to *P. katoptroides* (Yanez-Muñoz et al. 2014). However, these species with similar morphological characteristics differ in the properties of their advertisement calls. The

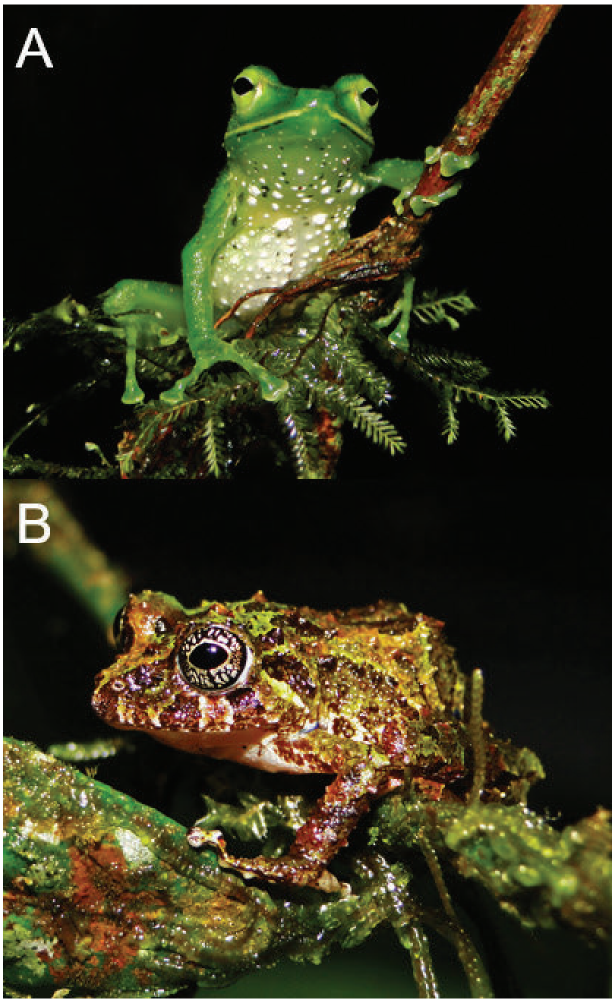

Figure 1. Individuals in their natural habitat. **A.** Adult male of *Pristimantis galdi* (DHMECN 12189, SVL 20 mm) from Sardinayacu, Parque Nacional Sangay, Ecuador; **B.** Adult male of *Pristimantis katoptroides* (DHMECN 12169, SVL 28.15 mm) from Danu, Parque Nacional Sangay, Ecuador.

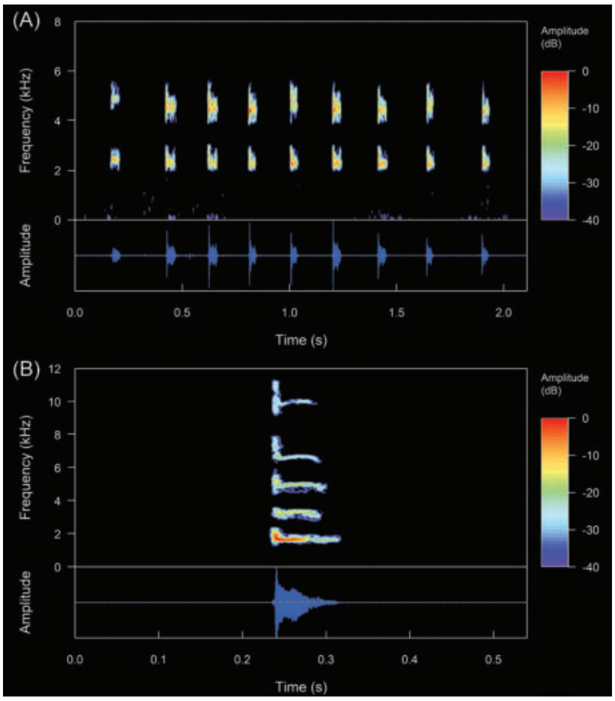

Figure 2. Spectograms and Oscillograms of the advertisement calls described in this study. **A.** *Pristimantis galdi* (DHMECN 12189, SVL 20 mm, 18 °C air temperature); **B.** *Pristimantis katoptroides* (DHMECN 12169, SVL 28.15 mm, 17.1 °C air temperature).

Table 1. Spectral and temporal values of *Pristimantis* calls belonging to the spiny green frog's ecotype (*sensu* Yánez-Muñoz et al. 2014). The abbreviations used in the parameters correspond to: kHz = kilohertz, ms = milliseconds, s = seconds. In the analyzed sample, n=number of specimens/calls/notes.

	P. galdi n=1/2/16	*P. katoptroides* n=3/30	*P. roni* n=1/9/50
Dominant frequency (kHz)	2.19–2.58 (2.39 ± 0.16)	1.60–2.06 (1.74 ± 0.11)	3.14–3.35 (3.32 ± 0.06)
Harmonic 1 (kHz)	4.35–5.34 (4.76 ± 0.36)	3.19–3.99 (3.43 ± 0.24)	–
Harmonic 2 (kHz)	–	4.94–5.09 (5.03 ± 0.05)	–
Harmonic 3 (kHz)	–	6.53–6.84 (6.65 ± 0.10)	–
Harmonic 4 (kHz)	–	8.80–9.91 (9.65 ± 0.42)	–
Call duration (ms)	1730–2017	42–64 (54.73 ± 5.69)	40–1247 (493.09 ± 341.67)
Interval between calls (s)	–	16.05–94.92 (44.84 ± 25.14)	2.09–7.93 (6.13 ± 1.71)
Call rate (calls/min)	–	0.64–3.72 (1.80 ± 0.99)	6.73–12.44 (8.79 ± 1.76)
Notes per call	7–9	1	1–5
Note duration (ms)	30–77 (44.62 ± 12.50)	–	29–45 (36.84 ± 4.68)
Interval between notes (ms)	151–325 (217.57 ± 54.16)	–	175–298 (249,56 ± 35.91)
Note rate (notes/s)	2.54–5.29 (4 ± 0.96)	–	–
Source	This work	This work	Yánez-Muñoz et al. 2014

call structure in both species is different, as *P. roni* emits several notes while *P. katoptroides* emits one. The calls of *P. katoptroides* are shorter, with longer intervals between calls and a lower number of emissions per minute. Finally, the dominant frequency in *P. katoptroides* is lower than that of *P. roni*. In the case of *P. galdi*, the call structure is similar (both species emit several notes), and the dominant frequency is lower than that of *P. roni* (see Fig. 3, Table 1 for further details). The advertisement calls from the three compared species share that they resemble a metallic sound (an onomatopoeic sound of a "Tic").

Among cryptic species, the use of acoustic diagnostic features for identification could be an alternative to morphometric and molecular diagnosis (Padial et al. 2009; Rivera-Correa et al. 2022). Even though morphologically similar, *P. galdi*, *P. katoptroides*, and *P. roni* have calls with different features, which supports the specificity of their acoustic signals at the species level. However, the magnitude of cryptic diversity and their advertisement calls remain largely unknown. (Funk et al. 2012; Reyes-Puig and Mancero 2022). Direct developing frogs, as is the case of *Pristimantis* species, do not conform to chorus aggregations and individuals are dispersed in their habitat (Duellman and Lehr 2009). Therefore, obtaining recordings of their calls could be more difficult due to their life history traits. In fact, the data collected in this study comes from two of the 17 expeditions conducted between 2010 to 2016 in Sangay National Park and buffer zones (Brito et al. 2017).

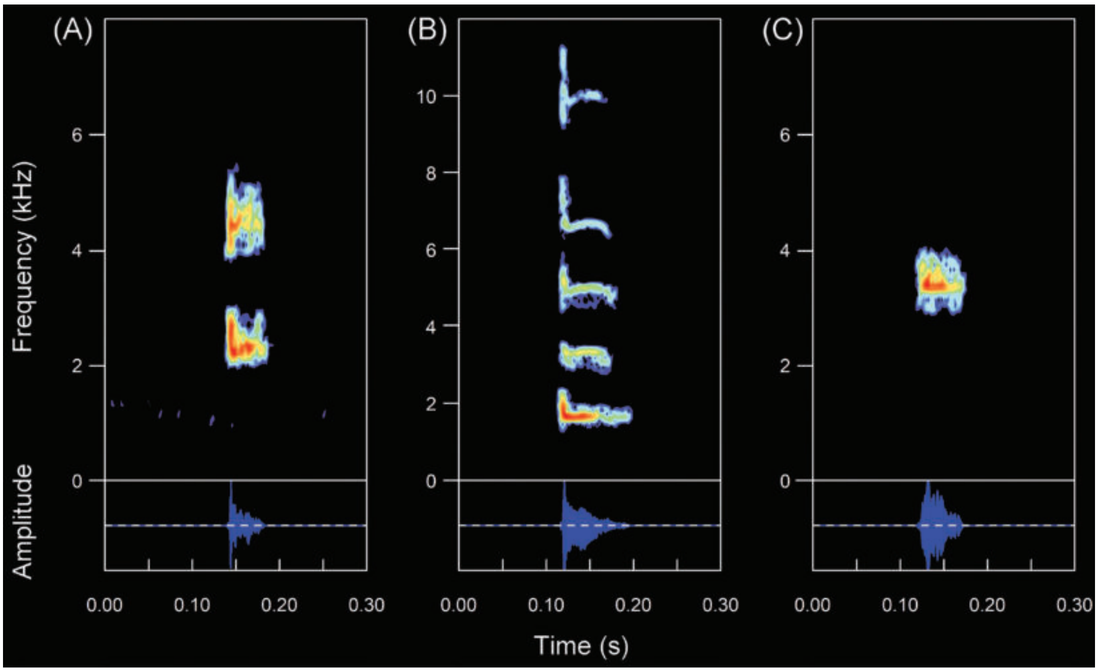

Figure 3. Comparative oscillograms and spectograms of advertisement calls of three *Pristimantis* species belonging to the spiny green frog's ecotype. **A.** *P. galdi* (DHMECN 12189, SVL 20 mm, 18 °C air temperature); **B.** *P. katoptroides* (DHMECN 12169, SVL 28.15 mm, 17.1 °C air temperature); **C.** *P. roni* (DHMECN 11322, SVL 16.55 mm, 15.6 °C air temperature).

Bioacoustics, behavioral and natural history information for species of the genus *Pristimantis* is very scarce, which contrasts with their abundant diversity (Hutter et al. 2016; Rivera-Correa et al. 2021). In Ecuador, such a contrast becomes more pronounced when taking into consideration the description rate of new species presented in recent years (Reyes-Puig and Mancero 2022). The fact that the call from a species such as *P. galdi*, which was described 153 years ago, has just been recorded for the first time suggests that recording the calls from *Pristimantis* species in Ecuador is difficult to obtain with short-term studies including direct visual encounters. Even though these methods are complementary and necessary for detecting and recording species in prospective phases (Boullhesen et al. 2021), complementary methos, such as passive acoustic monitoring, could help with recording the acoustic signals in one of Ecuador's most diverse and yet unknown groups of frogs.

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Appendix 1

Specimens recorded and collected:

Pristimantis galdi (Ecuador): Provincia de Morona Santiago, Complejo lacustre de Sardinayacu, Parque Nacional Sangay: DHMECN 12189, SVL 20 mm.46; FZ 11490–91

Pristimantis katoptroides (Ecuador): Provincia de Morona Santiago, Danu, Parque Nacional Sangay: DHMECN 12169, SVL 28.15 mm, FZ 11492; DHMECN 12170, SVL 24.05 mm, FZ 11493; DHMECN 12172, SVL 26.24 mm, FZ 11493.

Pristimantis roni (Ecuador): Provincia de Morona Santiago, Complejo lacustre de Sardinayacu, Parque Nacional Sangay: DHMECN 11322 SVL 16.55 mm, FZ 14208

Supplementary material 1

Pristimantis galdi recording 1. Fonozoo library code: FZ 11491

Authors: Diego Batallas, Jorge Brito

Data type: WAV file

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

Supplementary material 2

Pristimantis galdi recording 2. Fonozoo library code: FZ 11490

Authors: Diego Batallas, Jorge Brito

Data type: WAV file

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

Supplementary material 3

Pristimantis katoptroides recording 1. Fonozoo library code: FZ 11492

Authors: Diego Batallas, Jorge Brito

Data type: mp3 file

Link: https://doi.org/10.3897/herpetozoa.36.e97705.suppl3

Supplementary material 4

Pristimantis katoptroides recording 2. Fonozoo library code: FZ 11493

Authors: Diego Batallas, Jorge Brito

Data type: mp3 file

Link: https://doi.org/10.3897/herpetozoa.36.e97705.suppl4

Supplementary material 5

Pristimantis katoptroides recording 3. Fonozoo library code: FZ 11494

Authors: Diego Batallas, Jorge Brito

Data type: mp3 file

Link: https://doi.org/10.3897/herpetozoa.36.e97705.suppl5

Supplementary material 6

Pristimantis roni recording. Fonozoo library code: FZ 14208

Authors: Diego Batallas, Jorge Brito

Data type: WAV file

Link: https://doi.org/10.3897/herpetozoa.36.e97705.suppl6

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Microhabitat sharing for basking between squamate species in Poland

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https://zoobank.org/4B71350B-B328-44F0-81D6-1751FC5283E3

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Abstract

Aggregations (e.g. group basking) by snakes are usually limited to specific life cycle phases (e.g. mating) or are a consequence of drastic environmental changes (e.g. habitat destruction), high prey densities or highly limited resources within an environment (e.g. basking sites, wintering dens). Here, we report intra- and interspecific observations of four reptile species (primarily *Natrix natrix* and *Vipera berus*) sharing basking sites at the confluence of the rivers Dunajec and Poprad near the town of Stary Sącz in southern Poland. From a total of 84 records in the field between 2020–2022, there were 11 interactions from 24 July 2020 to 1 May 2022. Previous studies have indicated direct competition or interference in many species, which we did not observe. There is a noticeable lack of such observations of microhabitat sharing for basking between squamate species in scientific literature. Hence, the accumulation of such observations has the potential to reveal new insights into the behaviour and ecology of *N. natrix* and *V. berus*.

Key Words

adder, aggregations, *Anguis colchica*, behavioural thermoregulation, *Coronella austriaca*, ectothermy, grass snake, group behaviour, herpetofauna of Poland, reptiles

Records of a group or paired basking in snakes are infrequent; squamates usually lead a solitary life and are not sociable (Doody et al. 2021), although there are cases where they form groups in hibernacula, refugia sites or at optimal foraging locations (Costanzo 1986; Shah et al. 2006; Mebert 2011; Clark et al. 2012; Meek 2014; Christopoulos et al. 2022). These records are most often transient aggregations limited to certain life cycle phases, environmental changes and even high prey densities (Mouton 2011; Edgehouse et al. 2014; Gardner et al. 2015). In squamates, two types of aggregations are recognised: ecological (when individuals are attracted because of limited habitat availability due to external factors or too concentrated availability of resources, for example, food, shelter, basking sites) (Vasconcelos et al. 2017) and social (when individuals are attracted to conspecifics to avoid predators by group defence, increased thermoregulation (including European lizards) or mating behaviour (Graves and Duvall 1995; Kearney et al. 2001; Chapple 2003; Gardner et al. 2015; Meek and Luiselli 2022b; Turner 2022). Noteworthy and rare are observations of intra- or interspecific interactions outside the specific context that requires aggregating behaviour, often correlated with resource use, for example, heat source, mating partner, hibernacula, shelter or biotic or abiotic structures for predation avoidance (Graves and Duvall 1995; Kearney et al. 2001; Ajtić et al. 2013; Turner 2022).

Within thermoregulatory behaviour, basking in the sun is one of the most typical of thermoregulation in reptiles (Seebacher and Franklin 2005). The animals expose the

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entire or at least a portion of their body to thermal radiation while being immobile (Bulté and Blouin-Demers 2010). Thus, habitat selection is a critical component of reptilian ectothermy. Various thermal qualities of microhabitats/substrates influence a reptile's choice in selecting the most favourable basking sites in order to maintain preferred body temperatures (Meek and Luiselli 2022a). A poorly-understood type of behaviour is sharing basking microhabitats between individuals, in locations where there are many favourable sites and, thus, limited pressure from the lack of potential sources.

Our observations took place at the confluence of the Dunajec and Poprad rivers near the town of Stary Sącz in southern Poland (49°33'45"N, 20°38'11"E; Fig. 1), which is located south of the valley of Kotlina Sądecka. The observation area was 143 ha, including a wide variety of terrestrial and aquatic habitats suitable for the local herpetofauna. The landscape is dominated by deciduous trees (*Betula* sp., *Cornus* spp., *Populus* spp., *Salix* spp., *Quercus robur*) with a rich undergrowth (*Arrhenatherum* sp., *Dactylis glomerata*, *Salvia* spp., *Urtica dioica*) (Fig. 2). Open areas are characterised by a low percentage of shrub cover and an abundance of short herbaceous cover predominantly of *Rubus* spp. At one location (observation no. 7, Fig. 1), there were illegal landfills and trash dumping (anthropogenic refugia for snakes) covered by *Parthenocissus* sp. (Fig. 3).

As reptile predators influence the social behaviour in lizards (Chapple 2003), we recorded tentative predators; we often observed Hooded Crows (*Corvus cornix*), Common Ravens (*Corvus corax*), Common Pheasants (*Phasianus colchicus*) and rarely the Western Marsh Harriers (*Circus aeruginosus*), Common Buzzards (*Buteo buteo*) and the Common Kestrel (*Falco tinnunculus*). We also observed Red Foxes (*Vulpes vulpes*), Northern White-breasted Hedgehogs (*Erinaceus roumanicus*) and feral cats (*Felis catus*). However, during the fieldwork, predators were never observed in the immediate vicinity of the described records.

The field surveys were performed from February to October each year. Interactions were observed in the period from 24 July 2020 to 1 May 2022 (Table 1). Fieldwork was carried out 4 to 6 times a month, approximately every 4 to 5 days, from 08:30 h to 16:00 h, by one or two researchers. We applied a Visual Encounter Survey (VES), repeated a minimum of two times per survey day (Bartman et al. 2016; Boback et al. 2020). Each animal observed was photographed if possible and only positively identified species were recorded. No animals were captured or handled. All records were made in an area seemingly providing an abundance of suitable habitats. Environmental variability (e.g. scarcity of suitable habitats) can exert substantial pressure in rapidly switching between social and solitary behaviour in squamates (Rabosky et al. 2012; Regnet et al. 2017; Vasconcelos et al. 2017). We defined grouped, or paired basking, as direct contact between individuals or a distance not greater than 50 cm between them.

Overall, we observed *Vipera berus* on 58 occasions; basking aggregations were recorded five times (in four instances with another *V. berus* and in one with *N. natrix*).

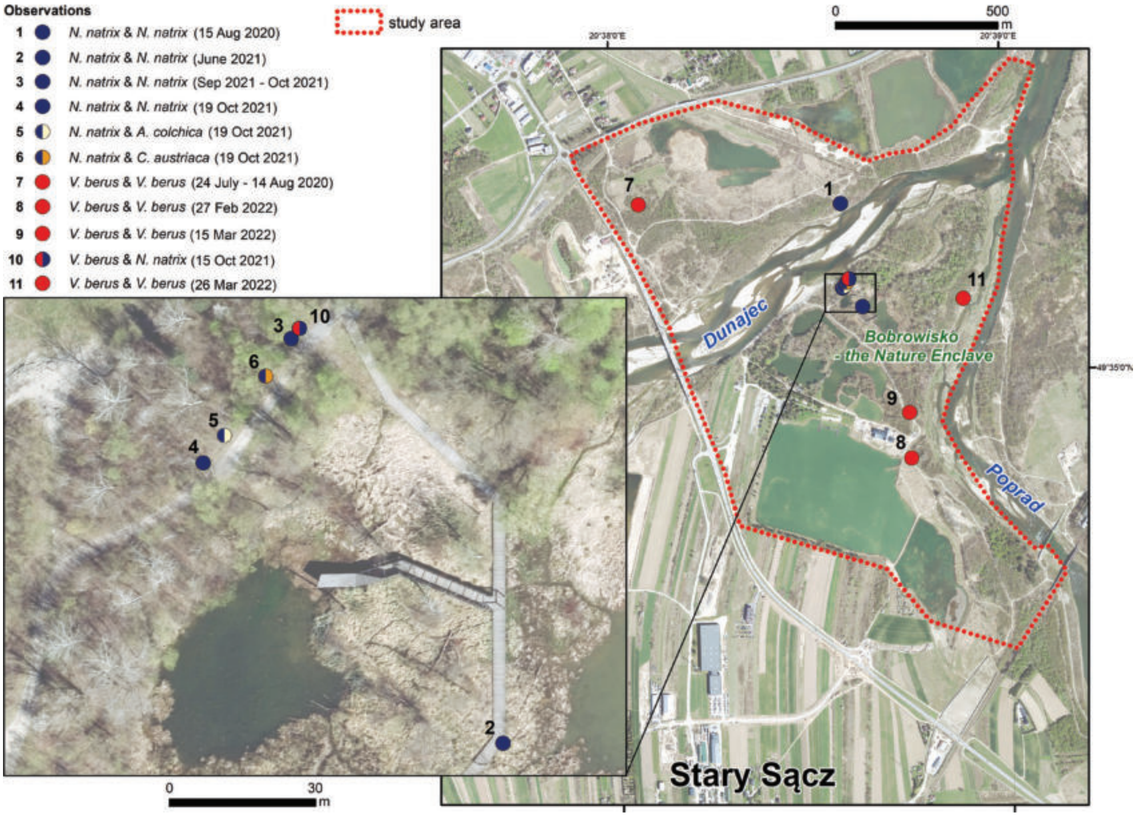

Figure 1. Records of microhabitat sharing during basking observed between July 2020 and March 2022 near the Dunajec and Poprad rivers confluence, Stary Sącz, Poland. Map created by Aleksandra Kolanek using base maps from https://mapy.geoportal.gov.pl/.

Figure 2. Examples of the habitats for observations no. 4, 5, 6, 3, 10 (see Fig. 1).

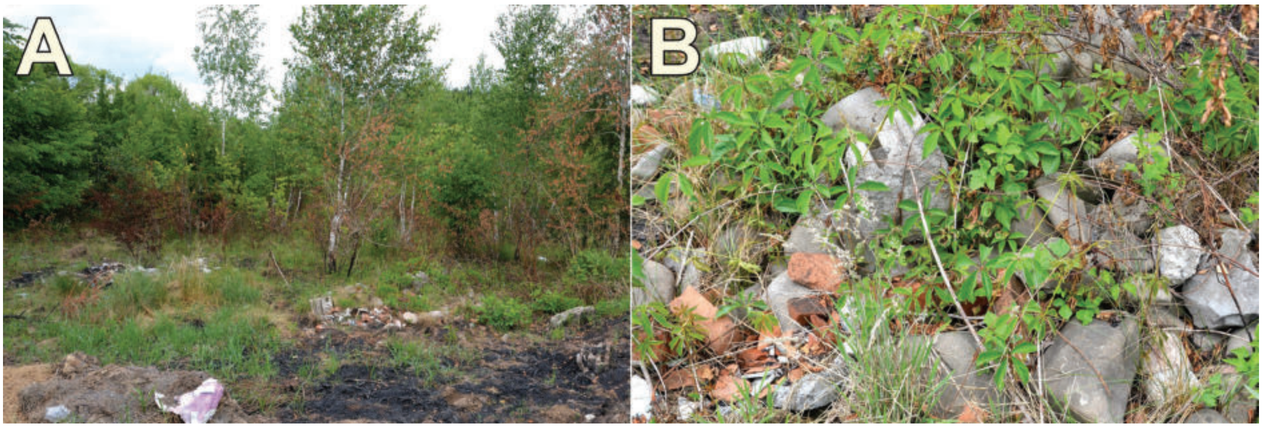

Figure 3. A pile of trash (**A**) and stones surrounded by grasses and thickets (**B**). The location is a basking site and refugia for *Vipera berus*.

For *N. natrix*, we observed communal basking on 7 out of 26 observations (four times with *N. natrix* and once each with *V. berus*, *Coronella austriaca* and *Anguis colchica*) (Table 1). In the case of adders, 8.62% share space for basking and 19.35% in the case of observed Grass Snakes.

First of our observations (and the only interaction from 2020) of *N. natrix* involving two adults basking next to each other (Fig. 4A). Subsequently, we observed snakes basking in three different locations regularly (Fig. 4B–D). *Natrix* spp. aggregates are frequently encountered during the breeding season, in limited areas and are rarely documented outside of this time period (Luiselli 1996; Meister et al. 2012; Ajtić et al. 2013).

We also observed communal basking of an adult and juvenile *N. natrix* alongside an adult Eastern Slowworm, *Anguis colchica* Nordmann, 1840 (Squamata, Anguidae) (Fig. 5A, B) and another individual alongside an adult Smooth Snake *Coronella austriaca* Laurenti, 1768 (Squamata, Colubridae) (Fig. 5C). These were one-time observations, the snakes were no longer observed together during the survey at this site. Only solitary Smooth Snakes were regularly seen at this location (no. 6) in later observations. *Coronella austriaca* are known for feeding on snakes and legless lizards (Čeirāns and Nikolajeva 2017; Di Nicola et al. 2020; Kolanek et al. 2020), although we did not observe specific predation events.

Table. 1. Observations of communal basking of squamates near Stary Sącz, Poland. Observation No. corresponds to localities indicated on Fig. 1.

No.	Date	Species	Times of day	Weather	Wind	Notes/Remarks	Coordinates
1	15/08/2020	*N. natrix* & *N. natrix*	Morning	partly sunny	light	2 adults often seen in wood pile	49.588028°N, 20.643076°E
2	06/2021	*N. natrix* & *N. natrix*	Morning/ afternoon	mostly cloudy	light	2 subadults basking on a root in a swamp	49.585155°N, 20.643923°E
3	09-10/2021	*N. natrix* & *N. natrix*	Afternoon	sunny	no wind	2 individuals basking in the bushes by the path	49.585911°N, 20.643346°E
4	19/10/2021	*N. natrix* & *N. natrix*	Morning	sunny	no wind	2 adults near the path	49.585685°N, 20.643088°E
5	19/10/2021	*N. natrix* & *A. colchica*	Morning	sunny	no wind	1 Juvenile and 1 adult *N. natrix* with an adult *A. colchica*	49.585735°N, 20.643150°E
6	19/10/2021	*N. natrix* & *C. austriaca*	Morning	sunny	no wind	2 adults snakes basking at 10:00 h in the sunny clearances	49.585843°N, 20.643271°E
7	24/06-14/08/2020	*V. berus* & *V. berus*	Morning/ afternoon	sunny	no wind	3 gravid females were regularly observed in the same place	49.588111°N, 20.634451°E
8	27/02/2022	*V. berus* & *V. berus*	Afternoon	partly sunny	light	2 adults with a melanistic pattern and with a classic pattern	49.580929°N, 20.645878°E
9	15/03/2022	*V. berus* & *V. berus*	Afternoon	sunny	no wind	2 adults with a melanistic pattern	49.582192°N, 20.645833°E
10	15/10/2021	*V. berus* & *N. natrix*	Morning	sunny	light	Juvenile *V. berus* with an adult *N. natrix*	49.585930°N, 20.643370°E
11	26/03/2022	*V. berus* & *V. berus*	Morning	partly sunny	light	2 adults with a classic pattern	49.585328°N, 20.648220°E

Figure 4. *Natrix natrix* communal basking. **A.** Observation no. 1, the same specimens were seen several times (without photographic documentation) in August 2020; **B.** Observation no. 2 in June 2021; **C.** Observation no. 3 in September/October 2021; **D.** Observation no. 4, a single record from 19 October 2021. Photographs by Maksymilian Jarmoliński.

In 2020, we observed three gravid *V. berus* Linnaeus, 1758 (Squamata, Viperidae) sharing a basking site (Fig. 6A), a behaviour also observed in *Crotalus horridus* Linnaeus, 1758 (Squamata, Viperidae) (Clark et al. 2012). To the best of our knowledge, this behaviour has not previously been published in Polish populations of adders. Observation no. 10 was of a juvenile *V. berus* basking directly on an adult *N. natrix* (Fig. 6B). In this case, the

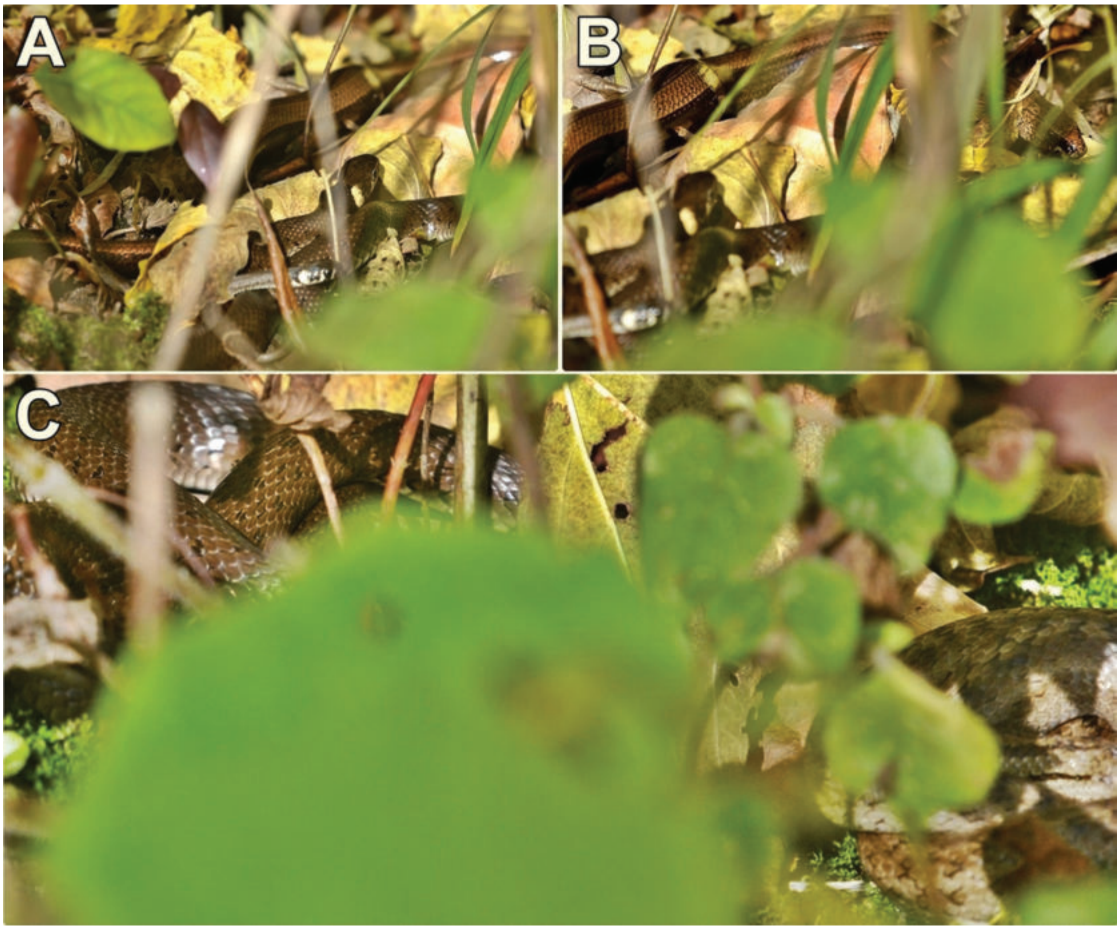

Figure 5. **A, B.** Two Grass Snakes (*Natrix natrix*) basking with an Eastern Slowworm (*Anguis colchica*); **C.** Basking *Natrix natrix* alongside a Smooth Snake (*Coronella austriaca*). Photographs by Maksymilian Jarmoliński.

Figure 6. **A.** Three gravid adders (*Vipera berus*) share a site for thermoregulation; **B.** Juvenile *V. berus* basking on top of a Grass Snake (*Natrix natrix*). Two adult adders with a classic (**C**), melanistic (**D**) and mixed (**E**) pattern thermoregulating together. Photographs by Maksymilian Jarmoliński.

direct contact between the two species was clearly visible (it was observed only on one occasion). In a separate observation (no photographic documentation was made), an adult *V. berus* was observed with a *N. natrix* at the same location (pers. obs.).

Observations 8, 9 and 11 of *V. berus* were of two adults with a classic pattern of thermoregulating and sharing a microhabitat (Fig. 6C), two melanistic adults basking together (Fig. 6D) and two snakes with a classic and a melanistic pattern after leaving the hibernaculum at the end of February 2022 (Fig. 6E). These few observations suggest that adders do not discriminate the body pattern of individuals with whom they bask. Of the 64 adders observed, 20 (31.75%) individuals were melanistic. Based on the hypothesis of thermal melanism in other species of snakes (*N. natrix*), the high rate of predation, as well as the benefits of climatic conditions, must be investigated (Bury et al. 2022). Additional observations might shed light on this interesting type of choice or the lack of it. Other factors need to be considered, such as age, sex, size and health of the animal, as well as possible effects from being gravid or digesting a meal.

Adders are typically solitary and social interactions are mostly observed between adult snakes and are limited to a short annual mating season (Nilson 1980; Otte et al. 2020). Adders hibernate individually or in small groups, infrequently basking together after emerging from the hibernaculum (Viitanen 1967; Phelps 2004; Dyugmedzhiev et al. 2019). Group basking and refugia sharing, apart from the above-mentioned situations, are only occasionally mentioned in scientific literature (Bauwens and Claus 2021).

Conclusions

Our observations indicate that there is more that needs to be discovered about inter- and intraspecific communal basking. Interspecific sharing of microhabitats/basking spots by squamate reptiles are rarely described in scientific literature and even less often in Polish language publications (Juszczyk 1974; Bauwens and Claus 2021). However, future studies should include additional records of this kind of behaviour that could help determine why there is no sharing of micro-habitats in other potentially-suitable localities. Supplementary data on body and microhabitat temperatures, substrate composition and type (Meek and Luiselli 2022b), habitat complexity and microhabitat characteristics, as well as local meteorological conditions, may be necessary to understand communal basking. More comprehensive research on specific populations sharing microhabitats opens up the possibility of studying in-depth different types of behaviour in the context of thermoregulation. Previous studies on reptiles show direct competition or interference in lizards and turtles (Cady and Joly 2003; Žagar et al. 2015; Hamilton 2021). However, so far there is a noticeable lack of such observations in snakes (Bauwens and Claus 2021) with the exception of garter snakes, *Thamnophis* spp. (Edgehouse et al. 2014). Here, we present data to aid in alleviating this lack of understanding of the relationship between animals in the context of habitat; this can significantly help in many cases, such as habitat management of protected species.

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The herpetofaunal diversity of Takhar Province, Afghanistan

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Abstract

Takhar Province of Afghanistan was previously known to harbour only two species of amphibians and eight species of reptiles with no record of snake species. From 2020 to 2021, we collected herpetological data from the Province and identified 14 species and specimens representing species complexes (*Bufotes viridis* complex and *Trapelus agilis* complex), with first records of *Testudo horsfieldii* (Testudinidae), *Paralaudakia badakhshana* (Agamidae), *Pseudopus apodus* (Anguidae), *Tenuidactylus caspius* (Gekkonidae), *Eumeces schneideri* (Scincidae) and *Varanus griseus* (Varanidae). In addition, we report the first province records of snakes, including *Eryx tataricus* (Erycidae), *Hemorrhois ravergieri* (Colubridae), *Natrix tessellata* (Natricidae) and the venomous species *Naja oxiana* (Elapidae). These records connect and supplement previously obtained data of these species from surrounding provinces and neighbouring countries. Our data reveal that Takhar Province is home to at least 20 species of amphibians and reptiles with a strong affiliation to the Turanian chorotype. All these data are a significant source of information for the diversity of herpetofauna in Afghanistan and subsequent biodiversity research in the Central Asiatic region.

Key Words

Anguidae, Central Asia, Colubridae, Elapidae, Hindu Kush, Natricidae, new records, range extension, zoogeography

Introduction

The recent research related to the herpetofauna of Afghanistan, as well as its overall biodiversity, is very rarely available (Jablonski et al. 2021b). The reasons to such a hiatus in biodiversity research in Afghanistan is well-known worldwide (long-term security situation in the war and later post-war turmoil) and, thus, the advancement in creating and sustaining research environment is at the backstage. Since the publication of a review compiling museum specimens and related distribution data for amphibians and reptiles of the country (Wagner et al. 2016), only several studies have brought new information on biogeography, distribution, species composition (Jablonski et al. 2019b; Jablonski and Masroor 2021; Jablonski et al. 2021a, c, 2022) and even to evolutionary history or molecular taxonomy of amphibians and reptiles from populations reaching the Afghan territory, particularly genera *Bufotes*, *Eremias*, *Natrix* and *Naja* (Dufresnes et al. 2019; Kazemi et al. 2021; Khan et al. 2021; Masroor et al. 2022; Jablonski et al. 2023). These preceding works were related to the overall species distribution and diversity of the country with a marginal impact on regional or provincial data. Only recently, a publication, based on random observations of herpetofauna in Kunduz Province has been published (Jablonski et al. 2021e). The study provided recent observations on five species of amphibians and reptiles with the first formal

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record of *Varanus griseus caspius* (Eichwald, 1831) for Kunduz Province. Detailed and recent data focusing on other provinces of Afghanistan are, however, almost non-existent (see Jablonski et al. (2019b)).

The overall knowledge of the species composition of amphibians and reptiles of Takhar Province comes from the collection of Richard Clark who visited Takhar in 1968 (Clark 1990). Between 28 and 29 April 1968, he collected specimens that are now stored at the California Academy of Sciences, USA (CAS). Earlier in 1964, a small collection of *Trapelus agilis* was made by Clas M. Naumann (1939–2004), lepidopterist and founder of the Zoological Museum in Kabul (Naumann and Nogge 1973). The collection is now in the Museum Koenig (ZFMK), Bonn, Germany under voucher numbers ZFMK 8594–97. As summarised in Wagner et al. (2016), Takhar Province is, according to the previous knowledge, inhabited by the amphibians, including the *Bufotes viridis* species complex (Bufonidae) (probably *Bufotes turanensis*), *Pelophylax terentivevi* (Ranidae) and reptiles represented by *Paralaudakia caucasia*, *P. lehmanni*, *Trapelus agilis*, *T. sanguinolentus* (Agamidae), *Tenuidactylus turcmenicus* (Gekkonidae), *Eremias nigrocellata,* and *E. velox* (Lacertidae). Recently, Jablonski et al. (2021a) provided data on the presence of *Laudakia nuristanica* (Anderson & Leviton, 1969) from the central parts of the Province. These published data suggest that the herpetofauna of Takhar Province is formally composed of two species of amphibians (although the *Bufotes viridis* complex is probably represented by several species; cf. Dufresnes et al. (2019) and comment below) and eight species of reptiles. Surprisingly, no snake species have been recorded for the Province so far. Whereas we recently herpetologically explored Takhar Province and collected valuable information, we present our results in the context of our recent findings and previously published field and museum data.

Materials and methods

Takhar Province lies in the north-eastern part of Afghanistan and borders Tajikistan (Fig. 1). The four Provinces, Badakhshan, Panjsheer, Baghlan and Kunduz share their borders with Takhar (Fig. 1). This Province is also unique in the sense that it is covered by different types of habitats with a large difference in altitude from lowlands of the Panj River (ca. 450 m a.s.l.) to Qulah-ye Shakarāw Mountain (5818 m a.s.l.) that is the highest point of the Province. Thus, habitats are formed by mountain landscapes (Hindu Kush) in the south and hilly or lowland areas (mostly of semi-desert or desert features, steppes and agricultural fields) in the central and northern parts. According to Olson et al. (2001), the main ecoregions in Takhar Province include Hindu Kush alpine meadows and *Paropamisus* xeric woodlands and border areas of the Province on the Panj River have been suggested as Darqad Wildlife Reserve (Johnson et al. 2012). Due to its geographic position and variety of different habitats and landscapes, Takhar Province represents an interesting, but underestimated area for biodiversity research.

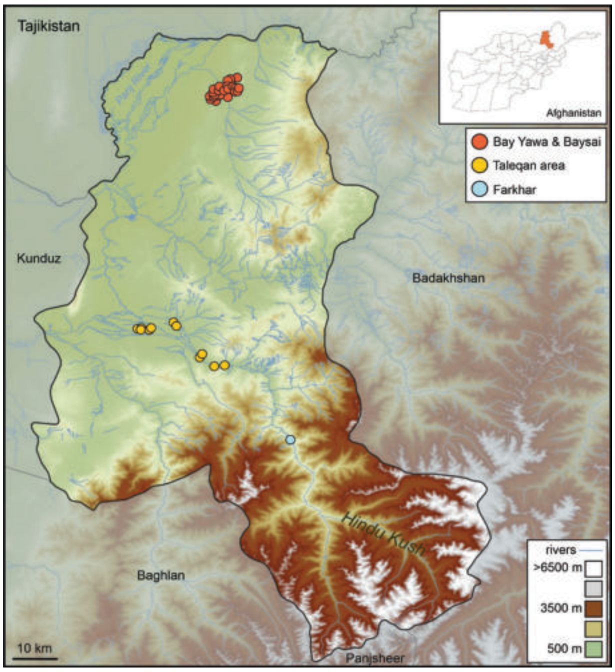

Figure 1. Herpetologically investigated localities of Takhar Province, Afghanistan.

During 2020 and 2021, we collected herpetological data from Takhar Province, northern Afghanistan (Fig. 1) in different areas (27 localities with 60 points of original coordinates) and habitats of the Province (Fig. 2). These data were collected by direct observations in the field, mostly from spring to autumn. Some data were supplemented from observations of local people if these observations were well supported with clear species descriptions and photos. Besides species identifications, we collected date of observation, the number of observed individuals, their age and sex, exact locality using geographic coordinates and the name of the closest village, elevation, type of the habitat, as well as other pertinent information. When possible, photographic vouchers were taken and archived in the Comenius University Herpetological Collection, Bratislava, Slovakia (CUHC-PA) under numbers CUHC-PA 160–222. The obtained data were compared with the correct and updated dataset of localities and coordinates of Afghan amphibians and reptiles (Jablonski, pers. data) published by Wagner et al. (2016), citizen-science data (GBIF–the Global Biodiversity Information Facility) and subsequent studies on Afghan herpetofauna published afterwards (see Results and Discussion). Due to significant differences in morphology and ecology, we follow the taxonomy of Baig et al. (2012) and Wagner et al. (2016) for genera *Laudakia* and *Paralaudakia*. This split is doubtful for some authors (Pyron et al. 2013), but in the same way genetically arguable and, thus, needs further investigations. The observed species were also classified according to the chorotype classification of Vigna Taglianti et al. (1999), and modified sensu Sindaco and Jeremčenko (2008) and Sindaco et al. (2013). The distribution data were visualised using QGIS Desktop 3.20.1 software (2022) and present-

Figure 2. Eco-physiographic habitats of Takhar Province, Afghanistan: **A.** Bay Yawa (Qizilqiye), habitat of *Naja oxiana*; **B.** Bay Yawa (Chanqutan), habitat of *Pseudopus apodus*, *Varanus griseus* and *Testudo horsfieldii*; **C.** Bay Yawa, agricultural fields, habitat of the *Bufotes viridis* complex and *Pelophylax terentievi*; **D.** vicinity of Bay Yawa Village.

ed into final maps, updated with new records for Takhar Province. The input data with geographic coordinates for species and source references, resulting to the here-presented map outputs, are provided in Suppl. material 1.

The following museum/collection abbreviations were used throughout the text (sensu Sabaj (2020)):

BMNH	(nowadays NHMUK) The Natural History Museum (formerly British Museum of Natural History), London, United Kingdom.
CAS	California Academy of Sciences, San Francisco, USA.
CUHC	Comenius University Herpetological Collection, Bratislava, Slovakia.
FMNH	Field Museum of Natural History, Chicago, USA.
MZLU	Zoologiska Museet, Lunds Universitet, Lund, Sweden.
NMW	Naturhistorisches Museum, Wien, Austria.
ZFMK	Leibniz-Institut zur Analyse des Biodiversitätswandels, former Zoologisches Forschungsmuseum Alexander Koenig, Bonn, Germany.
ZMUC	Københavns universitet Zoologisk Museum, Copenhagen, Denmark.

Results

AMPHIBIA

Anura

Bufonidae

Bufotes viridis (Laurenti, 1768) complex[*]

Figs 3, 4A–C

Takhar records. Taleqan (Gulayi Bagh), 36.4433°N, 69.3058°E; 792 m a.s.l., 31 January 2020, found dead in grassy habitat near stream, one individual of unknown sex (CUHC-PA 197); Taleqan (Takhar University Campus), 36.7427°N, 69.5162°E, 792 m a.s.l., 8 May 2020, garden near stream, one adult female (CUHC-PA 168); Taleqan (Takhar University Campus), 36.7420°N, 69.4860°E, 800 m a.s.l., 16 May 2020, open field, one subadult female (CUHC-PA 167); Taleqan (Pusthhur), 36.7607°N, 69.5793°E, 899 m a.s.l, 18 May 2020, semi-desert area, one subadult individual of unknown sex (CUHC-PA 189); Taleqan (Gulayi Bagh), 36.7386°N, 69.5101°E, 794 m a.s.l., 28 May 2020, garden near stream, two adult females (CUHC-PA 173).

Distribution in Afghanistan. Badakhshan, Badghis, Balkh, Bamiyan, Faryab, Ghazni, Herat, Kabul, Kandahar, Kunduz, Laghman, Logar, Nangarhar, Parwan,

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[*] In the studied area possibly including *Bufotes baturae* (Stöck, Schmid, Steinlein & Grosse, 1999); *Bufotes perrini* Mazepa, Litvinchuk, Jablonski & Dufresnes, 2019; *Bufotes pewzowi* (Bedriaga, 1898), *Bufotes turanensis* (Hemmer, Schmidtler & Böhme, 1978).

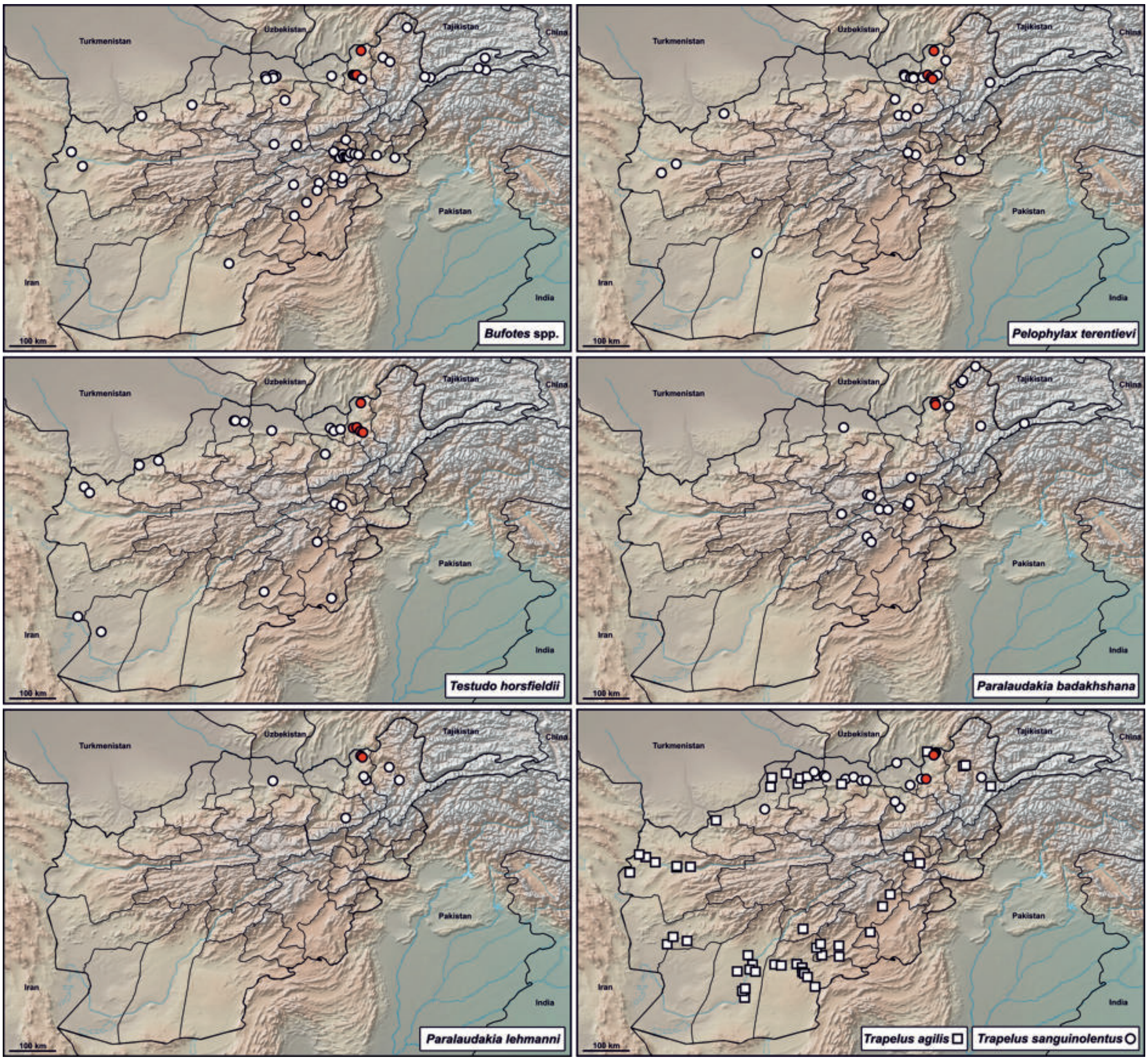

Figure 3. Herpetofaunistic records of Bufonidae, Ranidae, Testudinidae and Agamidae from Takhar Province in the context of updated records from Afghanistan (white dots or squares: published data (except for the data on *Bufotes oblongus* and *B. zugmayeri*, which are distributed in SW Afghanistan); red dots: new data).

Samangan, Takhar, Wardak (Wagner et al. 2016; Dufresnes et al. 2019; Jablonski et al. 2019a, b) Provinces, representing distribution in 50% of all Afghan Provinces.

Chorotype. *Bufotes turanensis* is the most likely species found in Takhar Province and belongs to the Turanian chorotype.

Remarks. The most comprehensive overview based on different types of data, but mostly genetics, has been provided by Dufresnes et al. (2019). According to these authors, *B. pewzowi*, *B. baturae*, *B. turanensis* and *B. perrini* are or may be present in northern Afghanistan and thus possibly in Takhar Province. Nevertheless, it is necessary to verify this through DNA-based approaches on a more comprehensive sampling of these toads collected from various locations within Afghanistan – most of data used by the published paper originated from the border areas of Tajikistan and Uzbekistan; see fig. S4 in Dufresnes et al. (2019). Individuals that we photographed resemble *B. turanensis* that was mentioned from Takhar Province as a single specimen from Taleqan (CAS 120973; Wagner et al. 2016). In such case, our observations provide the second confirmation of this species for the Province.

Ranidae

Pelophylax terentievi (Mezhzherin, 1992)

Figs 3, 4D–F

Takhar records. Taleqan (Gulayi Bagh), 36.4433°N, 69.3058°E; 792 m a.s.l., 31 January 2020, found dead in grassy habitat near stream, one individual of unknown sex (CUHC-PA 198); Bay Yawa (Baghi Mullah Gulmad), 37.4379°N, 69.7121°E, 670 m a.s.l., 31 March 2020, irrigation channels and pools, one adult female (CUHC-PA 186); Bya Yawa, 37.4311°N, 69.7185°E, 666 m a.s.l., 27 April

Figure 4. **A.** Adult individual of the *Bufotes viridis* complex from Taleqan (CUHC-PA 167); **B.** Adult female of the *B. viridis* complex from Taleqan (CUHC-PA 168); **C.** Adult female of the *B. viridis* complex from Taleqan (Gulayi Bagh) (CUHC-PA 173); **D.** Adult female of *Pelophylax terentievi* from Taleqan (Gulayi Bagh) (CUHC-PA 170); **E.** Adult male of *P. terentievi* from Takatuymaz (Tangi Farkhar) (CUHC-PA 171); **F.** Subadult individual of *P. terentievi* from Bay Yawa (Parchaw Khana) (CUHC-PA 215).

2020, rice field, one adult female (CUHC-PA 166); Taleqan (Khitayan), 36.6590°N, 69.6565°E, 927 m a.s.l., 10 May 2020, rice field, three adult males (CUHC-PA 169); Taleqan (Gulayi Bagh), 36.7426°N, 69.5162°E, 794 m a.s.l., 12 May 2020, during the middle of the night in the garden, one adult female (CUHC-PA 170); Takatuymaz (Tangi Farkhar), 36.6326°N, 69.6969°E, 982 m a.s.l., 13 May 2020, irrigation channels and pools, one adult and three juveniles of both sexes (CUHC-PA 11); Bay Yawa (Parchaw Khana), 37.4272°N, 69.7209°E, 680 m a.s.l., 29 May 2020, Prut River vicinity; one subadult of unknown sex (CUHC-PA 187); Bay Yawa (Parchaw Khana), 37.4287°N, 69.7199°E, 675 m a.s.l., 4 September 2021, canal, one subadult of unknown sex (CUHC-PA 204); Bay Yawa (Parchaw Khana), 37.4254°N, 69.7215°E, 688 m a.s.l., 14 September 2021, canal, one subadult of unknown sex (CUHC-PA 215).

Distribution in Afghanistan. Badakhshan, Badghis, Baghlan, Helmand, Herat, Kabul, Kunduz, Nangarhar and Takhar Provinces (Wagner et al. 2016; Jablonski et al. 2021c, e), representing 26% of all provinces.

Chorotype. Turanian.

Remarks. *Pelophylax terentievi* is one of the ranid frogs that was originally reported from Takhar Province under the name *Rana ridibunda* Pallas, 1771 (Clark 1990). It was mentioned as an extremely abundant species living in streams, ponds and irrigation ditches (Clark 1990) which corresponds to our observations. The distribution of this species follows the Panj River, a tributary of the Amu Darya River. However, this species has also been reported from other parts of Afghanistan, even south of the Hindu Kush range [for review, see Jablonski et al. (2021c)].

REPTILIA
Testudines
Testudinidae

Testudo horsfieldii Gray, 1844

Figs 3, 5A, B

Takhar records. Bay Yawa (Khagebilendi), 37.4058°N, 69.6853°E, 1124 m a.s.l., 2, 4, 12, 21 and 22 April 2020, grassy habitat, tens of individuals from juveniles to adults of both sexes (CUHC-PA 179); Taleqan (Takhar University Campus), 36.7405°N, 69.4803°E, 792 m a.s.l., 5 May 2020, grassy habitat, tens of individuals from juveniles to adults of both sexes (CUHC-PA 178); Taleqan (Khitayan), 36.6638°N, 69.6580°E, 980 m a.s.l., 13 May 2020; grassy habitat, tens of individuals from juveniles to adults of both sexes (CUHC-PA 181); Tangi Farkhar, 36.6353°N, 69.7264°E, 1039 m a.s.l., 13 May 2020, grassy habitat, tens of individuals from juveniles to adults of both sexes (CUHC-PA 182); Taleqan (Pusthhur), 36.7492°N, 69.5869°E, 814 m a.s.l., 18 May 2020, grassy habitat, tens of individuals from juveniles to adults of both sexes (CUHC-PA 180).

Distribution in Afghanistan. Badghis, Baghlan, Balkh, Farah, Ghazni, Herat, Jowzjan, Kabul, Kunduz, Nimroz, Paktika and Zabul Provinces (Wagner et al. 2016; Jablonski et al. 2019b, 2021e), representing 35% of all provinces.

Chorotype. Turanian.

Remarks. We provide the first record of *T. horsfieldii* for Takhar Province. These are currently the easternmost records of the species in Afghanistan. The geographically closest records are from Kunduz Province, ca. 30 km by air distance westwards (Jablonski et al. 2021e).

Squamata
Agamidae

Paralaudakia badakhshana (Anderson & Leviton, 1969)

Figs 3, 5C–E

Takhar records. Khilyazi bala Village, 37.4684°N, 69.7585°E, 897 m a.s.l., 13 September 2021, rocky habitat, one adult male (CUHC-PA 212); Khilyazi bala, 37.4682°N, 69.7609°E, 927 m a.s.l., 13 September 2021, rocky habitat, one adult individual of unknown sex (CUHC-PA 213); Khilyazi bala Village, 37.4252°N, 69.7648°E, 884 m a.s.l., 15 September 2021, one subadult individual of unknown sex (CUHC-PA 218).

Distribution in Afghanistan. Badakhshan, Baghlan, Balkh, Bamiyan, Ghazni, Kabul, Parwan, Takhar, Wardak Provinces (Moheb and Mostafawi 2012; Wagner et al. 2016; Jablonski et al. 2019a), representing 26% of all provinces.

Chorotype. Western Central-Asiatic Mountains.

Remarks. We provide the first species records of this taxon for Takhar Province. It is an endemic species for Afghanistan and has never been genetically studied. It is possible that some historical distribution points (e.g. from Ghazni, specimens ZFMK 8608–12 and 13315–16) may represents different species and the range may change in future. Additionally, the type locality “Mazar-i-Sharif, northern Afghanistan, 36°34'N, 67°05'E, 457 m elevation” is challenged by some authors (Baig et al. 2012). Thus, DNA genotyping and detailed morphological examination of Afghan populations (presumably belonging to *P. badakhshana*), are necessary to further investigations.

Paralaudakia lehmanni (Nikolskii, 1896)

Figs 3, 5G–I

Takhar records. Bay Yawa Sharqi (Tundi Aman), 37.4223°N, 69.7543°E, 794 m a.s.l., 15 September 2021, rocky habitat, one adult male (CUHC-PA 216); Bay Yawa Sharqi, 37.4248°N, 69.7633°E, 871 m a.s.l., 15 September 2021, rocky habitat, one adult male (CUHC-PA 217); Bay Yawa Sharqi (Latakhali), 37.4322°N, 69.7399°E, 737 m a.s.l., 15 September 2021, rocky habitat, one subadult individual of unknown sex (CUHC-PA 222).

Distribution in Afghanistan. Badakhshan, Baghlan, Balkh (Wagner et al. 2016) and Takhar Provinces, representing 12% of all provinces.

Chorotype. Western Central-Asiatic Mountains.

Remarks. This study provides the first records of this species for Takhar Province. Same as for *P. badakhshana*, we have no information on genetics of this species from Afghanistan. It is a widely distributed lizard in Central Asia, distributed from middle to high elevations (Baig et al. 2012). The geographic coordinates 33.422272°N, 63.662567°E given for the locality “Darrah-e-Andarab, vic. of Bani [Baghlan Prov., 2100 m]” located in Ghor Province (Wagner et al. 2016) are wrong and, thus, are corrected here to Andarab Valley in Baghlan Province, approx. 35.6265°N, 69.2152°E.

Paralaudakia sp.

Fig. 5F

Takhar records. Khilyazi bala Village, 37.4687°N, 69.7584°E, 912 m a.s.l., 13 September 2021, rocky habitat, one adult male (CUHC-PA 214).

Figure 5. A, B. Individuals of *Testudo horsfieldii* from Bay Yawa (Khagebilendi) of different sex and ages (CUHC-PA 179); **C.** Adult male of *Paralaudakia badakhshana* from Khilyazi bala (CUHC-PA 212); **D, E.** Ventral and dorsal view on the individual of *P. badakhshana* from Khilyazi bala (CUHC-PA 213); **F.** Individual of *Paralaudakia* sp. from Khilyazi bala (CUHC-PA 214); **G, H.** Adult males (CUHC-PA 216 and 217) of *Paralaudakia lehmanni* from Khilyazi bala; **I.** Subadult individual of *P. lehmanni* from Bay Yawa (CUHC-PA 222).

Remarks. The observation of this individual is from the area where we observed *P. badakhshana* and *P. lehmanni*. However, the morphology of the specimen does not correspond to that of any of the two mentioned species. Until the species status is confirmed, we keep this record as *Paralaudakia* sp. It is a speculative explanation now, but this specimen may represent a hybrid between the two species of *Paralaudakia* distributed in the area. This needs further field investigations and subsequent genetic research.

Trapelus agilis (Olivier, 1804) complex*

Figs 3, 7A, B

Takhar records. Taleqan (Taqhar University Campus), 36.7408°N, 69.4858°E, 794 m a.s.l., 26 March 2019, grassy habitat, one adult individual of unknown sex (CUHC-PA 190); Bay Yawa (Tally Dere), 37.4128°N, 69.6939°E, 910 m a.s.l., 4 April 2020, semi-desert habitat in rocky area, one adult individual of unknown sex (CUHC-PA 191); Bay Yawa (Tashqutan), 37.4234°N, 69.6975°E, 877 m a.s.l., 2 September 2021, semi-desert habitat, one subadult individual of unknown sex found under a stone (CUHC-PA 202); Bay Yawa (Baghi Mullah Gulmad), 37.4292°N, 69.7210°E, 736 m a.s.l., 10 September 2021, semi-desert habitat, one adult male (CUHC-PA 207); Khilyazi payan near the primary school, 37.4552°N, 69.7361°E, 756 m a.s.l., 13 September 2021, rocky habitat near small stream, one adult individual of unknown sex (CUHC-PA 210).

Distribution in Afghanistan. This species complex (Shahamat et al. 2020) is distributed through Afghanistan. *Trapelus agilis* is mentioned from the Provinces of Badakhshan, Badghis, Balkh, Farah, Faryab, Ghazni, Helmand, Herat, Jowzjan, Kabul, Kandahar, Takhar, Uruzgan and Zabul (Wagner et al. 2016; Jablonski et al. 2019a, b), representing 41% of all provinces. *Trapelus sanguinolentus* is exclusively known from provinces north of Hindu Kush Mountains, i.e., Badakhshan, Baghlan, Balkh, Faryab, Jowzjan, Kunduz, Samangan and Takhar (Wagner et al. 2016; Jablonski et al. 2021e), representing 24% of all provinces.

Chorotype. Iranian + Turanian, and Sindhian (*T. agilis*).

Remarks. Based on external morphology and distribution of individuals, the observations from Takhar Province represent *T. sanguinolentus*. However, due to morphological similarities and the fact that both species are known in the literature from Takhar Province (specimens CAS 120275 and ZFMK 8594–97; Wagner et al. (2016)), we consider our identification as preliminary, pending genetic confirmation that is missing from this part of Central Asia (cf. Shahamat et al. (2020)). The geographic position for specimens ZMUC R-36133, ZMUC R-36161, ZMUC R-36150–55, ZMUC 36204–05, ZMUC R-36146–48 and 36156–57 was corrected from those mentioned by Wagner et al. (2016), based on the database of ZMUC. Additionally, the geographic position of specimens CAS 90765–66 and CAS 90777 is corrected, based on the data of CAS (Jablonski, pers. data).

Anguidae

Pseudopus apodus ssp. *apodus* (Pallas, 1775)

Figs 6, 7C, D

Takhar records. Bay Yawa, 37.4309°N, 69.7289°E, 681 m a.s.l., 11 April 2019, garden, two adult females (CUHC-PA 185); Bay Yawa, 37.4218°N, 69.6955°E, 911 m a.s.l., 12 April 2020, grassland, one juvenile of unknown sex (CUHC-PA 160); Bay Yawa (Shamal Dere), 37.4288°N, 69.6938°E, 968 m a.s.l., 15 April 2020, observed early in the day in and close to holes of the highly cultivated, open area, adult male (CUHC-PA 161); Bay Yawa (Shamal Dere), 37.4249°N, 69.6929°E, 936 m a.s.l., 15 April 2020, observed early in the day in and close to holes of the highly cultivated, open area, one adult of unknown sex (CUHC-PA 162); Bay Yawa (Tashqutan), 37.4186°N, 69.7083°E, 797 m a.s.l., 21 April 2020, cultivated, open area, one adult female (CUHC-PA 163); Bay Yawa (Keserkul), 37.4259°N, 69.7161°E, 731 m a.s.l., 21 April 2020, cultivated, open area, one adult male (CUHC-PA 164); Bya Yawa (Taqcha), 37.4005°N, 69.6980°E, 956 m a.s.l., 23 April 2020, cultivated, open area, one adult female (CUHC-PA 165).

Distribution in Afghanistan. The species is known from Badakhshan, Badghis, Baghlan, Farah and Herat Provinces, representing 15% of all provinces.

Chorotype. Mediterranean + Iranian + Turanian.

Remarks. The present record is the first-ever observation of this species for Takhar Province. The importance of this record is that it represents a connection to the unexpected, highly elevated record of the species from Badakhshan Province (locality Zebak, 2653 m a.s.l. from 18 August 1965; specimens FMNH 161121–22; Anderson and Leviton (1969)). It suggests that *P. apodus* could colonise warmer river valleys in the mountains from areas with higher population densities. More records are, thus, expected in the future from the north Provinces, such as Balkh, Faryab, Jowzjan or Kunduz, where this steppe and grassland species, surprisingly, has never been recorded (Jablonski et al. 2021e). The subspecies *P. a. apodus* (Pallas, 1775) is present in Central Asia and Afghanistan (see Jablonski et al. (2021d)).

Gekkonidae

Tenuidactylus caspius (Eichwald, 1831)

Figs 6, 7E

Takhar record. Bay Yawa, 37.4272°N, 69.7109°E, 725 m a.s.l., 31 January 2021, semi-desert habitat with bushes and burrows, hiding under stone, one subadult individual (CUHC-PA 192).

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* Including *Trapelus sanguinolentus sanguinolentus* (Pallas, 1827).

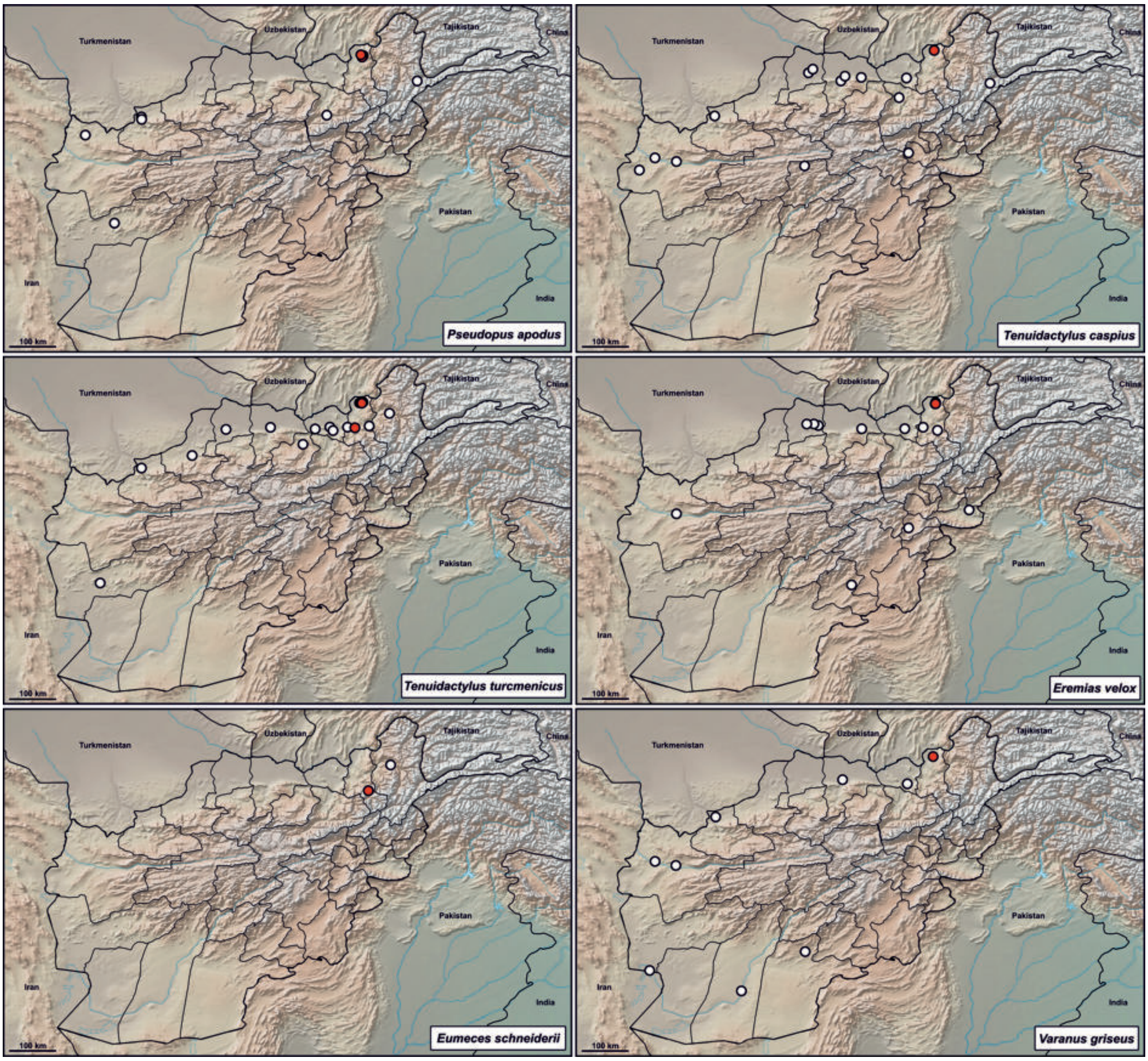

Figure 6. Herpetofaunistic records of Anguidae, Gekkonidae, Lacertidae, Scincidae and Varanidae from Takhar Province in the context of updated records from Afghanistan (white dots: published data; red dots: new data).

Distribution in Afghanistan. It is known from the Provinces of Badakhshan, Badghis, Baghlan, Balkh, Daykundi, Herat, Jowzjan, Kabul, Kunduz (Wagner et al. 2016; Jablonski et al. 2019) and Takhar, representing 26% of all provinces.

Chorotype. Turanian.

Remarks. We report this species for the first time from Takhar Province. The species is distributed mostly in northern provinces. Records from Badakhshan (CAS 115945, FMNH 161130), Daykundi (MZLU L957/3792) and Kabul (FMNH 161063) need further clarification as they represent highly elevated or records out of the main distribution range of the species. On the other hand, we identified that the specimen ZFMK 94240 represents *T. caspius* that was not mentioned in Wagner et al. (2016). This specimen represents the first record for Kunduz Province (36.6735°N, 68.9034°E, Kunduz army camp; cf. Jablonski et al. (2021e)) and a connection point to record from Takhar Province and possibly to record from Zebak (2653 m a.s.l.), Badakhshan (FMNH 161130, CAS 115945; Anderson and Leviton (1969)).

Tenuidactylus turcmenicus (Szczerbak, 1978)

Figs 6, 7F–H

Takhar records. Taleqan (Gulayi Bagh), 36.7426°N, 69.5163°E, 791 m a.s.l., 31 May 2020, wall of a house in the village, one adult individual of unknown sex (CUHC-PA 183); Bay Yawa (Qizilqiya), 37.4301°N, 69.7150°E, 694 m a.s.l., 2 September 2021, sandy cave, one adult individual of unknown sex (CUHC-PA 200); Bay Yawa (Qizilqiya), 37.4289°N, 69.7136°E, 703 m a.s.l., 2 September 2021, sandy cave, one adult individual of unknown sex (CUHC-PA 201); Bay Yawa (Sukhtedere), 37.4084°N, 69.6967°E, 834 m a.s.l., 2 September 2021,

Figure 7. **A.** Adult individual of the *Trapelus agilis* complex from Bay Yawa (Tally Dere) (CUHC-PA 191); **B.** Adult individual of the *T. agilis* complex Khilyazi payan (CUHC-PA 210); **C.** Juvenile individual of *Pseudopus apodus* from Bay Yawa (CUHC-PA 160); **D.** Adult male of *P. apodus* from Bay Yawa (Shamal Dere) (CUHC-PA 161); **E.** Subadult individual of *Tenuidactylus caspius* from Bay Yawa (CUHC-PA 192); **F.** Adult individual of *Tenuidactylus turcmenicus* from Taleqan (Gulayi Bagh) (CUHC-PA 183); **G.** Adult individual of *T. turcmenicus* from Bay Yawa (Qizilqiya) (CUHC-PA 200); **H.** Adult individual of *T. turcmenicus* from Khilyazi payan (CUHC-PA 211).

stony habitat, five observed individuals of unknown sex (CUHC-PA 203); Khilyazi Payan, 37.4384°N, 69.7319°E, 705 m a.s.l., 13 September 2021, sandy stones, one subadult individual of unknown sex (CUHC-PA 208); Bay yawa sharqi (Tundi Aman), 37.4568°N, 69.7385°E, 777 m a.s.l., 13 September 2021, sandy stones, one adult individual (CUHC-PA 211); Khilyazi payan, 37.4273°N, 69.7650°E, 947 m a.s.l., 15 September 2021, rocky habitat, two adult individuals of unknown sex (CUHC-PA 219); Khilyazi payan near primary school, 37.4358°N, 69.7461°E, 765 m a.s.l., 15 September 2021, rocky habitat, one subadult individual (CUHC-PA 221).

Distribution in Afghanistan. It is known from Badakhshan, Badghis, Balkh, Farah, Faryab, Jowzjan, Kunduz, Samangan and Takhar Provinces (Wagner et al. 2016; Jablonski et al. 2019a, b, 2021), representing 26% of all provinces.

Chorotype. Turanian.

Remarks. The species is already known from Takhar Province (specimens CAS 120317-21, CAS 121069). This species could be confused with *T. caspius* or other species of the genus. Thus, the extralimital record of the species from Farah Province (NMW 15879) should be verified and genetic research covering different populations of northern Afghanistan is needed. In this context, we corrected the locality and coordinates mentioned by Wagner et al. (2016) for ZMUC R-34128. This specimen is from Fayzabad in Badakhshan Province, collected on 21 March 1949 (D. Scherz, pers. comm.).

Lacertidae

Eremias velox ssp. *velox* (Pallas, 1771)

Figs 6, 8

Takhar records. Bay Yawa (Sukhtedere), 37.4316°N, 69.7145°E, 692 m a.s.l., 27 February 2019, observed on the ground, under stones, in holes or near vegetation in semi-desert habitat, dozens of adults of both sexes (CUHC-PA 172); Bay Yawa (Sukhtedere), 37.4219°N, 69.7124°E, 822 m a.s.l., 4 April 2020, observed on the ground, under stones, in holes or near vegetation in semi-desert habitat, dozens of adults of both sexes (CUHC-PA 174); Bay Yawa (Sukhtedere), 37.4148°N, 69.7125°E, 733 m a.s.l., 17 March 2020, observed on the ground, under stones, in holes or near vegetation in semi-desert habitat, dozens of adults of both sexes (CUHC-PA 184); Bay Yawa (Qizilqiya), 37.4273°N, 69.7109°E, 711 m a.s.l., 31 January 2021, semi-desert habitat, one adult female (CUHC-PA 193); Bay Yawa, 37.4268°N, 69.7063°E, 812 m a.s.l., 31 January 2021, semi-desert habitat, one adult male (CUHC-PA 194); Bay Yawa, 37.4130°N, 69.7094°E, 747 m a.s.l., 31 January 2021, semi-desert habitat, one adult female (CUHC-PA 195); Khilyazi Payan, 37.4384°N, 69.7319°E, 705 m a.s.l., 13 September 2021, rocky habitat and dry stream, one adult male (CUHC-PA 209).

Distribution in Afghanistan. It is known from the Provinces of Balkh, Herat, Jowzjan, Kunduz, Logar, Nangarhar, Takhar and Zabol (Wagner et al. 2016), representing 24% of all provinces.

Chorotype. Turanian + Iranian.

Remarks. This species has high phenotype variability (Fig. 8) and is considered a species complex with un-nested taxonomy (Rastegar-Pouyani et al. 2012; Khan et al. 2021). Populations originating in Iran that are showing high levels of genetic diversity can be expected also from the Afghan territory. The disjunct character of the species' distribution in Afghanistan (north and south of the Hindu Kush; Fig. 5) suggests a geographic isolation with possible subsequent ecological or genetic divergence that should be tested. From the list of specimens of *E. velox* provided by Wagner et al. (2016), we excluded the specimen ZFMK 8584 (locality "Ost-Afghanistan, Prov. Ghazni, Dasht-e-Nawar, 3000 m N.N.") that is the holotype of *Eremias afghanistanica* Böhme & Szczerbak, 1991.

Scincidae

Eumeces schneideri (Daudin, 1802) ssp. *princeps* (Eichwald, 1839)

Fig. 6

Takhar records. Farkhar, Chashmay-e-Garmak, 36.4223°N, 69.9155°E, 1459 m a.s.l., 12 June 2020, rocky habitat in the river valley, one subadult individual of unknown sex (CUHC-PA 196).

Distribution in Afghanistan. The genus *Eumeces* (see Remarks) is known from Badakhshan, Badghis, Helmand, Kandahar (Wagner et al. 2016) and Takhar Provinces. This represents 15% of all provinces in the country.

Chorotype. Mediterranean + Arabian + Iranian + Turanian.

Remarks. This is the first-ever record of this species from Takhar Province. However, it is not exactly clear what species it is and where it is distributed in Afghanistan. According to recently published phylogeography of *E. schneiderii* (Faizi et al. 2021), two species of this group should be present in Afghanistan: *E. zarudnyi* (Nikolskii, 1900) [elevated to species status by Faizi et al. (2021)] in southern Afghanistan and *E. schneiderii princeps* in northern parts of the country (Fig. 1 in Faizi et al. (2021)). This suggests that Takhar Province is inhabited by the latter taxon. However, to come to this conclusion, we are missing good morphological or genetic data that could confirm our assumption. Wagner et al. (2016) report *E. blythianus* (Anderson, 1871) for Afghanistan, based on a single record and specimen (ZFMK 41118) from "Seberghan" (36.66°N, 65.75°E) in the northern part of the country. This record should be verified since we expect that the specimen could represent the *E. schneiderii* group due to its record north of the Hindu Kush mountains (*E. blythianus* is known mostly from Pakistan; Masroor (2009); Faizi et al. (2017)).

Figure 8. The phenotype diversity in the *Eremias velox* complex from Tahkar Province: **A.** Adult male from Bay Yawa (Khilyazi Payin) (CUHC-PA 209); **B.** Subadult female from Bay Yawa (Sukhtedere) (CUHC-PA 172); **C, F.** Adult male from Bay Yawa (CUHC-PA 194); **D.** Adult female from Bay Yawa (CUHC-PA 193); **E.** Subadult male from Bay Yawa (Sukhtedere) (CUHC-PA 174), adult female from Bay Yawa (CUHC-PA 195).

Varanidae

Varanus griseus (Daudin, 1803) ssp. *caspius* (Eichwald, 1831)

Fig. 6

Takhar records. Bay Yawa (khujabilandi), 37.4203°N, 69.6961°E, 954 m a.s.l., 13 June 2014, grassy, semi-desert habitat, one adult individual of unknown sex.

Distribution in Afghanistan. The species has been recorded in Badghis, Balkh, Farah, Helmand, Herat, Kandahar, Kunduz (Eiselt and Adametz 1977, NMW; Wagner et al. 2016; Jablonski et al. 2021e) and Takhar Provinces. This represents 21% of all provinces in Afghanistan.

Chorotype. Saharan + Arabian + Iranian + Turanian + Indian.

Remarks. It is the first record of this species from Takhar Province. Although we only have a single documentation of this species, local people report *V. griseus* as common and it is regularly observed in the Bay Yawa area. The first record from the Kandahar Province is represented by the museum specimen NMW 34781/1, which is not mentioned in Wagner et al. (2016).

Erycidae

Eryx tataricus (Lichtenstein in Eversmann, 1823)

Figs 9, 10A, B

Takhar records. Bay Yawa (Keserkul), 37.4280°N, 69.7195°E, 685 m a.s.l., 29 May 2020, found in the arid habitat near water (CUHC-PA 188); Taleqan (Takhar University Campus), 36.7388°N, 69.4852°E, 792 m a.s.l., 17 September 2020, hilly area with the steppe vegetation, one juvenile individual of unknown sex (CUHC-PA 175); Bay Yawa Sharqi, 37.4337°N, 69.7538°E, 958 m a.s.l., 15 September 2021, rocky habitat, one adult individual of unknown sex (CUHC-PA 220).

Distribution in Afghanistan. This species has been recorded in Provinces of Badakhshan, Badghis, Faryab, Ghor, Helmand, Herat, Jowzjan, Kabul, Kunduz, Laghman, Nangarhar (Wagner et al. 2016) and Takhar. It represents 35% of all provinces.

Chorotype. Turanian.

Remarks. We bring the first record from Takhar Province that creates a connection to so-far isolated and surprisingly high elevated record (FMNH 161123) from Zebak "102.4 kilometres east of Faizabad", (2653 m a.s.l.; 18 August 1965), Badakhshan Province (Anderson and Leviton 1969). More data are needed regarding the morphology and genetics of the species from Afghanistan to resolve its distribution. It can be confused with *E. elegans* (Gray, 1849), a similar species inhabiting mostly rocky habitats. Record provided by Boulenger (1889), i.e. "Afghanistan" cannot be exactly georeferenced.

Colubridae

Hemorrhois ravergieri (Ménétriés, 1832)

Figs 9, 10C, D

Takhar records. Bay Yawa (Keserkul), 37.4264°N, 69.7127°E, 734 m a.s.l., 1 April 2020, dead on the road, killed by local people, one subadult male (CUHC-PA 177).

Distribution in Afghanistan. The species is known from the Provinces of Badakhshan, Bamiyan, Herat, Kabul, Kandahar, Kunduz, Nuristan, Paktia (Wagner et al. 2016; Jablonski et al. 2019b) and Takhar. It represents 26% of all provinces.

Chorotype. West Asian Mountains transition zone + Turanian + Iranian.

Remarks. The here-presented record is the first of this species for Takhar Province. It is a common Central Asiatic snake, often found in a variety of habitats (Szczerbak 2003). Some Central Asiatic populations or individuals are characterised by a black head (Tuniyev et al. 1997) which was also the case for the observed individual from Takhar.

Natricidae

Natrix tessellata (Laurenti, 1768)

Figs 9, 10E, F

Takhar records. Taleqan (Takhar University Campus), 36.7388°N, 69.4852°E, 775 m a.s.l., 14 September 2020, observed near the irrigation canal and rice fields around 11 p.m., one adult female eating *Pelophylax terentievi* (CUHC-PA 176); Bay Yawa (Parchaw Khana), 37.4088°N, 69.7346°E, 720 m a.s.l., 4 September 2021, stony shore near the water, juvenile individual of unknown sex (CUHC-PA 205); Bay Yawa, 37.4291°N, 69.7208°E, 681 m a.s.l., 9 September 2021, stony shore near water, one adult female (later killed by the local farmer).

Distribution in Afghanistan. The species is known from the Provinces of Badakhshan, Balkh, Bamiyan, Faryab, Herat, Jowzjan, Kabul, Kunduz (Wagner et al. 2016) and Takhar, representing 26% of all provinces.

Chorotype. Turanian + Siberian-European + Mediterranean + West Asian Mountains transition zone.

Remarks. Jablonski et al. (2023) studied populations of dice snakes from Central Asia including Afghanistan and found that their previously uncovered mitochondrial lineages (Guicking et al. 2009) are affiliated with specific river systems. The population from adjacent Kunduz Province has been affiliated to so-called Uzbekistan lineage distributed in the Amu Darya Basin. Moreover, Central Asiatic populations show deep divergence from populations from Europe and, thus, further taxonomical changes are possible with additional data. Our here-provided records are the first-ever documentation of this species for Takhar Province. The geographic origin of specimens BMNH 1938.2.4.12-14 mentioned by Wagner et al. (2016) is unknown. Thus, they cannot be included to the georeferenced dataset of the species from Afghanistan.

Elapidae

Naja oxiana (Eichwald, 1831)

Figs 9, 10G

Takhar record. Bay Yawa (Qizilqiya), 37.4267°N, 69.7085°E, 736 m a. s. l., 1 September 2019, observed on the sandy road during the evening and in the morning next day.

Distribution in Afghanistan. According to Wagner et al. (2016), this species is known from different parts of the country, mostly from the northern and south-eastern Provinces, i.e. Badghis, Herat, Kabul, Kunduz, Logar and Nangarhar (Fig. 9). It represents 18% of all provinces.

Chorotype. Iranian + Turanian + Palearctic-Oriental transition zone.

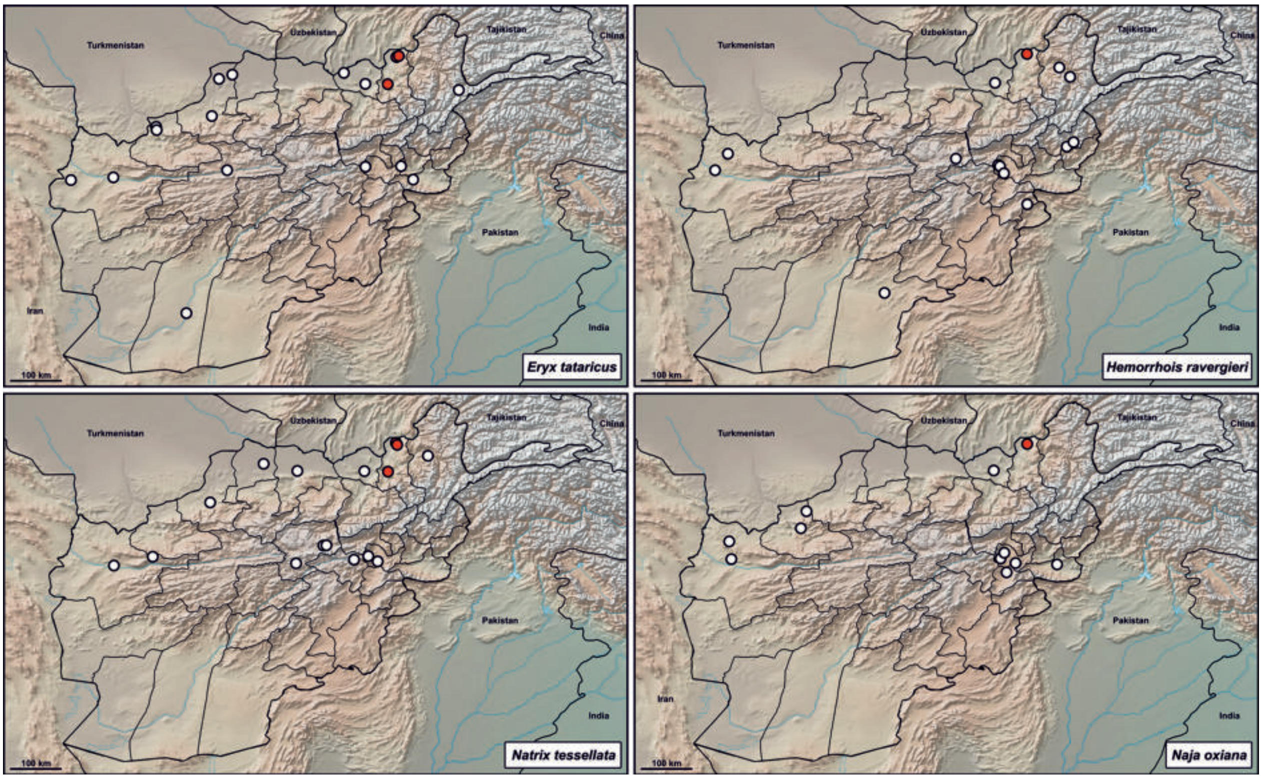

Figure 9. Herpetofaunistic records of Erycidae, Colubridae, Natricidae and Elapidae from Takhar Province in the context of updated records from Afghanistan (white dots: published data; red dots: new data).

Remarks. This is the first record of this species from Takhar Province. Kazemi et al. (2021) compared the genetic data of populations from northern Afghanistan with those from Iran and Turkmenistan and highlighted a shallow pattern of genetic variability suggesting ecological plasticity (the species is inhabiting variety of habitats) connected with rapid colonisation events in the past. However, populations south of the Hindu Kush Mountains were not studied and, due to this significant geographic barrier, we can expect the deeper genetic structure of the species.

Discussion

Our contribution brings a significant source of data for amphibians and reptiles from the poorly studied regions of Afghanistan, Takhar Province. Overall, we observed 14 species, two species complexes (*Bufotes viridis* complex and *Trapelus agilis* complex) and a population of *Paralaudakia* sp. that could not be assigned to any known taxa, based on available data. In the context of the overall lack of data on the species diversity and composition in the country (Jablonski et al. 2021b), such a dataset may contribute to better evaluation of ecologically important areas for conservation purposes sensu Johnson et al. (2012), primarily for the purposes of the National Environmental Protection Agency of Afghanistan.

The significance and simultaneously the rarity of data and poor field investigations are highlighted by the fact that our study brings the first published records of snakes for Takhar Province, particularly for four species in four families: *Eryx tataricus* (Erycidae), *Hemorrhois ravergieri* (Colubridae), *Natrix tessellata* (Natricidae) and the highly venomous species *Naja oxiana* (Elapidae). Except for these species, we also recorded for the first time *Testudo horsfieldii* (Testudinidae), *Paralaudakia badakhshana* (Agamidae), *Pseudopus apodus* (Anguidae), *Tenuidactylus caspius* (Gekkonidae), *Eumeces schneideri* (Scincidae), and *Varanus griseus* (Varanidae). The most frequently recorded species were *Pelophylax terentievi* (nine localities), *Tenuidactylus turcmenicus* (eight localities), *Pseudopus apodus* and *Eremias velox* (both with seven localities). Other observed species were recorded in five or less localities. High abundances were recorded for *T. horsfieldii* (tens of individuals). Although none of these amphibians or reptiles is new to Afghanistan, our records supplement our previous knowledge and better explain the distribution patterns of several species (e.g. *P. apodus*, *E. tataricus*; cf. Wagner et al. (2016)). According to our data and comparison of known records, all recorded species are distributed only in 50% or less of Afghan Provinces, including Takhar. We also increased the knowledge of the herpetofauna of the Province by 50% (10 species). Finally, our results highlight that the herpetofauna of Takhar Province is composed of two species of amphibians and 18 species of reptiles (see Appendix 1) that strongly connect to the so-called Turanian or related chorotypes. The Turanian chorotype was exclusively recorded in six species (30%) and part-

Figure 10. **A.** Juvenile individual of *Eryx tataricus* from Taleqan (CUHC-PA 175); **B.** Adult female of *E. tataricus* from Bay Yawa (Kaserkul) (CUHC-PA 188); **C, D.** Dorsal and ventral view on subadult male of *Hemorrhois ravergieri* from Bay Yawa (Kaserkul) (CUHC-PA 177); **E.** Ventral view on adult female of *Natrix tessellata* from Taleqan (CUHC-PA 176); **F.** Adult female of *N. tessellata* from Bay Yawa (CUHC-PA 206); **G.** Juvenile individual of *Naja oxiana* from Bay Yawa (CUHC-PA 199).

ly in 10 species (50%). This composition reflects the steppes and semi-desert habitats of the Province, typical for many parts of northern Afghanistan. Other species are represented by the Western Central-Asiatic Mountains chorotype (three species, 15%), Iranian chorotype (one species, 5%) or a combination of different chorotypes (see above).

Our data from the study area highlight the overall poor state of herpeto-faunistic investigations from Afghanistan. The country lies between Central and South Asia and is positioned to play a crucial role for understanding of the past historical dynamics related to current composition and diversity of amphibians and reptiles in the region. In this perspective, as an example to elaborate the in-depth understanding, few genera in the family Colubridae (*Oligodon*, *Ptyas*, *Lycodon*) have the core of their distribution in the Oriental zoogeographic realm, but these reach deeply into the territory of Central Asia (Tajikistan, Turkmenistan, Uzbekistan; Sindaco et al. (2013)) with limited or no records in the intervening areas of northern Afghanistan. We thus expect that the isolated distribution of these genera in Central Asia can be only a bias of data (or currently fragmented distribution), particularly their lack from Afghanistan (see Wagner et al. (2016); Jablonski et al. (2019b)). This is supported by a recently published study on populations of *Lycodon bicolor* (Nikolskii, 1903) from the southern and central Asiatic range as genetically closely related (Amarasinghe et al. 2023)

Besides, the importance of thermally suitable river and mountain valleys for species distribution and spreading in Central Asia is also highlighted in this study. A good example is *P. apodus* and *E. tataricus* and their possible ability to colonise high elevation areas as indicated, so far, by the isolated and almost 60 years old record in mountainous Badakhshan Province (Anderson and Leviton 1969). With the here-provided data from Takhar Province, we can say that such a record represents again a lack of data and thermophilic species may be present also in apparently less suitable areas. Thus, the comprehensive distribution and ecological limits of amphibians and reptiles in Afghanistan and Central Asia should be studied in the future in a broader sense. Despite Afghanistan being one of the most complicated countries for basic field research with many areas inaccessible, further biodiversity research combining basic field investigations and morphology with genetics is indispensable (Jablonski et al. 2021).

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Appendix 1

The updated list of amphibians and reptiles of Takhar Province (*this study).

AMPHIBIA
Anura
Bufonidae
1. ***Bufotes turanensis*** (Hemmer, Schmidtler, & Böhme, 1978)

Ranidae
2. ***Pelophylax terentievi*** (Mezhzherin, 1992)

REPTILIA
Testudines
Testudinidae
1. ***Testudo horsfieldii*** Gray, 1844 *

Squamata
Agamidae
2. ***Laudakia nuristanica*** (Anderson & Leviton, 1969)
3. ***Paralaudakia badakhshana*** (Anderson & Leviton, 1969) *
4. ***Paralaudakia caucasia*** (Eichwald, 1831)
5. ***Paralaudakia lehmanni*** (Nikolskii, 1896)
6. ***Trapelus agilis*** (Olivier, 1804)
7. ***Trapelus sanguinolentus*** ssp. ***sanguinolentus*** (Pallas, 1827)

Anguidae
8. ***Pseudopus apodus*** ssp. ***apodus*** (Pallas, 1775) *

Gekkonidae
9. ***Tenuidactylus caspius*** (Eichwald, 1831) *
10. ***Tenuidactylus turcmenicus*** (Szczerbak, 1978)

Lacertidae
11. ***Eremias nigrocellata*** Nikolskii, 1896
12. ***Eremias velox*** ssp. ***velox*** (Pallas, 1771)

Scincidae
13. ***Eumeces schneideri*** (Daudin, 1802) ssp. ***princeps*** (Eichwald, 1839) *

Varanidae
14. ***Varanus griseus*** (Daudin, 1803) ssp. ***caspius*** (Eichwald, 1831) *

Erycidae
15. ***Eryx tataricus*** (Lichtenstein in Eversmann, 1823) *

Colubridae
16. ***Hemorrhois ravergieri*** (Ménétriés, 1832) *

Natricidae
17. ***Natrix tessellata*** (Laurenti, 1768) *

Elapidae
18. ***Naja oxiana*** (Eichwald, 1831) *

Supplementary material 1

The source distribution data for species maps (without new data obtained from Takhar Province in this study)

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First record of a male-male aggressive interaction in the golden Alpine salamander *Salamandra atra aurorae* (Caudata, Salamandridae)

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Abstract

The golden Alpine salamander *Salamandra atra aurorae* Trevisan, 1982 is an endemic subspecies found in Sette Comuni and Vezzena plateau in Veneto, Italy. We describe an aggressive interaction between two males which fought for four minutes, trying to go on top of each other and rubbing their chin on the antagonist's head. This is the first documented case of aggressive behaviour in *Salamandra atra aurorae*.

Key Words

Amphibians, behaviour, ecology, male-male interaction

It is important to understand the behavioural patterns that allow amphibians to exploit their terrestrial habitats so we can plan proper management actions and enhance conservation policies. Intraspecific interactions recorded on members of the genus *Salamandra* in terrestrial environments show a relatively wide range of sexual and territorial behaviours, which, apart from mating, include homing, site fidelity, displaying postures to protect territories and detect conspecifics (Werner et al. 2014; Manenti et al. 2017; Di Nicola et al. 2022).

Several male-male interactions have been documented, but mostly in the grey literature. Although they are difficult to interpret because they could be linked to territoriality, mating competition, mistaken mating attempts or sex recognition (Guex and Grossenbacher 2004; Di Nicola et al. 2022), such observations are worth reporting on as they increase the understanding of salamanders' behavioural and ecological requirements. A recent review showed that male-male interactions have been reported for *S. algira*, *S. lanzai* and several subspecies of *S. salamandra* (Di Nicola et al. 2022). Although also male-male interactions for *S. atra atra* are known (Di Nicola et al. 2022), no records for the highly threatened golden Alpine salamander *Salamandra atra aurorae* Trevisan, 1982 exist so far.

Salamandra atra aurorae is endemic to a narrow area ranging from Sette Comuni and Vezzena plateau (Romanazzi and Bonato 2014) in Veneto region (north-east Italy). It can be found mostly in mixed woods with prevalence of beech and white fir, but there are populations in woods with prevalence of spruce and shrubbed meadow (Bonato and Fracasso 2003). If the environment is suitable the golden Alpine salamander can reach high abundances. For example, Bonato and Fracasso (2003) reported a density up to 475 individuals/ha with a sex-ratio of 1:1 and home range of only a few square meters. In the present note we report the first documented case of a male-male aggressive interaction in the golden Alpine salamander.

The observation was made on 4 June 2022, in Bosco del Dosso, near Asiago (Province of Vicenza), the type locality of the golden Alpine salamander (Bonato and Grossenbacher 2000), while making a survey focused on the colouration of *S. a. aurorae*. The survey started at 6.45 am, the temperature was 14 °C and the relative humidity was 76%, measured with a Walfront HT-86 Humidity meter (resolution of 0.01% rh, 0.01 °C). At 7.00 am we found two individuals fighting near a white fir. The two salamanders had a highly visible swollen cloaca, a distinctive male characteristic, and unique dorsal patterns that made them recognizable throughout the duration of the interaction. The first salamander, hereafter 'sal1', had a heavy golden colouration on the back, head and limbs; almost all of its dorsal pattern was gold. In contrast, the second one, hereafter 'sal2', had a golden colouration only in the central part of the back, the first part of the limbs and on the head.

The whole interaction was filmed using a Canon EOS 5D Mark III, mounting a 24–70 mm f/2.8 L USM lens. Immediately after spotting the two males, we started recording, capturing four videos of 47, 116, 66 and 5 seconds each for a total of little less than 4 minutes. The videos were cut and merged in order to create a single movie (accessible at https://youtu.be/sVpBYo-E_y0) without change of focus, adjustment of the camera and movements of the operator.

At the beginning of the video (Fig. 1A) sal1 was on top of sal2 trying to rub its chin on the rival's head. After a brief stop sal1 went on trying to grab the competitor's forelimbs and to rub its chin on the opponent's head. During this time sal2 was moving, trying to escape from the rival.

After a quick interruption, during which sal1 seemed to leave the opponent, sal1 turned around and resumed the attack towards sal2 by climbing on top of him and rubbing its chin on the head of sal2. Also this time sal2 was moving, and this was effective in making the opponent fall, even if sal1 was able to quickly recover and get back on top. A few moments later sal2 managed to free itself from the hold of sal1, and sal2 was able to grasp the opponent's forelimbs after getting on top of sal1 using both anterior and posterior legs (Fig. 1B).

After sal1 was able to escape briefly, sal2 continued its attack which was made easier by sal1 being completely still. Even if sal2 was not able to completely get on top of sal1, the head rubbing was very intense since sal2 was in a good position to firmly use its legs to sustain the weight. After some time sal1 escaped the grasp of sal2, which

Figure 1. Stages in the encounter of two male salamanders. **A.** Start of the interaction: sal1 climbing on top of sal2; **B.** Roles are inverted: sal2 is now on top of sal1; **C.** The interaction has ended.

immediately stopped its chase: the two salamanders stayed near each other (30 cm apart) for about one minute (Fig. 1C), after which the two salamanders began to move in opposite directions. This led us to assume that the interaction was over. Only at this point sal2 was collected: it had a total length of 12.14 cm and weighed 11 g. The other individual was not collected, but had a similar size.

The sequence of behaviours displayed by the two males is similar to that reported in the other species of the genus *Salamandra* (Di Nicola et al. 2022) and in several North American salamanders, which, in addition to mounting attempts, forelimb grasping and snout-rubbing behaviours, also display more aggressive behaviours such as bite, bite-hold, and cannibalism as the ultimate aggressive response (Staub 1993; Camp and Lee 1996; Davis 2002; Deitloff et al. 2014). The biting behaviour was associated with *Salamandra salamandra* by Verrell (1989) but without mention of any occurrence. As of today, more research is needed to confirm this behaviour in the genus *Salamandra*. It can now be confirmed that aggressive male-male interactions are present in the golden Alpine salamander. Considering the rarity of information about this behaviour in this species, and more generally in the genus *Salamandra*, it is important to address the factors that enhance the probability of male-male aggressive interactions, to assess the frequency of these interactions and their biological meaning in terms of advantages for mating and trophic resources access.

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A novel species of piping frog *Eleutherodactylus* (Anura, Eleutherodactylidae) from southern Mexico

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Abstract

We describe a new species of *Eleutherodactylus* (subgenus *Syrrhophus*) from Guerrero, Mexico, based on morphological and molecular data, as well as advertisement call analysis. *Eleutherodactylus franzi* **sp. nov.** has unique features including widely expanded fingertips, indistinct, but visible lumbo-inguinal glands, an immaculate white venter and dark reticulations on a cream dorsal background colouration. The new species belongs to the *Eleutherodactylus nitidus* species group. *Eleutherodactylus franzi* **sp. nov.** is micro-endemic, restricted to a small range in karstic hillsides on the southern extreme of the Mexican Transverse Ranges. We discuss conservation needs of this species, which we provisionally classify as Critically Endangered.

Resumen

Describimos una especie nueva de *Eleutherodactylus* (subgénero *Syrrhophus*) de Guerrero, México basado en datos morfológicos y moleculares, así como análisis de llamadas de apareamiento. *Eleutherodactylus franzi* **sp. nov.** tiene características únicas, incluyendo puntas de los dedos ampliamente expandidas, glándulas lumbo-inguinales indistintas pero visibles, un vientre blanco inmaculado y una coloración dorsal de reticulaciones oscuras sobre un fondo crema. La especie nueva pertenece al grupo de especies de *Eleutherodactylus nitidus*. *Eleutherodactylu. franzi* **sp. nov.** es micro-endémica, restringida a un pequeño rango en laderas kársticas en el extremo sur del Eje Neovolcánico. Presentamos mapas de distribución y gráficos de llamadas de apareamiento de los machos de la nueva especie y sus parientes más cercanos, así como recomendaciones de conservación.

Kurzfassung

Wir beschreiben eine neue Art von *Eleutherodactylus* (Untergattung *Syrrhophus*) aus Südmexiko, auf der Grundlage morphologischer und molekularer Daten sowie einer Analyse des Anzeigerufs. *Eleutherodactylus franzi* **sp. nov.** ist einzigartig in der Gattung durch eine Kombination von Merkmalen, darunter weit verbreiterte Fingerspitzen, undeutliche, aber sichtbare lumbo-inguinale Drüsen, ein makellos weißer Bauch und eine einzigartige Rückenfärbung aus dunklen Netzen auf einem cremefarbenen Hintergrund.. Die neue Art gehört zur *Eleutherodactylus nitidus* Artengruppe. *Eleutherodactylus franzi* **sp. nov.** ist mikroendemisch und auf ein kleines Verbreitungsgebiet in Karsthängen am südlichen Ende der Mexikanischen Querketten beschränkt. Wir präsentieren eine Verbreitungskarte der neuen Art, analysieren ihren Anzeigeruf und geben Empfehlungen für ihren Schutz.

PENSOFT.

Key Words

amphibians, Anura, conservation, Guerrero, Mexican Transverse Ranges, taxonomy

Palabras clave

anfibios, Anura, conservación, Eje Neovólcanico, Guerrero, taxonomía

Schlüsselwörter

Amphibien, Anura, Eje Neovólcanico, Guerrero, Naturschutz, Taxonomie

Introduction

Frogs of the genus *Eleutherodactylus* Duméril & Bibron, 1841 from continental North America are amongst the least understood and most taxonomically challenging groups of New World anurans (Hedges et al. 2008). The subgenus *Syrrhophus* Cope, 1878, in particular, has received little attention until recently. Recent studies that sampled all of the currently recognised species of *Syrrhophus* from continental North America led to the description of twelve new species. Reyes et al. (2015) identified two distinctive species of *Eleutherodactylus* from western Mexico using morphological and molecular data. Grünwald et al. (2018) analysed the morphological and molecular data of all known species within the subgenus *Syrrhophus* and assigned the continental North American species to three species groups contained in two species series: the *Eleutherodactylus longipes* species series (including the *E. longipes* species group) and the *E. nitidus* species series (including the *E. nitidus* and *E. modestus* species groups). They described six new species and synonymised one, while also providing data on advertisement calls for species in the *E. modestus* species group.

Recent studies have expanded the diversity within the *E. nitidus* species group. Palacios-Aguilar and Santos-Bibiano (2020) described a new species of *Eleutherodactylus* from the foothills of Guerrero, while Grünwald et al. (2021) reviewed the group and described two new species from the Sierra Madre del Sur of Guerrero. In addition, Hernández-Austria et al. (2022) presented a more detailed phylogeny of the *E. longipes* species group and described one new species of *Eleutherodactylus* and re-elevated one species from synonymy. Finally, Devitt et al. (2023) published a further review of the *E. modestus* and *E. nitidus* species groups, in which they named one of the species closely related to *E. petersi* and described a new species from the Eje Neovólcanico. These rearrangements resulted in the recognition of 43 named species as valid within the subgenus *Syrrhophus*.

The present study provides evidence, based on molecular, morphological and mating call data, for the occurrence of an additional undescribed species of *Syrrhophus* in the State of Guerrero, in southern Mexico. Our molecular phylogenetic analysis indicates that the species belongs to the *Eleutherodactylus nitidus* species group and is most closely related to *E. humboldti*. We analyse the male advertisement call of the new species, compare it to closely-related species and discuss its limited distribution. Finally, we make conservation recommendations.

Materials and methods

Taxonomic sampling

We examined specimens of all currently recognised species of the subgenus *Syrrhophus* (Frost, 2020) and measured specimens of all species, except for the enigmatic *E. verruculatus* (Peters, 1870), whose existence has been questioned by several authors (Firschein 1954; Lynch 1970; Grünwald et al. 2018, 2021).

We photographed all specimens used in this study alive, including dorsal, lateral and ventral profiles, as well as photographs of each showing colours of flanks and flash colours on the groin and thigh. We then euthanised the frogs with 10% ethanol or with topical benzocaine and took tissue samples from the thigh muscle or liver upon death and preserved them in 96% ethanol. We preserved specimens in 10% formalin and transferred them to 70% ethanol for storage. We measured additional specimens of the subgenus *Syrrhophus* in the Museo de Zoología, Facultad de Ciencias (MZFC) of the Universidad Nacional Autónoma de México (UNAM) and in the Amphibian and Reptile Diversity Research Center (ARDRC) of the University of Texas at Arlington (UTA).

We did not measure type specimens of some previously-described taxa so we used the measurements of the type specimens provided in their original descriptions and published literature. Measurements of *Eleutherodactylus dilatus* (Davis & Dixon, 1955), *E. maurus* (Davis & Dixon, 1955) (= *E. fuscus*), *E. albolabris* (Taylor, 1943) are given in their original descriptions and in Dixon (1957a, 1957b). Measurements for *E. nitidus* (Peters, 1870) and *E. petersi* (Duellman, 1954) were taken from Dixon (1957a, b), while measurements for *E. orarius* (Duellman, 1958) and *E. syristes* (Hoyt, 1965) were taken from their original descriptions. In the case of *Eleutherodactylus pipilans* (Taylor, 1940), *E. nebulosus* (Taylor, 1943) and *E. rubrimaculatus* (Taylor & Smith, 1945), we used the measurements provided in their original descriptions and from Lynch (1970). Measurements of *E. erythrochomus* Palacios-Aguilar & Santos-Bibiano, 2020), *E. maculabialis* Grünwald, Reyes-Velasco, Franz-Chávez, Morales-Flores, Ahumada-Carrillo, Rodriguez & Jones, 2021 and *E. sentinelus* Grünwald, Reyes-Velasco, Franz-Chávez, Morales-Flores, Ahumada-Carrillo, Rodriguez & Jones, 2021 were taken from the descriptions. Measurements of each one of the above species were also taken from specimens collected during this study from or near the type locality.

The material collected was deposited at the Instituto de Investigaciones sobre los Recursos Naturales (INIRENA), which is now officially known as Colección Herpetológica de la Universidad Michoacana (CHUM) of the Universidad Michoacana de San Nicolás de Hidalgo in Morelia, Michoacán, Mexico; at the Museo de Zoología, Facultad de Ciencias (MZFC) of the Universidad Nacional Autónoma de México (UNAM) in Mexico City; and at the University of Texas at Arlington, Texas (UTA). While we formally catalogued the specimens we collected, several specimens examined from both the MZFC and UTA collections have not been catalogued, in which case we list the original field numbers and the respective museum in which they were deposited. Furthermore, several of the sequences generated were submitted to GenBank using the original field numbers and not final museum catalogue numbers. Original field number abbreviations are as follows: CIG (Christoph I. Grünwald) to be catalogued at MZFC; ENS (Eric N. Smith) to be catalogued at UTA; JAC (Jonathan A. Campbell) to be catalogued at UTA; JRV (Jacobo Reyes-Velasco) to be catalogued at UTA; RHA (Raquel Hernández-Austria) and GP (Gabriela Parra-Olea) both to be catalogued at the Colección Nacional de Anfibios y Reptiles, Instituto de Biología (CNAR) at the Universidad Nacional Autónoma de México (UNAM) in Mexico City. Specimen numbers for all materials examined are provided in Appendix 1.

Morphological measurements

The characters and terminology we use herein follow those of Lynch and Duellman (1997), Savage (2002) and Grünwald et al. (2018, 2021). We took the following measurements for each specimen (abbreviations listed in parentheses): snout-vent length (SVL); head length (HL); head width (HW); eyelid width (EW); interorbital distance (IOD); internarial distance (IND); eye-naris distance (END); diameter of eye (ED); width of tympanum (TW); height of tympanum (TH); eye-tympanum distance (ETD); upper arm length (UpL); forearm length (FoL); palm length (PaL), total hand length (HaL); length of 1st finger (F1L); width of pad on 1st finger (F1PW); width of 1st finger (F1W); length of 2nd finger (F2L); width of pad on 2nd finger (F2PW); width of 2nd finger (F2W); length of 3rd finger (F3L); width of pad on 3rd finger (F3PW); width of 3rd finger (F3W); length of 4th finger (F4L); width of pad on 4th finger (F4PW); width of 4th finger (F4W); inner palmar tubercle length (IPTL); middle palmar tubercle length (MPTL); outer palmar tubercle length (OPTL); femur length (FeL); tibia length (TL); tarsal length (TaL), foot length (FL), total foot length (TotFL); length of 2nd toe (T2L); width of pad on 2nd toe (T2PW); width of 2nd toe (T2W); length of 3rd toe (T3L); width of pad on 3rd toe (T3PW); width of 3rd toe (T3W); length of 4th toe (T4L); width of pad on 4th toe (T4PW); width of 4th toe (T4W); length of 5th toe (T5L); width of pad on 5th toe (T5PW); width of 5th toe (T5W); inner metatarsal tubercle length (IMTL); and outer metatarsal tubercle length (OMTL). We measured hand length (HA) from the tip of the longest finger to the base of the palm and foot length (FL) from the tip of the longest toe to the base of the tarsus. The outer palmar tubercle refers to a small tubercle on the outer surface of the palm, but is not one of the larger supernumerary tubercles. While these tubercles usually are present in *Syrrhophus*, they generally are absent in some species and their presence is variable in others.

Measurements were made with Truper (Mexico) brand digital calipers and rounded to the nearest 0.1 mm. The sex of adult specimens was determined by presence of vocal slits.

Molecular analysis

DNA extraction and PCR amplification

A detailed description of the DNA extraction and PCR amplification protocols can be found in Grünwald et al. (2018). In brief, we extracted DNA from tissue samples by using a standard potassium acetate protocol and sequenced a fraction of the 16s rRNA mitochondrial gene by using the primers LX12SN1a (forward) and LX16S1Ra (reverse) of Zhang et al. (2013) or with the modified primers 16Sar and 16Sbr of Bossuyt and Milinkovitch (2000). We then shipped ExoSap purified PCR products to Eurofins Genomics (Lexington, KY, USA) for sequencing.

Sequence alignment and phylogenetic analysis

We included additional sequences of multiple members of the subgenus *Syrrhophus* obtained from GenBank to infer the phylogenetic relationships of the new individuals sequenced in this study. We have included all sequences used in this study with their accession numbers in GenBank in Appendix 2. As our main interest is to understand the evolutionary relationships of the new species to members of the *Eleutherodactylus nitidus* species group, we included all of the species currently recognised in that group. We also included two members each of the *E. longipes* and the *E. modestus* species groups in our analysis.

We removed regions with poor-quality base calls by manually trimming the 5' and 3' ends of all sequences using the programme Geneious v.6.1.6 (Biomatters Ltd., Auckland, NZ). We then aligned all sequences in Muscle (Edgar 2004), with a final alignment of 560 base pairs. Our final alignment included 83 samples, of which 13 are new.

We performed Bayesian Inference of phylogeny (BI) in MrBayes v.3.2.2 (Ronquist et al. 2012), implemented on the CIPRES Science Gateway server (Miller et al. 2010). First, we selected the best-fit models of nucleotide substitution for the 16s rRNA mitochondrial gene using the Bayesian Information Criterion (BIC) implemented in PartitionFinder v.1.1.1 (Lanfear et al. 2012). Our Bayesian analysis consisted of four runs of 10 million generations each, with four chains (one cold and three heated), sampling every 1,000 generations. We used Tracer v.1.6 (Drummond and Rambaut 2012) to confirm convergence of the independent runs, based on overlap in likelihood and parameter estimates amongst runs, as well as effective sample size (ESS) and Potential Scale Reduction Factor value estimates (PSRF). PSRF indicated that individual runs had converged by 100,000 generations, so we discarded the first 25% of the runs as burn-in. Finally, we annotated posterior probability values on the resulting topology using the programme TreeAnnotator v.1.8.3 (Rambaut et al. 2014) and collapsed all nodes with less than 0.50 posterior support. Additionally, we obtained genetic distances for the members of the group with the use of Mega X (Kumar et al. 2018).

Bioacoustic analysis

We recorded vocalisations of several individuals of the new species described here, as well as all other members of the *Eleutherodactylus* (*Syrrhophus*) *nitidus* species group (sensu Grünwald et al. (2018, 2021)). We recorded the frogs while they were actively calling in the field, using the WavePad free recording software (NCH Software 2015) on various Apple iPhones. We recorded the calls at distances ranging from 50–150 cm, although when possible, we tried to be within 100 cm of the frog. Ambient temperatures were not taken at the time of recording, but we did record the time of day the recordings were made. The calls were recorded at a sampling rate of 44.1 kHz and an amplitude resolution of 16 bits.

We isolated the individual calls from other calls and background noise using Adobe Audition CC, using default settings in the application. We then analysed the calls using the software Raven Pro 1.5 (The Cornell Lab of Ornithology 2014). Spectrograms were constructed using a Blackman-type window with a size of 5 ms, 80% overlap and DFT of 512 samples. Temporal parameters were measured from the oscillogram in ms. Spectrogram and oscillogram graphics were generated using Seewave v.1.6 (Sueur et al. 2008) in RStudio v.1.1.423 (RStudio Team 2016). Values in the call descriptions are given as mean ± standard deviation. 2D spectrograms were visualised using a sliding window analysis of short-term Fourier transform calculations.

Results

Molecular phylogenetic results

Our phylogenetic results (Fig. 1) are mostly in concordance with other molecular phylogenies of the subgenus *Syrrhophus* (Grünwald et al. 2018, 2021; Hernandez-Austria et al. 2022; Devitt et al. 2023). The novel species is nested within the *Eleutherodactylus nitidus* species group as defined by Grünwald et al. (2018, 2021). Our analysis recovered all members of the *E. nitidus* species group as sister to the *E. modestus* species group, with strong support (posterior support (pp) = 1). Within the *E. nitidus* species group, there was a trichotomy with one strongly-supported clade (pp = 1) comprised of *E. dilatus*, *E. sentinelus*, *E. maculabialis*, *E. syristes*, *E. maurus*, *E. humboldti*, the novel species described here, *E. nitidus*, *E. petersi*, *E. orarius*, *E. jamesdixoni* and *E. albolabris*. The relationship of the remaining members of the *E. nitidus* species group including *E. pipilans*, *E. erythrochomus*, *E. rubrimaculatus* and *E. nebulosus* to one another and to the larger supported clade were not well resolved, except that *E. rubrimaculatus* and *E. nebulosus* are closely related to each other. The new species was recovered as a sister species to the sympatric *E. humboldti*, although with low support (pp = 0.61).

Systematic account

***Eleutherodactylus franzi* sp. nov.**

https://zoobank.org/18CE837E-C4B3-4468-BA37-AFB9213A9509

Figs 2–4, 5A

Franz's Piping Frog / Rana gaitera de Franz

Type material. ***Holotype*.** INIRENA 2900 (CIG 01725). Adult male (Fig. 2), El Cucharillo, Municipio de Ixcateopan de Cuauhtémoc (18.5331, -99.7335, 2,315 m a.s.l.; datum = WGS84), Guerrero, Mexico, collected on 27 June 2020 by Héctor Franz-Chávez, Christoph I. Grünwald, André J. Grünwald and Kimberly Montelongo-Chávez.

***Paratypes* (n = 14; Fig. 3).** INIRENA 2901–10 (CIG 01716–24, 01726), 10 adult males, collected at same locality and on same date as holotype; INIRENA 2895–97 (CIG 01727–29), 3 adult males, 3 km E of Ixcateopan de Cuauhtémoc, Municipio de Ixcateopan de Cuauhtémoc

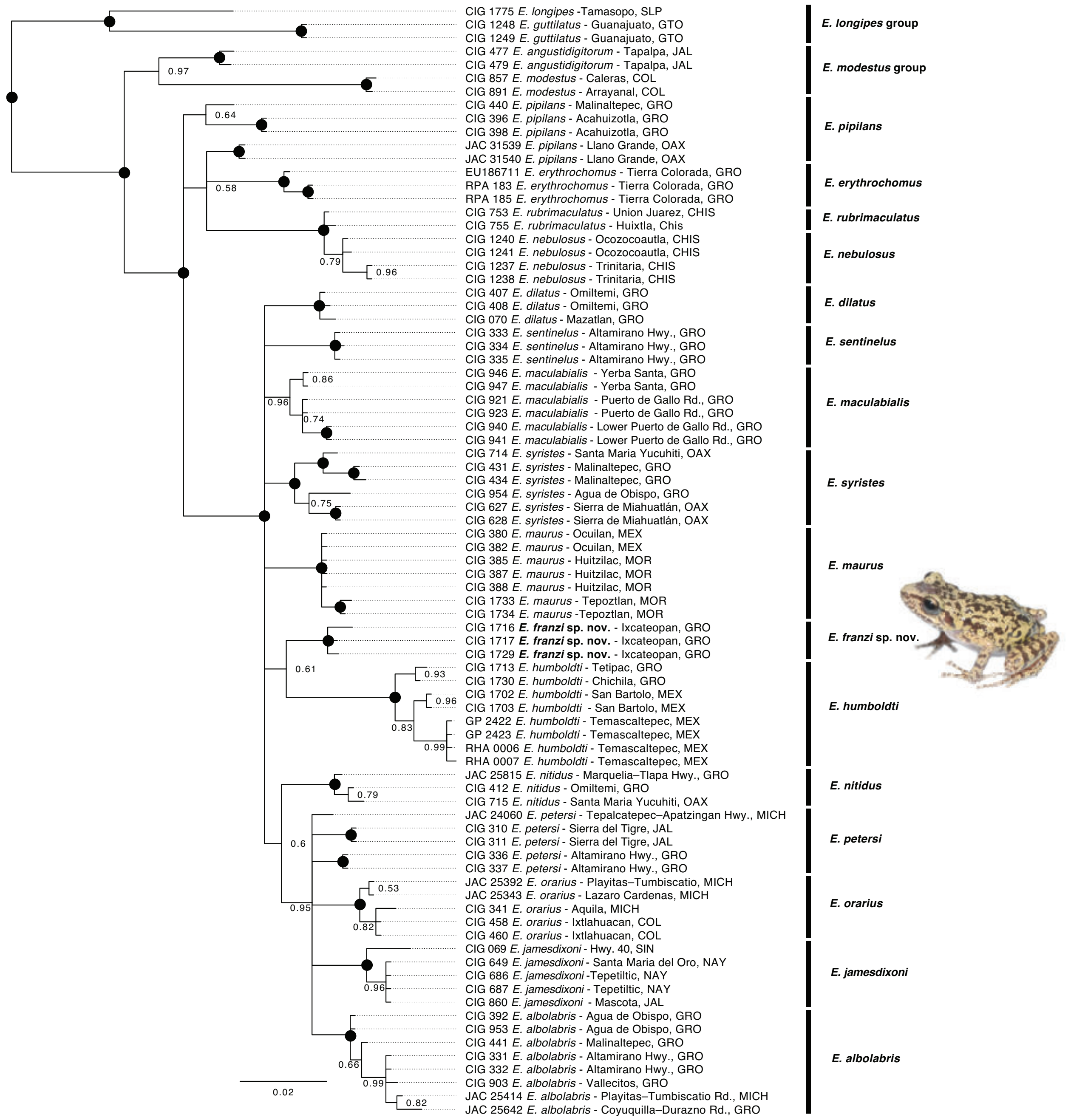

Figure 1. Bayesian phylogenetic inference of members of the *Eleutherodactylus* subgenus *Syrrhophus*, with a focus on the *E. nitidus* species group, based on the mitochondrial locus 16S rRNA. Black circles represent nodes with a posterior support of 1. All nodes with support of less than 0.5 are collapsed.

(18.5076, -99.7329, 2,060 m a.s.l.; datum = WGS84), Guerrero collected on 27 June 2020 by Héctor Franz-Chávez, Christoph I. Grünwald, André J. Grünwald and Kimberly Montelongo-Chávez; INIRENA 2898 (CIG 01731), 1 adult male, between El Cucharillo and Chichila, Municipio de Taxco de Alarcón, (18.5335, -99.7117, 2,260 m a.s.l.; datum = WGS84), Guerrero, Mexico, collected on 27 June 2020 by Héctor Franz-Chávez, Christoph I. Grünwald, André J. Grünwald and Kimberly Montelongo-Chávez.

Diagnosis. Based on our phylogenetic analysis, this is a member of the genus *Eleutherodactylus*, subgenus *Syrrhophus*, as defined by Hedges et al. (2008). In the *Eleutherodactylus (Syrrhophus) nitidus* species series and the *Eleutherodactylus* (*Syrrhophus*) *nitidus* species group as defined by Grünwald et al. (2018), based on the condition of the tympanic annuli, ventral epidermis and visceral peritoneum. A small frog, but relatively large *Syrrhophus*, adult males measure 25.6–29.5 mm SVL; vocal slits are present in males, readily visible under partially translucent ventral epidermis; digital tips are widely expanded, 1.8–2.6 times the width of the narrowest part of the finger on the third and fourth fingers; fingers moderately long, finger lengths are I-II-IV-III with third finger

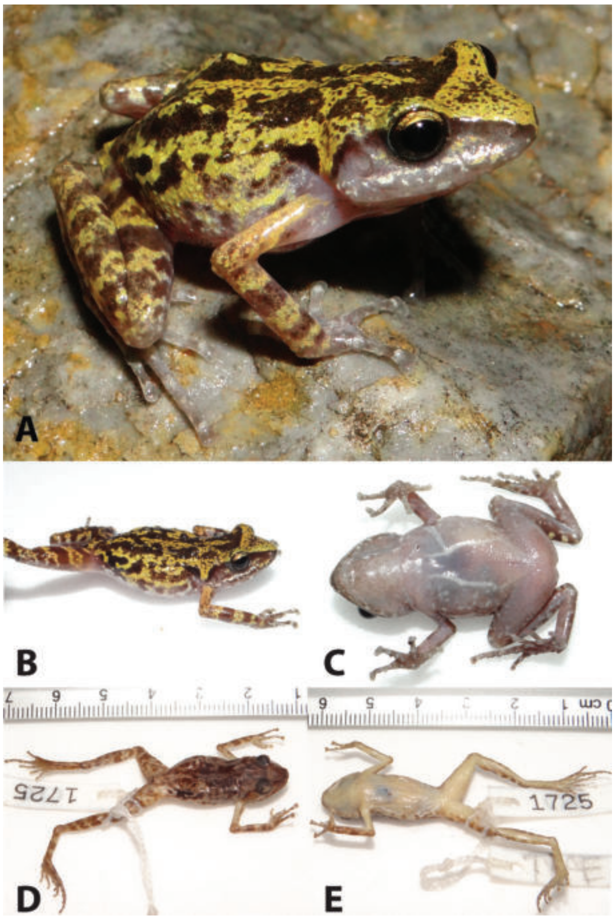

Figure 2. Holotype of *Eleutherodactylus franzi*, sp. nov., INIRENA 2900 (CIG 01725), El Cucharillo, Municipio de Ixcateopan de Cuauhtémoc, Guerrero, Mexico in life (**A–C**) and in preservative (**D, E**).

length ranging from 15–18% of SVL; compact lumbo-inguinal gland above the inguinal region present, raised, indistinct, but visible in live specimen; ventral epidermis is partially translucent and visceral peritoneum is clear, not white, thus abdominal vein is not clearly visible against a white background on the venter of live specimens and viscera is partially visible through translucent dark grey ventral epidermis; limbs moderate, TL/SVL ratio is 0.40–0.50, FeL/SVL ratio is 0.31–0.38 and TotFL/ SVL ratio is 0.50–0.60; snout relatively short, END/ SVL ratio is 0.08–0.11; tympanum small, indistinct and round, tympanic annuli not visible in live specimen; TW/ED ratio is 0.40–0.60. The dorsal and lateral skin is slightly shagreened, whereas the ventral skin is smooth. Dorsal colouration cream or tan, with darker brown reticulations on dorsal and lateral portions of head and back; loreal and region dark brown, from snout to behind tympanum, with some pale spots and speckling present on labial region same colour as pale dorsal colouration; pale interorbital bar variable, may be present or absent, when present same colour as pale dorsal colouration of head and body; distinct irregular-edged dark brown transverse bands present on legs; upper arms same colouration and pattern as forearms; no pale mid-dorsal stripe; upper flanks same colour as dorsum, lower flanks whitish with some grey marbling; venter pale lavender with some sparse white spotting and pale grey on throat. No inguinal flash colours present on thighs and groin. The mating call of adult males is a short low-pitched pipe (see below; Fig. 4).

Comparisons. *Eleutherodactylus franzi* can be distinguished from all species in the *Eleutherodactylus* (*Syrrhophus*) *longipes* species series by: possessing a small, indistinct tympanum with no tympanic annulus visible and with a diameter less than 50% of the diameter of the eye; by possessing a ventral epidermis which is semi-translucent and combined with a visceral peritoneum which is not white, an abdominal vein on the venter is not clearly evident against a white background in life; by possessing indistinct, but visible raised lumbo-inguinal gland above the inguinal region.

Eleutherodactylus franzi can be distinguished from most species of the *Eleutherodactylus* (*Syrrhophus*) *modestus* species group by the combination of possessing a compact, protruding lumbo-inguinal gland above the inguinal region, digital tips which are expanded more than 1.8 times the width of the narrowest part the finger on the third and fourth fingers and the lack of a distinct interorbital bar a colour distinct from the dorsal ground colouration. It can further be distinguished from the superficially similar *E. grunwaldi* by its smaller body size, 25.6–29.5 mm (vs. 28.4–32.4 mm), less expanded fingertips, 1.8–2.5 times the width of the narrowest part of the finger on fingers three and four (vs. 2.8–3.2) and the presence of a visible raised lumbo-inguinal gland. From the superficially very similar *E. saxatilis*, it can be distinguished by snout shape, eye size and head colouration. These two frogs, although not closely related, are very similar in appearance, but come from two widely-separated mountain ranges in central Mexico and are genetically distinct. *Eleutherodactylus franzi* has an angular canthus rostralis, with a shorter snout that is distinctly truncated from a lateral profile. It has a larger eye, with a larger ETD and, generally, there is a pale interorbital region that lacks dark markings. In *E. saxatilis*, the canthus rostralis is noticeably rounded and the snout is acuminate from a lateral profile. The eyes are smaller and located closer to the tympanum and there is no noticeable lack of dark dorsal markings in the interorbital area.

Within its own species group, *E. franzi* can be distinguished from most species by possessing a compact inguinal gland that is indistinct, but visible in live specimens. This character may or may not be visible in preserved specimens depending on how they were preserved. This species differs from *E. pipilans*, *E. erythrochomus* and *E. nebulosus*, which lack visible compact lumbo-inguinal glands altogether. All other known species in the *E. (Syrrhophus) nitidus* species group have readily visible compact lumbo-inguinal glands above the inguinal region, except *E. maculabialis*, which has similarly visible, but indistinct lumbo-inguinal glands. *Eleutherodactylus franzi* can be

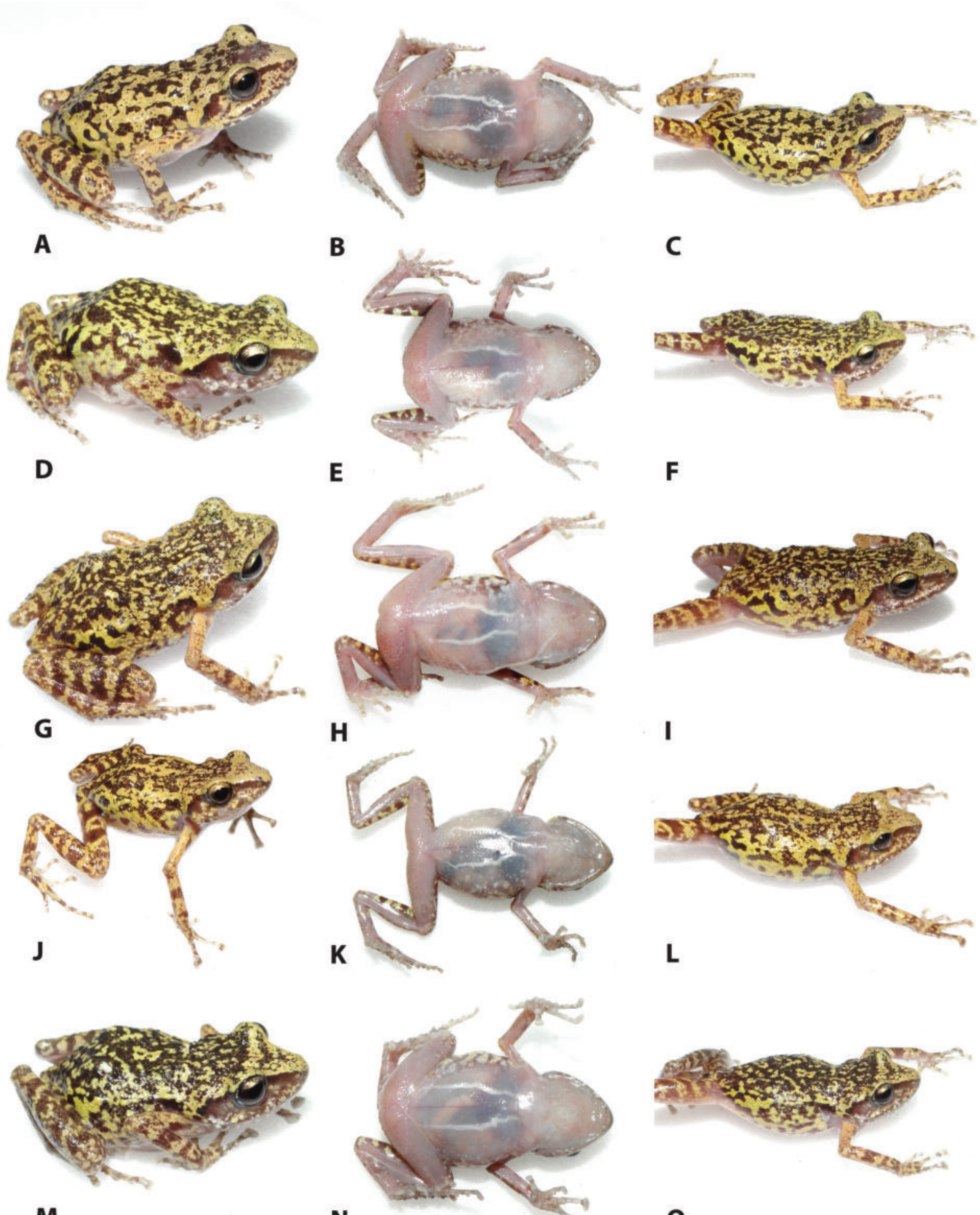

Figure 3. Some of the paratypes of *Eleutherodactylus franzi* sp. nov., in life. **A–C.** INIRENA 2902 (CIG 01717) El Cucharillo, Municipio de Ixcateopan de Cuauhtémoc, Guerrero, Mexico; **D–F.** INIRENA 2910 (CIG 01726) El Cucharillo, Municipio de Ixcateopan de Cuauhtémoc, Guerrero, Mexico; **G–I.** INIRENA 2895 (CIG 01727), 3 km E of Ixcateopan de Cuauhtémoc, Municipio de Ixcateopan de Cuauhtémoc, Guerrero, Mexico; **J–L.** INIRENA 2896 (CIG 01728), 3 km E of Ixcateopan de Cuauhtémoc, Municipio de Ixcateopan de Cuauhtémoc, Guerrero, Mexico; **M–O.** INIRENA 2898 (CIG 01731), between El Cucharillo and Chichila, Municipio de Taxco de Alarcón, Guerrero, Mexico.

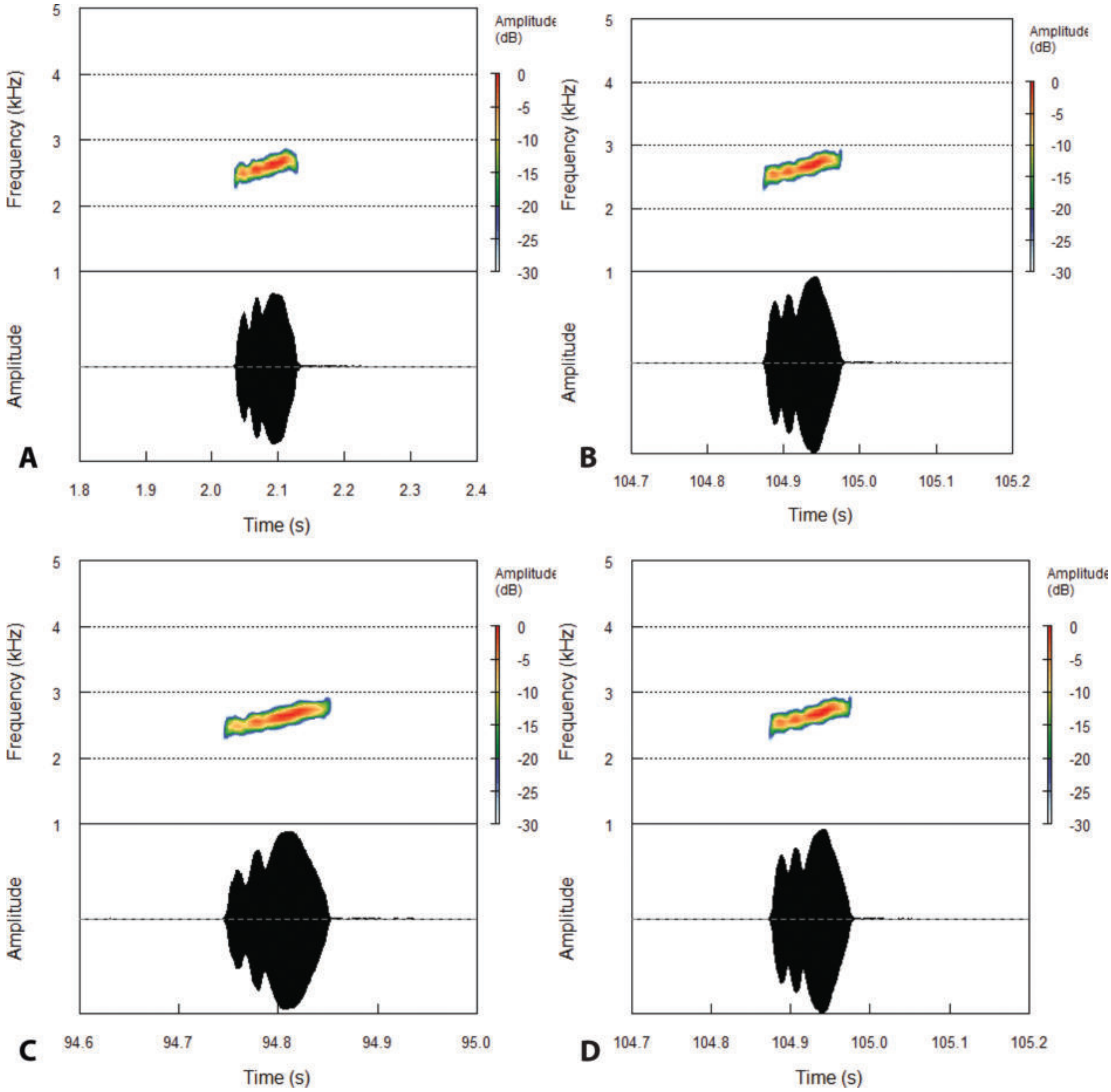

Figure 4. Spectrograms (top) and oscillograms (bottom) of the advertisement calls of *Eleutherodactylus franzi* sp. nov., recorded at El Cucharillo, Municipio de Ixcateopan de Cuauhtémoc, Guerrero, Mexico (**A, B**) and at 3 km E of Ixcateopan de Cuauhtémoc, Guerrero, Mexico (**C, D**).

further distinguished from *E. pipilans* and *E. nebulosus* by possessing digital tips which are expanded more than 1.8 times the width of the narrowest part of the finger and from *E. erythrochomus* by possessing digital tips which are more than 1.5 times, but less than 3.0 times, the width of the narrowest part of the finger. It is distinguished from *E. albolabris*, *E. nitidus*, *E. petersi*, *E. jamesdixoni* and *E. orarius* by the combination of larger size, smoother skin, longer limbs and tips of digits which are expanded more than 1.8 times the narrowest part of the finger on the third and fourth fingers. This species is distinguished from *E. dilatus*, *E. humboldti*, *E. maurus* and *E. sentinelus* by its larger size, smoother skin, pale dorsal colouration with dark reticulations and lack of a pale interorbital bar which is paler than the pale dorsal colouration. Furthermore, all these species, except *E. humboldti*, present multi-note whistles, while *E. franzi* has a call that consists of a single note, low-pitched pipe. *Eleutherodactylus franzi* may be distinguished from *E. maculabialis* and *E. syristes* by its larger size, more expanded fingertips, lack of inguinal flash colouration, as well as those species' unique advertisement call which consists of a trill rather than a short pipe. In Guerrero, two other species of saxicolous *Eleutherodactylus* (*E. pipilans* and *E. erythrochomus*) have similar colouration, similar smooth skin and widely expanded digital pads. Both are readily distinguishable from *E. franzi* by possessing an indistinct lumbo-inguinal gland which is barely visible in life and by a male advertisement call which is a peep instead of a pipe. Furthermore, *E. franzi* can be distinguished from *E. pipilans* by its more expanded digital tips on the third and fourth fingers, 1.8–2.5 times the width of the narrowest part of the finger (vs. 1.3–1.9) and distinct lack of dark markings in the interorbital region (vs. no difference from rest of dorsum). It can further be distinguished from *E. erythrochomus* by the conspicuous dark pattern on a pale dorsal colouration, lesser expanded digital tips no more than 2.5 times the width of the narrowest part of the digit on the third and fourth fingers (vs. 2.3–3.8) and a less distinct tympanum. General characteristics for the *Eleutherodactylus nitidus* species group are given in Table 1.

Description of the holotype. Adult male, relatively large (26.2 mm SVL); head as wide (9.5 mm) as long (9.5 mm), head wider than body; snout rounded from a dorsal view and rounded to slightly truncate from a lateral profile; tympanum indistinct, rounded with no supra-tympanic fold present; tympanum small, oval, greatest width of tympanum 1.4 mm; greatest diameter of eye 2.8 mm; tympanum width to eye-diameter 0.51; eyelid width 1.6 mm, approximately 38% of the IOD; first finger shorter than second finger; finger lengths from shortest to longest I-II-IV-III; digital pads on fingers two, three and four expanded, 2.1 times the narrowest point of the digit on fingers three and four; expanded finger pads widely expanded, truncate, three palmar tubercles; inner palmar tubercle 70% of middle palmar tubercle and outer palmar tubercles about 60% as large as middle palmar tubercle, (Fig. 5A); toe lengths from shortest to longest I-V-II-III-IV, TL1 and TL5 very similar; outer metatarsal conical with a round base moderate, approximately 56% of inner metatarsal tubercle; inner metatarsal tubercle spherical shape with oval base, large, approximately 1.1 mm in length. Dorsal skin smooth, lateral skin slightly shagreened with some low tubercles; ventral skin smooth. Vocal slits present.

In life, the holotype had a yellowish-tan dorsal colouration on the back, with darker brown blotches on the back and flanks. Head yellowish-tan with some dark brown speckling, no pale interorbital bar; however, the interorbital region mostly lacks darker brown speckling giving it the resemblance of a pale interorbital bar. Labial region pale grey with some tan and some white speckling. Three and four white-tipped tubercles present at the rictal. A dark brown stripe present from the tip of the snout posteriorly through loreal region, eye and tympanum to right above rictal tubercles. Forearms, thighs, femur and tarsus tan with indistinct pale brown banding. The upper arms were unmarked, tan to slightly orange. No inguinal flash colouration was present on groin or thighs. Ventral colouration was lavender with some sparse white and on sides, ventral colouration on throat grey. Ventral skin was slightly translucent and visceral peritoneum clear, so no visible red abdominal vein and viscera were visible in life.

Colouration in preservative is pale tan on dorsum, with darker brown reticulations. Pale tan interorbital bar present. Dark canthal bar is dark brown. Unmarked upper arms are cream to white. Limbs cream with dark brown cross-banding. The dorsal surfaces of the legs are light

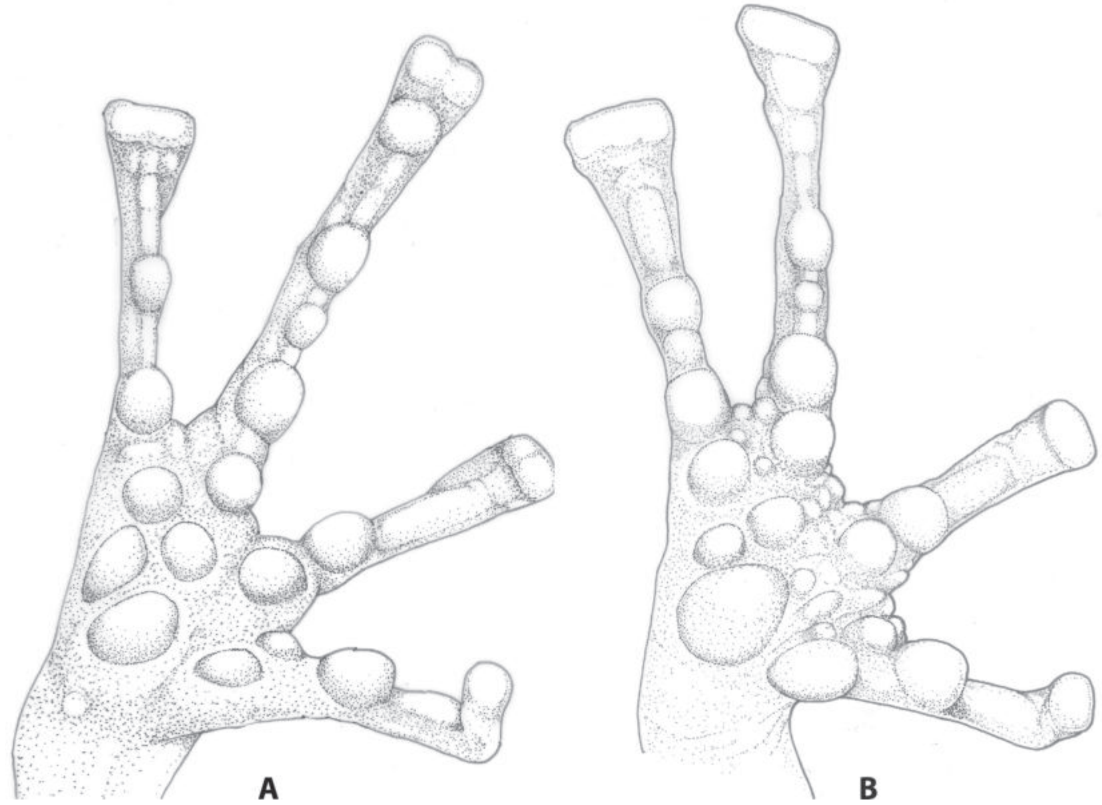

Figure 5. A. Ventral aspect of the hand of the holotype of *Eleutherodactylus franzi* sp. nov., INIRENA 2900 (CIG 01725) from El Cucharillo, Municipio de Ixcateopan de Cuauhtémoc, Guerrero, Mexico; **B.** Ventral aspect of the hand of a *Eleutherodactylus humboldti*, INIRENA 2911 (CIG 01703) from 9.4 km N of Valle de Bravo junction on Valle de Bravo – Toluca toll road, near San Bartolo, Municipio de Amanalco, Estado de México, Mexico.

Table 1. Key Comparative Characters of the *Eleutherodactylus nitidus* species group.

	E. albolabris	***E. dilatus***	***E. erythrochomus***	***E. franzi* sp. nov.**	***E. humboldti***	***E. jamesdixoni***	***E. maculabialis***	***E. maurus***	***E. nebulosus***	***E. nitidus***	***E. orarius***	***E. petersi***	***E. pipilans***	***E. rubrimaculatus***	***E. sentinelus***	***E. syristes***
Size	Medium	Medium	Large	Large	Medium	Medium	Small	Small	Medium	Medium	Medium	Medium	Large	Small	Medium	Small
SVL adult males (range) in mm	23.0–26.8	23.8–25.7	24.9–30.0	25.6–29.5	23.4–25.7	24.3–26.3	17.9–24.7	20.7–24.3	22.9–28.3	24.3–26.3	24.6–28.0	23.9–26.3	25.5–29.6	18.2–23.5	23.3–25.3	21.7–24.6
Condition of Interorbital Bar	Indistinct, Pale	Present, Pale	Absent	Absent	Usually Present, Pale	Indistinct, Pale	Present, Pale	Present, Pale	Absent	Indistinct, Pale	Indistinct, Pale	Indistinct, Pale	Absent	Absent	Present, Pale	Present, Pale
Pale Mid-dorsal Blotch	Sometime Present	Absent	Absent	Absent	Present	Present	Absent	Absent	Absent	Present	Sometimes Present	Present	Absent	Absent	Present	Absent
Colouration of Lip	White	Dark with pale flecking	As head	Grey, with flecking of dorsal colouration	Pale, with white flecking,	Mottled	Dark with pale spots	Dark with pale flecking	Dark with pale spots	Mottled	White, mottled with dark	Mottled	As head	Dark with white or pale spots	Dark with pale flecking	Dark, variedly spotted
Inguinal Flash Colouration	Orange	Yellow	Absent	Absent	Yellow or orange	Faint, yellow-orange	Varied, Yellow / Orange	Varied, usually Absent	Absent	Faint, yellow-orange	Faint, yellow-orange	Faint, yellow-orange	Absent	Absent	Yellow	Varied, Yellow / Orange
Ventral Colouration	White with black spots	Grey with white and black	Transparent	White or pinkish	Grey, with white and black	White with dark mottling	Grey with white and black	Grey with white and black	Transparent	White with dark mottling	White with dark mottling	White with dark mottling	Transparent	Pale grey with white spotting	Transparent with white and black	Transparent and White, with black spots
Dorsal Skin Texture	Smooth	Not Smooth	Smooth	Smooth	Not Smooth	Not Smooth	Smooth	Not Smooth	Smooth	Not Smooth	Not Smooth	Not Smooth	Smooth	Smooth	Smooth	Smooth
Ventral Skin Texture	Slightly Rugose	Rugose	Smooth	Smooth	Rugose	Slightly Rugose	Smooth	Rugose	Smooth	Slightly Rugose	Slightly Rugose	Slightly Rugose	Smooth	Smooth	Smooth	Smooth
Condition of Lumbo-Inguinal Gland	Very distinct	Very distinct	Indistinct	Distinct	Very distinct	Very distinct	Distinct	Very distinct	Indistinct	Very distinct	Very distinct	Very distinct	Indistinct	Indistinct	Very distinct	Distinct
3FPW/3FW	1.3–1.9	1.5–1.7	2.3–3.8	1.8–2.5	1.9–2.4	1.1–1.5	1.4–2.1	1.3–1.6	1.1–1.5	1.1–1.5	1.2–1.4	1.1–1.7	1.5–1.9	1.5–1.9	1.7–2.3	1.1–1.9
4FPW/4FW	1.3–1.9	1.5–1.8	2.3–3.5–	1.8–2.5	1.3–2.1	1.1–1.5	1.4–2.1	1.3–1.7	1.1–1.5	1.1–1.5	1.2–1.4	1.1–1.7	1.5–2.0	1.5–1.9	1.5–2.3	1.2–1.8
TW/ED	0.27–0.32	0.25–0.35	0.33–0.51	0.40–0.61	0.37–0.52	0.25–0.29	0.25–0.28	0.28–0.31	0.35–0.38	0.25–0.29	0.25–0.29	0.25–0.29	0.30–0.36	0.25–0.36	0.26–0.28	0.25–0.29
Call	Whistle	Peep	Peep	Pipe	Pipe	Whistle	Trill	Pipe	Peep	Whistle	Whistle	Whistle	Peep	Peep	Peep	Trill

brown and the groin and posterior surfaces of the thighs are brown. Ventral surfaces yellowish-cream, unmarked, slightly darker brownish pigmentation on throat and chin. Ventral surfaces of hands and feet brown, with dark brown spots. (Fig. 2D, E).

Measurements of the holotype (in millimetres). IND 2.2, IOD 4.2, END 2.5, ETD 0.9, UpL 6.4, FoL 7.5, PaL 2.3, HaL 6.7, F1L 2.3, F1PW 0.5, F1W 0.4, F2L 2.8, F2PW 0.8, F2W 0.4, F3L 4.7, F3PW 1.1, F3W 0.5, F4L 3.5, F4PW 1.1, F4W 0.5, IPTL 0.7, MPTL 1.0, OPTL 0.6, FeL 10.0, TL 11.0, TaL 7.1, TotFL 11.4, T1L 2.4, T1PW 0.62, T1W 0.5, T2L 3.6, T2PW 0.7, T2W 0.5, T3L 4.4, T3PW 0.7, T3W 0.5, T4L 6.0, T4PW 0.7, T4W 0.5, T5L 2.5, T5PW 0.6, T5W 0.4, IMTL 1.1, OMTL 0.6, FeL/SVL 38%, TL/SVL 42%, HaL/SVL 26%, TotFL/SVL 56%, HL/SVL 35%, HW/SVL 36%.

Variation. SVL from 25.6–29.5 mm (27.15 ± 1.77). Expanded finger pads vary from 1.8–2.5 times the narrowest part of the digit on the third finger and from 1.8–2.6 times the narrowest part of the digit on the fourth finger, with average 2.0 ± 0.21 on the third finger and average 2.2 ± 0.21 on the fourth finger. Dorsal ground colouration cream or tan, but varied with some greenish, reddish or yellowish tinge, always with darker brown blotches or reticulations. The extent of the darker brown blotches or reticulation varied greatly. The condition of the interorbital area ranged from unmarked and same colour as ground colouration to heavily marked by dark speckling same colour as dark dorsal blotches or reticulation. Venter always lavender, but with varying amounts of white spots. Morphological variation of *E. franzi* is presented in Table 2.

Advertisement call. The advertisement call of the males of this species consists of a single, short, low-pitched pipe that lasts about 106 ms and has a dominant frequency of 2612.7 ± 40.6 Hz (Fig. 4A–D). The note has limited amplitude modulation and the highest energy is displayed at the end of the note. The call is similar to that of *E. pipilans* and *E. erythrochomus*. The call differs from the nearby *E. maurus* and the sympatric *E. humboldti*, which also have a pipe, by being shorter and lower pitched. We present call data for both *E. franzi* and the sympatric *E. humboldti* in Table 3. The advertisement call also differs from the sympatric or near sympatric *E. petersi* and *E. nitidus* as these two species have a call that consists of a multi-note whistle rather than a single-note pipe.

Distribution and ecology. *Eleutherodactylus franzi* appears to be endemic to the Sierra de Taxco Region of northern Guerrero (Fig. 6). This is an extension of the Mexican Transverse Volcanic Belt which extends south into the Balsas Basin. This frog occurs at high elevations between 2,000–2,400 m a.s.l. and has been collected in tropical deciduous forest, oak woodland and pine-oak forest, as well as mixtures of these assemblages. It is restricted to areas of karstic rock outcroppings and the associated sinkholes and caves systems. This species may also occur in nearby Estado de México, as habitat is continuous and the State line is near the type locality. Old reports of *E. pipilans* from that State may be referable to this species; however, we have made attempts to examine these specimens at MZFC and were not able to find the specimens (C. Grünwald, pers. obs.)

Etymology. This species is named after Héctor Franz-Chávez, Mexican herpetologist and avid field collector

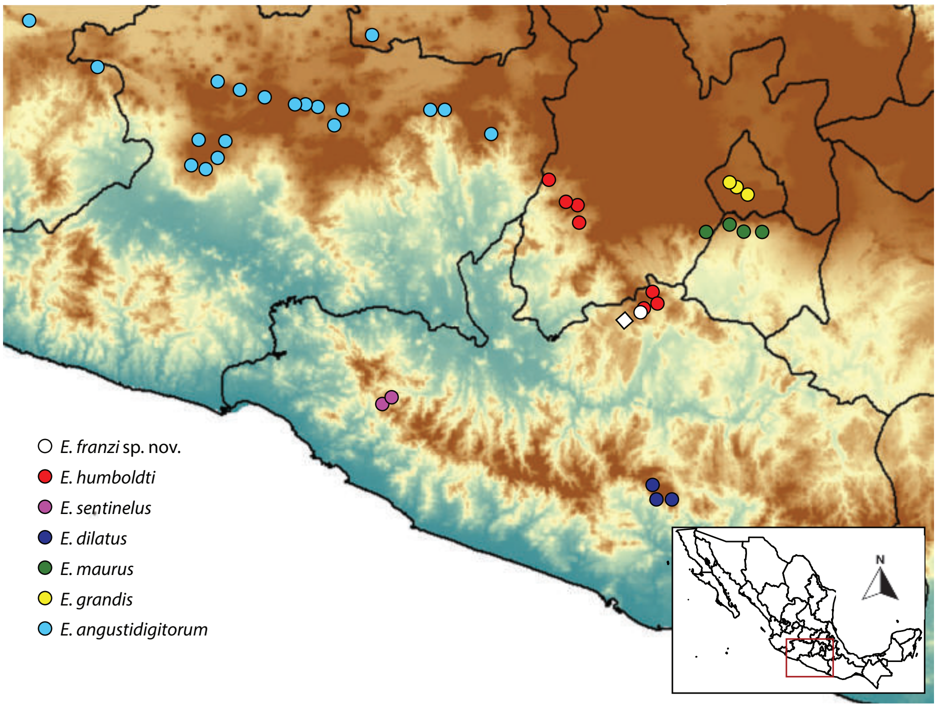

Figure 6. Map showing the type localities and distribution of *Eleutherodactylus* which are either closely related or superficially similar to the species described herein from southern Mexico. White diamond represents the type locality of *E. franzi* sp. nov. and white circle represents additional locality.

Table 2. *Eleutherodactylus franzi* sp. nov. Morphological Measurements (in millimetres).

	INIRENA 2901	INIRENA 2902	INIRENA 2903	INIRENA 2904	INIRENA 2905	INIRENA 2906	INIRENA 2907	INIRENA 2908	INIRENA 2909	INIRENA 2900	INIRENA 2910	INIRENA 2895	INIRENA 2896	INIRENA 2897	INIRENA 2898
SVL	27.72	25.58	27.72	26.86	26.4	26.04	28.62	26.68	25.55	26.24	27.97	28.7	27.12	29.51	26.56
HL	9.08	8.76	9.35	9.1	8.61	8.33	8.76	8.73	8.68	9.12	9.38	9.06	8.79	9.04	8.61
HW	9.78	9.03	9.81	9.78	9.82	9.07	9.4	9.41	9.42	9.5	9.99	9.68	9.67	9.66	9.61
TW	1.53	1.36	1.52	1.88	1.74	1.45	1.38	1.47	1.32	1.4	1.39	1.12	1.39	1.34	1.42
ED	3.74	2.62	2.79	3.08	2.9	2.63	2.74	2.78	2.81	2.76	2.77	2.78	2.38	2.8	2.94
EIW	1.13	1.39	1.52	1.68	1.19	1.45	1.49	1.67	1.69	1.59	1.35	1.78	1.51	1.62	1.9
IOD	5.1	5.02	5.19	5.09	5.04	4.32	5.06	5.09	5.05	4.24	4.4	5.09	5.14	5.08	5.37
IND	2.25	2.29	2.42	2.31	2.28	2.16	2.32	2.38	2.12	2.17	2.53	2.6	2.37	2.21	2.3
END	3.15	2.61	2.51	2.53	2.7	2.77	2.78	2.96	2.79	2.47	2.61	2.69	2.33	2.55	2.37
ETD	0.96	0.92	0.98	1	0.86	1.02	0.82	0.85	0.89	0.89	0.76	0.84	0.76	0.95	0.98
UpL	7.1	6.19	6.31	6.02	6.5	6	6.08	6.62	6.31	6.43	6.39	6.31	6.39	6.88	6.58
FoI	7.86	7.39	7.78	7.49	7.28	7.28	7.32	7.58	7.56	7.51	7.82	7.51	7.58	7.92	7.62
HaL	7.57	6.82	7.3	6.41	6.23	6.6	6.72	6.28	6.46	6.86	6.93	6.7	6.58	7.24	7.53
F3PW/F3W	1.78	1.85	1.94	2.33	2.23	1.77	1.97	2.13	2.08	2.09	1.75	2.43	1.87	2.12	2.53
F4PW/F4W	1.77	2.34	2.15	2.38	2.10	1.84	1.94	2.12	2.17	2.13	2.42	2.44	2.46	1.97	2.64
FeL	10	9.4	9.11	9.24	9.19	9.87	9.57	9	9.32	9.95	9.81	9.82	9.34	9.22	9.9
TL	12.78	11.79	11.88	11.82	11.46	11.5	11.42	12.41	12.69	11.05	11.81	11.47	11.21	12.39	11.34
TaL	7.41	7.47	7.36	7.51	7.31	7.18	7.93	6.84	7.92	7.14	7.42	7.59	7.42	7	7.83
FL	4.49	4.33	4.97	4.31	3.9	4.22	4.49	4.12	4.21	4.58	4.82	4.35	4.25	4.58	4.75
3TL	3.72	3.74	3.64	3.59	3.18	3.16	3.44	3.18	3.17	3.53	3.42	3.34	3.48	3.38	3.71
TotFL	11.9	11.37	11.08	11.41	11.15	10.82	12.44	11.16	11.44	11.42	11.47	11.31	11.42	11.96	12.46
IPT	0.68	0.89	0.86	0.88	0.86	0.64	0.92	0.91	0.86	0.68	0.62	0.94	0.8	0.8	0.78
MPT	1.09	1.19	1.1	1.12	1.16	1.18	1.17	1.06	1.14	1	1.06	1.02	1.08	1.18	1.26
OPT	0.52	0.56	0.58	0.54	0.62	0.58	0.76	0.66	0.68	0.58	0.52	0.6	0.71	0.76	0.64
IMTL	1.02	1.2	1.36	1.2	1.14	1.01	1.12	1.26	1.18	1.1	1.16	1.15	1.16	1.13	1.1
OMTL	0.72	0.77	0.6	0.78	0.74	0.92	0.75	0.68	0.74	0.62	0.78	0.65	0.91	0.81	0.98
TW/ED	0.41	0.52	0.54	0.61	0.60	0.55	0.50	0.53	0.47	0.51	0.50	0.40	0.58	0.48	0.48
F3PW	1.14	1.26	1.28	1.26	1.36	1.12	1.42	1.32	1.25	1.13	1.1	1.36	1.12	1.4	1.62
F3W	0.64	0.68	0.66	0.54	0.61	0.67	0.72	0.62	0.6	0.54	0.63	0.56	0.6	0.66	0.64
F4PW	1.1	1.36	1.33	1.31	1.3	1.14	1.4	1.44	1.41	1.13	1.26	1.34	1.23	1.38	1.48
F4W	0.62	0.58	0.62	0.55	0.62	0.62	0.72	0.68	0.65	0.53	0.52	0.55	0.5	0.7	0.56

Table 3. Advertisement call data of *Eleutherodactylus franzi* sp. nov. and the sympatric *Eleutherodactylus humboldti*.

	***Eleutherodactylus franzi* sp. nov.**	***Eleutherodactylus humboldti***
Individuals	4	3
Call type	Pipe	Pipe
Dominant frequency (kHz)	2.61 ± 0.04	2.89 ± 0.23
Call length (ms)	106.0 ± 5.2	256.8 ± 12.8
Call rate (/m)	6.88 ± 0	2.16 ± 1.73
Call rise time (ms)	63.64 ± 4.06	90.82 ± 45.37
Pulse Rate	–	2.05 ± 0.62
Call Interval	13.03 ± 3.35	39.75 ± 14.77

who collected the type material and who helped collect an extensive sampling of the *Eleutherodactylus* specimens to be used in our succession of studies.

Discussion

In recent years, the subgenus *Syrrhophus* has received increasing attention from researchers. With the description of the new species here, the number of the taxa in the subgenus *Syrrhophus* increases to 44, with two in Cuba and 42 in mainland North America. Guerrero remains the most diverse State for the subgenus with 11 species occurring within its borders. The assignation of the novel species to the *E. nitidus* species group raises the number of species in that group to 16 (Grünwald et al. 2021; Devitt et al. 2023). This brings the west Mexican clades of the *E. modestus* species group (16 species) and the *E. nitidus* species group (16 species) to be equally diverse as currently understood. The east Mexican clade, the *E. longipes* group, currently is understood to contain nine species, although molecular results suggest that several undescribed species exist within this group as well (Hernández-Austria et al. 2022).

One of the most pressing questions regarding the taxonomy of these frogs is whether the subgenus *Syrrhophus* should be considered a genus. The genus *Eleutherodactylus* currently includes over 200 species distributed throughout North and Central America, including the Caribbean (Frost 2020). *Eleutherodactylus* was once the largest genus of vertebrates, but Heinicke, Duellman and Hedges (2007) split it into multiple genera. Currently, five subgenera are recognised in *Eleutherodactylus*: *Eleutherodactylus*, *Euhyas* Fitzinger, 1843, *Pelorius* Hedges, 1989, *Schwartzius* Hedges, Duellman & Heinicke, 2008 and *Syrrhophus*. Each of these subgenera is monophyletic and can be defined morphologically. For an in-depth review of *Eleutherodactylus*, see Hedges, Duellman and Heinicke (2008). The distinction between a genus and subgenus is arbitrary and a solid case can be made for *Syrrhophus* to be recognised as its own genus. *Syrrhophus* is a monophyletic group that diverged from its closest relatives, members of the subgenus *Euhyas*, over 20 million years ago (Heinicke et al. 2007). We suggest that a more detailed study of the macro-taxonomic relationships of these frogs should be performed and the current taxonomic status of the subgenera should be assessed in detail.

Conservation priorities

The species described herein has a very limited range, known only from near the type locality and restricted to one biogeographical formation as defined by Grünwald et al. (2015), namely the Central Mexican Transverse Range Pine-Oak Woodland (#44 on p. 409 in Grünwald et al. (2015).

Eleutherodactylus franzi is known from a karstic mountain range that has about 75 km^2 of habitat at elevations sufficiently high enough to support populations. It has not been collected anywhere else and may be restricted to the immediate vicinity of the type locality (Fig. 7). Other saxicolous specialised species of *Eleutherodactylus* have similarly only been collected near the type locality, including *E. erythrochomus*, *E. grunwaldi*, *E. manantlanensis*, *E. colimotl* and *E. saxatilis*. Due to its limited distribution and potential endemism near the type locality, we propose this species be provisionally classified as Critically Endangered B1ab(iii), based on the IUCN Red List Criteria that its occurrence is less than 100 km^2, it occurs in only one threat-defined location and there is ongoing decline in the extent and quality of its habitat due to small-scale cattle ranching, illegal logging and varied agricultural practices.

The frog subgenus *Syrrhophus* is the fastest-growing group of frogs in Mexico, in terms of the number of new taxa being described. Despite the recently augmented interest in the group, we believe that the current diversity may still be underestimated and might continue to increase in the coming years. Apart from collecting in regions currently under-sampled, like the mountains of Guerrero and Oaxaca, we suggest that it is important to re-examine specimens already housed in herpetological collections, as there are multiple errors in the identification of specimens of *Eleutherodactylus* and un-identified new taxa may be hiding in collections.

Additionally, we believe that it is of great importance that future molecular studies include additional markers,

Figure 7. Type locality of *Eleutherodactylus franzi* sp. nov., at El Cucharillo, Municipio de Ixcateopan de Cuauhtémoc, Guerrero, Mexico.

as this allows for a much better understanding of the relationships within this group of frogs. Finally, our data suggest that it is time to revise the status of the subgenus *Syrrhophus* and to determine if it should be elevated to generic level.

Competing interests

The authors have declared that no competing interests exist.

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Appendix 1

Specimens examined

Eleutherodactylus albolabris (*n* = 20): MEXICO: Guerrero: MZFC 33025–33030 (CIG 00327–00332), 33082–33085 (CIG 00390– 00393), MZFC 33108–33109 (CIG 00441–00442), MZFC 33230 (CIG 00668), MZFC 33300–33301 (CIG 00903–00904), MZFC 33323 (CIG 00953), MZFC 33325–33326 (CIG 00955–00956), JAC 25586, 25642.

Eleutherodactylus. angustidigitorum (*n* = 20): MEXICO: Jalisco: MZFC 33127–33130 (CIG 00476–00479), MZFC 33224–33225 (CIG 00662–00663), MZFC 33386–33388 (CIG 00991–00993), JAC 24912; Michoacán: MZFC 33015–33017 (CIG 00316– 00318), MZFC 33065–33070 (CIG 00373–00378), JAC 26977.

Eleutherodactylus campi (*n* = 13): MEXICO: Nuevo León: MZFC 33195–33198 (CIG 00606–00609); UNITED STATES: Texas: JHM 1390–1394.

Eleutherodactylus colimotl (*n* = 20): MEXICO: Colima: MZFC 29282 (CIG 00468), MZFC 33115–33120 (CIG 00462–00467), MZFC 33237–33239 (CIG 00682–00684), MZFC 33299 (CIG 00901), MZFC 33329–3330 (CIG 00960–00961), JAC 30498–30499, 30631; Michoacán: MZFC 33036 (CIG 00340), JAC 23999–24001.

Eleutherodactylus cystignathoides (*n* = 6): MEXICO: Veracruz: MZFC 33351–33353 (CIG 01163–01165), MZFC 33354 (CIG 01170), JAC 30000–30001.

Eleutherodactylus dennisi (*n* = 13): MEXICO: Tamaulipas: MZFC 33255–33261 (CIG 00822–00828), UTA 59516–59521.

Eleutherodactylus dilatus (*n* = 19): MEXICO: Guerrero: MZFC 33089–33094 (CIG 00405–00410), MZFC 33097 (CIG 00428), MZFC 33231 (CIG 00669), UTA 4017–4020, 4023–4024, 5269, 5276–5279.

Eleutherodactylus erendirae (*n* = 25): MEXICO: Jalisco: MZFC 33000–33008 (CIG 00300–00309), MZFC 33226–33229 (CIG 00664–00667), MZFC 33232 (CIG 00673), MZFC 33234–33235 (CIG 00679–00681); Michoacán: MZFC 29274, 33019–33024 (CIG 00319–00325).

Eleutherodactylus erythrochomus (*n* = 2): MEXICO: Guerrero: INIRENA 2923–24 (CIG 01922–01923).

***Eleutherodactylus franzi* sp. nov.** (*n* = 15): MEXICO: Guerrero: INIRENA 2895–98, 2900–2910 (CIG 01716–01729, 01731).

Eleutherodactylus floresvillelai (*n* = 12): MEXICO: Michoacán: MZFC 33053–33064 (CIG 00361–00372).

Eleutherodactylus grandis (*n* = 1): MEXICO: Ciudad de Mexico: UTA 56845.

Eleutherodactylus grunwaldi (*n* = 12): MEXICO: Colima: MZFC 27467–27475, MZFC 27484, MZFC 33298 (CIG 00898); JRV 00230.

Eleutherodactylus guttilatus (*n* = 10): MEXICO: Guanajuato: MZFC 33367–33369 (CIG 01248–01250); San Luis Potosí: MZFC 33200–33206 (CIG 00619–00625).

Eleutherodactylus humboldti (*n* = 17): MEXICO: Estado de México: INIRENA 2911–2916 (CIG 01703, 01702, 01704–01710); Guerrero: INIRENA 2899, 2920–2922 (, 01730, CIG 01713–01715); CIG 00962–00965.

Eleutherodactylus interorbitalis (*n* = 7): MEXICO: Sinaloa: MZFC 33186–33187 (CIG 00584–00585), MZFC 33190–33194 (CIG 00600–00604).

Eleutherodactylus jaliscoensis (*n* = 15): MEXICO: Jalisco: MZFC 33131–33141 (CIG 00480–00490), MZFC 33274–33276 (CIG 00861–00863), MZFC 33280 (CIG 00876).

Eleutherodactylus jamesdixoni (*n* = 14): MEXICO: Jalisco: MZFC 33010–33014 (CIG 00310–00314), MZFC 33034–33035 (CIG 00336–00337), MZFC 33110 (CIG 00457), MZFC 33273 (CIG 00860), JAC 28612; Nayarit: MZFC 33211 (CIG 00649), MZFC 33240–33242 (CIG 00685–00687).

Eleutherodactylus leprus (*n* = 7): MEXICO: Veracruz: MZFC 33345–33350 (CIG 01139–01144), CIG 01270.

Eleutherodactylus longipes (*n* = 3): MEXICO: Nuevo León: MZFC 33199 (CIG 00611); Querétaro: UTA 59421–59422.

Eleutherodactylus maculabialis (*n* = 27): MEXICO: Guerrero: MZFC 33307–33319 (CIG 00916–00923, 00940–00941, 00945–00947), MZFC 33321 (CIG 00949), MZFC 33323 (CIG 00953), CIG 01484–01485, 01501, JAC 25643–25646.

Eleutherodactylus marnocki (*n* = 3): USA: Texas: JHM 1427–1429.

Eleutherodactylus manantlanensis (*n* = 14): MEXICO: Colima: MZFC 33372–33377 (CIG 00530–00535), MZFC 33379–33381 (CIG 00646–00648), MZFC 33292–33296 (CIG 00892–00896).

Eleutherodactylus maurus (*n* = 19): MEXICO: Estado de México: MZFC 33071–33076 (CIG 00379–00384), MZFC 33355 (CIG 01174); Morelos: MZFC 33077–33080 (CIG 00385–00388), INIRENA 2925–30 (CIG 01733–01737, 01742).

Eleutherodactylus modestus (*n* = 34): MEXICO: Colima: MZFC 26888–26889, MZFC 33263–33270 (CIG 00850–00857), MZFC 33291 (CIG 00891), MZFC 33297 (CIG 00897); Jalisco: MZFC 33144–33149 (CIG 00493–00498), MZFC 33150–33154 (CIG 00505–00509), MZFC 33161 (CIG 00522), MZFC 33183–33185 (CIG 00570–00572), MZFC 33217–33223 (00655–00661).

Eleutherodactylus nebulosus (*n* = 6): MEXICO: Chiapas: MZFC 33361–33366 (CIG 01236–01241).

Eleutherodactylus nietoi (*n* = 13): MEXICO: Michoacán: MZFC 33121 (CIG 00299), MZFC 33042–33045 (CIG 00346–00349), MZFC 33050–33052 (CIG 00355–00357), MZFC 33336–33337 (CIG 00974–00975), MZFC 33342–33343 (CIG 00983–00984), MZFC 33344 (CIG 00994).

Eleutherodactylus nitidus (*n* = 31): MEXICO: Estado de México: JAC 27237; Guerrero: MZFC 33096–33097 (CIG 00411–00412), MZFC 33104–33105 (CIG 00437–00438), JAC 25815; Morelos: MZFC 33081 (CIG 00389); Oaxaca: MZFC 33357–33358 (CIG 01211–01212); Puebla: MZFC 33356 (CIG 01181), JAC 27256–27276.

Eleutherodactylus orarius (*n* = 13): MEXICO: Colima: MZFC 26890, MZFC 33262 (CIG 00849); Michoacán: MZFC 33037 (CIG 00341), MZFC 33335 (CIG 00973), JAC 24020, 25526, 25563–25564, 29107, 30500–30501, 30517, 30625.

Eleutherodactylus pallidus (*n* = 13): MEXICO: Jalisco: MZFC 33271–33272 (CIG 00858–00859); Nayarit: MZFC 33189 (CIG 00588), MZFC 33212–33216 (CIG 00650–00654), MZFC 33243–33245 (CIG 00688–00690), MZFC 33018 (CIG 00995); Sinaloa: MZFC 33188 (CIG 00586).

Eleutherodactylus petersi (*n* = 11): MEXICO: Guerrero: MZFC 33034–33035 (CIG 00336–00337); JAC 25219, 25265–25266, 25299; Jalisco: MZFC 33010–33014 (CIG 00310–00314), MZFC 33034–33035 (CIG 00336–00337), MZFC 33110 (CIG 00457), MZFC 33273 (CIG 00860), JAC 28612; Michoacán: MZFC 33382–33385 (CIG 00675–00677), JAC 26947; Nayarit: MZFC 33211 (CIG 00649), MZFC 33240–33242 (CIG 00685–00687).

Eleutherodactylus. pipilans (*n* = 15): MEXICO: Guerrero: MZFC 33086–33088 (CIG 00396–00398), MZFC 33106–33107 (CIG 00439–00440), MZFC 33322 (CIG 00952), CIG 1465; Oaxaca: MZFC 33210 (CIG 00645), JAC 24283, 25809–25811.

Eleutherodactylus rubrimaculatus (*n* = 3): MEXICO: Chiapas: MZFC 33249–33251 (CIG 00753, 00755–00756),

Eleutherodactylus rufescens (*n* = 40): MEXICO: Jalisco: MZFC 33122–33126 (CIG 00471–00475), MZFC 33162–33164 (CIG 00527–00529), MZFC 33165–33174 (CIG 00544–00553), MZFC 33385 (CIG 00678); Michoacán: MZFC 33038–33041 (CIG 00342–00345), MZFC 33046–33049 (CIG 00350–00353), MZFC 33175–33182 (CIG 00559–00566), MZFC 33233 (CIG 00674), MZFC 33338 (CIG 00976), MZFC 33339–33341 (CIG 00980–00982).

Eleutherodactylus saxatilis (*n* = 4): MEXICO: Sinaloa: MZFC 26893, 26896, 26898–26899.

Eleutherodactylus sentinelus (*n* = 8): MEXICO: Guerrero: MZFC 33031–33033 (CIG 00333–00335), MZFC 33302– 33306 (CIG 00907–00913).

Eleutherodactylus syristes (*n* = 21): MEXICO: Guerrero: ANMO 2999; MZFC 33098–33103 (CIG 00431–00436), MZFC 33324 (CIG 00954), MZFC 33327–33328 (CIG 00957–00958) JAC 25701–25703; Oaxaca: MZFC 33207–33208 (CIG 00627–00628), MZFC 33209 (CIG 00644), 33378 (CIG00643), MZFC 33246–33247 (CIG 00713–00714), MZFC 33359–33360 (CIG 01232– 01233).

Eleutherodactylus teretistes (*n* = 5): MEXICO: Jalisco: MZFC 33142–33143 (CIG 00491–00492), MZFC 33277–33279 (CIG 00864–00866).

Eleutherodactylus verrucipes (*n* = 3): MEXICO: Tamaulipas: MZFC 33253–33254 (CIG 00813–00814); Querétaro: CIG 01273.

Eleutherodactylus wixarika (*n* = 3): MEXICO: Jalisco: MZFC 27477–27479.

Appendix 2

Table A1. Genetic accession numbers.

Field number	Organism	Museum number	Locality	GenBank number
CIG-00953	*E. albolabris*	MZFC-33323	Mexico: Guerrero	MG856956
JAC-25642	*E. albolabris*	UTA-61578	Mexico: Guerrero	MT872448
CIG-00392	*E. albolabris*	MZFC-33084	Mexico: Guerrero	MT872468
CIG-00441	*E. albolabris*	MZFC-33108	Mexico: Guerrero	MT872476
CIG-00331	*E. albolabris*	MZFC-33029	Mexico: Guerrero	MT872482
CIG-00332	*E. albolabris*	MZFC-33030	Mexico: Guerrero	MT872483
CIG-00477	*E. angustidigitorum*	–	Mexico: Jalisco	MG856963
CIG-00479	*E. angustidigitorum*	–	Mexico: Jalisco	MG856964
CIG-00407	*E. dilatus*	MZFC33091	Mexico: Guerrero	MG856973
CIG-00408	*E. dilatus*	MZFC33092	Mexico: Guerrero	MG856974
CIG-00070	*E. dilatus*	–	Mexico: Guerrero	**OP895113**
EU186711	*E. erythrochomus*	MZFC 16254	Mexico: Guerrero	EU186711
RPA-0183	*E. erythrochomus*	–	Mexico: Guerrero	MZ203201
RPA-0185	*E. erythrochomus*	–	Mexico: Guerrero	MZ203202
CIG-01716	*E. franzi sp. nov.*	INIRENA-2901	Mexico: Guerrero	**OP888987**
CIG-01717	*E. franzi sp. nov.*	INIRENA-2902	Mexico: Guerrero	**OP888988**
CIG-01729	*E. franzi sp. nov.*	INIRENA-2897	Mexico: Guerrero	**OP888989**
CIG-01248	*E. guttilatus*	–	Mexico: Guajuato	**OP895114**
CIG-01249	*E. guttilatus*	–	Mexico: Guajuato	**OP895115**
CIG-01702	*E. humboldti.*	INIRENA-2912	Mexico: Mexico	**OP888990**
CIG-01703	*E. humboldti.*	INIRENA-2913	Mexico: Mexico	**OP888991**
CIG-01713	*E. humboldti*	INIRENA-2920	Mexico: Guerrero	**OP888992**
CIG-01730	*E. humboldti*	INIRENA-2899	Mexico: Guerrero	**OP888993**
GP-2422	*E. humboldti*	–	Mexico: Mexico	**OP888994**
GP-2423	*E. humboldti*	–	Mexico: Mexico	**OP888995**
RHA-0006	*E. humboldti.*	–	Mexico: Mexico	**OP888996**
RHA-0007	*E. humboldti*	–	Mexico: Mexico	**OP888997**
CIG-00649	*E. jamesdixoni*	MZFC-33211	Mexico: Nayarit	MT872469
CIG-00687	*E. jamesdixoni*	–	Mexico: Nayarit	MG857035
CIG-00686	*E. jamesdixoni*	–	Mexico: Nayarit	**OP895111**
CIG-00860	*E. jamesdixoni*	–	Mexico: Jalisco	**OP895112**
CIG-00921	*E. maculabialis*	MZFC-33312	Mexico: Guerrero	MT872460
CIG-00923	*E. maculabialis*	MZFC-33314	Mexico: Guerrero	MT872461
CIG-00940	*E. maculabialis*	MZFC-33315	Mexico: Guerrero	MT872462
CIG-00941	*E. maculabialis*	MZFC-33316	Mexico: Guerrero	MT872463
CIG-00946	*E. maculabialis*	MZFC-33318	Mexico: Guerrero	MT872464
CIG-00947	*E. maculabialis*	MZFC-33319	Mexico: Guerrero	MT872465
CIG-00893	*E. manantlanensis*	MZFC-33293	Mexico: Colima	MG857007
CIG-00388	*E. maurus*	MZFC-33080	Mexico: Morelos	MG857010
CIG-00380	*E. maurus*	MZFC-33072	Mexico: Mexico	MG857011
CIG-00382	*E. maurus*	MZFC-33074	Mexico: Mexico	MT872478
CIG-00385	*E. maurus*	MZFC-33077	Mexico: Morelos	MT872479
CIG-0-387	*E. maurus*	MZFC-33079	Mexico: Morelos	MT872480
CIG-01733	*E. maurus*	INIRENA-2925	Mexico: Morelos	**OP888998**
CIG-01734	*E. maurus*	INIRENA-2926	Mexico: Morelos	**OP888999**
CIG-00857	*E. modestus*	MZFC33270	Mexico: Colima	MG857021
CIG-00891	*E. modestus*	–	Mexico: Colima	MG857012
CIG-00753	*E. nebulosus*	MZFC-33249	Mexico: Chiapas	MG857056
CIG-00755	*E. nebulosus*	MZFC-33250	Mexico: Chiapas	MG857057
CIG-01237	*E. nebulosus*	MZFC-33362	Mexico: Chiapas	MT872429
CIG-01238	*E. nebulosus*	MZFC-33363	Mexico: Chiapas	MT872430
CIG-01240	*E. nebulosus*	MZFC-33365	Mexico: Chiapas	MT872431
CIG-01241	*E. nebulosus*	MZFC-33366	Mexico: Chiapas	MT872432
EU186712	*E. nitidus*	AMCC-118239	Mexico: Puebla	EU186712
CIG-00715	*E. nitidus*	MZFC-33248	Mexico: Oaxaca	MG857030
CIG-00412	*E. nitidus*	MZFC-33096	Mexico: Guerrero	MG857031
CIG-00336	*E. nitidus*	MZFC-33034	Mexico: Guerrero	MG857032
CIG-00311	*E. nitidus*	MZFC-33011	Mexico: Jalisco	MG857033
JAC-25815	*E. nitidus*	UTA-61584	Mexico: Guerrero	MT872459
CIG-00341	*E. orarius*	MZFC-33037	Mexico: Michoacán	MG857041
CIG-00460	*E. orarius*	MZFC-33113	Mexico: Colima	MG857042
JAC-24020	*E. orarius*	UTA-59508	Mexico: Michoacán	MT872434
JAC-25343	*E. orarius*	UTA-62402	Mexico: Michoacán	MT872442
JAC-25344	*E. orarius*	UTA-62403	Mexico: Michoacán	MT872443
CIG-00458	*E. orarius*	MZFC-33111	Mexico: Colima	MT872477
JAC-25392	*E. orarius*	–	Mexico: Michoacan	**OP895116**
CIG-00337	*E. petersi*	MZFC-33035	Mexico: Guerrero	MT872473
CIG-00310	*E. petersi*	MZFC-33010	Mexico: Jalisco	MT872487
CIG-00396	*E. pipilans*	MZFC-33086	Mexico: Guerrero	MG857054
CIG-00398	*E. pipilans*	MZFC-33088	Mexico: Guerrero	MG857055
CIG-00440	*E. pipilans*	MZFC-33107	Mexico: Guerrero	MT872475
JAC-31539	*E. pipilans*	–	Mexico: Oaxaca	**OP895117**
JAC-31540	*E. pipilans*	–	Mexico: Oaxaca	**OP895118**
CIG-00334	*E. sentinelus*	MZFC-33032	Mexico: Guerrero	MT872485
CIG-00335	*E. sentinelus*	MZFC-33033	Mexico: Guerrero	MT872486
CIG-00333	*E. sentinelus*	MZFC-33031	Mexico: Guerrero	MT872484
CIG-00954	*E. syristes*	MZFC-33324	Mexico: Guerrero	MG857070
CIG-00714	*E. syristes*	MZFC-33247	Mexico: Oaxaca	MG857072
CIG-00628	*E. syristes*	MZFC-33208	Mexico: Oaxaca	MG857073
CIG-00627	*E. syristes*	MZFC-33207	Mexico: Oaxaca	MT872467
CIG-00431	*E. syristes*	MZFC-33098	Mexico: Guerrero	MT872471
CIG-00434	*E. syristes*	MZFC-33101	Mexico: Guerrero	MT872472

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New survey data on abundance and movements for two poorly known Asian Spiny Frogs

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Abstract

We present new natural history data on abundance and movements (daily and seasonal) during the pre-breeding (March-June) and breeding-post-breeding season (July-September) of two poorly studied frog species of the Himalayas, *Nanorana vicina* and *Allopaa hazarensis*. We estimated 185 Murree Hills Frogs and 90 Hazara Frogs within the study area (0.79 ha). The daily and seasonal movement data showed that the two species moved either between neighboring ponds or remained in an array of smaller ponds (within an area of 120 m^2) along the stream bank. About 75% of movements were < 29.5 m in *N. vicina* and < 50.87 m in *A. hazarensis* during pre-breeding season while < 41.5 m in *N. vicina* and < 81 m in *A. hazarensis* during breeding-post-breeding season. We suggest inclusion of amphibian habitat requirements and ensuring stream connectivity in urban planning and development projects in the area to prevent the local extinction of the endemic species. In the future, more robust and long-term studies, encompassing more streams situated within a wider area, would help clarify dispersal, colonization, metapopulation structure, and dynamics of these endemic frogs of the forested montane streams in the Himalayan Foothills.

Key Words

Dicroglossidae, Highland Frogs, Himalaya, Lincoln Index, Paini tribe

Introduction

The temporal and spatial aspects of animal movement are considered important for population control, metapopulation dynamics and life-long sustainability of wildlife species. The impact of land use, the spread of invasive species, diseases, and responses to global climate change, can be better understood if we comprehend animal movement and dispersal (Smith and Green 2006). Movement is essential for the reproduction and survival of local populations and in a wider landscape or regional scale, mobility among populations is necessary for recolonization after local extinction and for the maintenance of metapopulations (Marsh and Trenham 2001). Young amphibians seldom disperse more than 2–3 kilometers (Petranka and Holbrook 2006) and rarely move more than five kilometers (Peter 2001). Amphibian dispersal is vital for the maintenance of amphibian populations and yet this is often significantly compromised in anthropogenically impacted landscapes (Ribeiro et al. 2019).

Currently, 21 species of amphibian (all anurans) have been documented in Pakistan (Rais et al. 2021) of which nine are believed to be endemic to Pakistan (Ali et al. 2018). *Allopaa hazarensis*, Family Dicroglossidae, is endemic to the springs and streams of Northern Pakistan (> 1,195 m elevation) while *Nanorana vicina*, Family Dicroglossidae, is endemic to Pakistan (> 1,765 m elevation) and India (Khan 2006; Ahmed et al. 2020) (Fig. 1). The two frog species are listed as Least Concern in IUCN Red List of Threatened Species. The major threats to these frog species include habitat degradation, urbanization, and climate change (Ohler and Dutta 2004; Khan et al. 2008).

The description of their adults and tadpoles is available (Dubois and Khan 1979; Khan and Malik 1979; Rais et al. 2014; Gill et al. 2020; Ikram et al. 2022). Further information about molecular taxonomy and phylogeny (Hofmann et al. 2019, 2021; Akram et al. 2021), impact of climate change (Saeed et al. 2021, 2022a), and occupancy modeling and population monitoring through eDNA (Saeed et al. 2022b) have been carried out recently. The post-breeding radiotelematry (VHF) revealed that the two species show little movement (< 3 m) (Akram et al. 2022). These two frog species breed during summer and monsoon: July-August (Saeed et al. 2021). There is no study available on what triggers them to breed and regarding their clutch size and age structure. The temperature had a positive correlation with increase in the concentrations of sex hormones (Saeed et al. 2021). Since these frogs inhabit forested streams having hard and rocky substrate (Khan 2006), these are believed to lay eggs under the heavy boulders where early development takes place. Under laboratory conditions, *A. hazarensis* completed metamorphosis during 12th-18th week while *N. vicina* completed metamorphosis during 18th-22th week with 22 °C as the most stable temperature for their growth. Although higher temperatures (>26 °C) were associated with early onset and completion of metamorphosis, they caused reduction in the body size, more frequent developmental complications or deformities such as edema and tail kinks, lower fitness and higher mortality (Saeed et al. 2021).

Since not much is known about the natural history of these two Himalayan endemics, we aimed to provide abundance data and examine daily and seasonal movements of the *N. vicina* (Murree Hills Frogs) and the *A. hazarensis* (Hazara Frogs) along a continuous habitat- montane forested freshwater stream. Our findings will help survey anurans associated with similar habitats elsewhere in the world. Our data on relative abundance and movement patterns of the two Himalayan endemics help increase understanding of their habitat requirements, metapopulation structure, dispersal, and colonization.

Materials and methods

Study area system and species

We conducted the study at a permanent freshwater stream (Fig. 2) located in Murree Tehsil, Rawalpindi District, Punjab Province, Pakistan. The study area is a part of the Western Himalayan Ecoregion which is included in Global 200 Ecoregions of Ecological Significance. Murree spreads over an area of about 697.5 km^2 with an elevation of 550–2,600 m (Shahzad et al. 2015). The climate is subtropical highland (Cwb as per Koppen climate classification) (Beck et al. 2018). The mean maximum temperature of the area is 25 °C while the mean annual precipitation is 1,789 mm (EPDP 2010). The precipitation generally occurs in the form of snow during the winter (December, January, and February) and rain during the summer. The study area features subtropical pine forest with Chir Pine (*Pinus roxburghii*) as the dominant plant species of the area (Shahzad et al. 2015).

The study sites consisted of six ponds, connected by a permanent stream at an elevation of between 1,660 and 1,705 m (Fig. 3). It cascades over rapids and the nearest adjacent streams are 370 m and 1,750 m (straight linear distance) away over low ridges. We selected a section of 133 m of the stream and surveyed frogs 30 m on either side of it (0.79 ha.).

Study duration, sampling, tagging, estimation of distance moved and population size

We gathered data from March, 2019, to September, 2019, during pre-breeding (March-June), and breeding-post-breeding seasons (July-September). We performed an eight-day field session in each season. During the first field season (May 17–24, 2019), we captured and marked 74 (33 ♂, mean snout-vent length, SVL: 67.02 ± 11.67; 41 ♀, SVL: 68.02 ± 10.62) individuals of *N. vicina* and 15 (8 ♂, SVL: 54.18 ± 19.15; 7 ♀, SVL: 64.41 ± 24.34) of *A. hazarensis* (Suppl. material 1: table 1a). We measured the straight distance (meters) between the ponds using measured rope to determine the distance between the pond where the frog was released to the pond where it was recaptured. We flagged rocks and trees using pre-measured ropes of different lengths (10 m, 20 m, and 30 m) on either side of the stream to note the distance moved by the specimens away from the stream (Fig. 3). This eliminated the need to measure distance moved by the frogs, manually or through a GPS each time. The survey team consisted of 8–10 observers. The surveyors moved upstream (from first pond to the last identified), capturing adults for tagging and estimating the distance moved. We carried out the surveys 4–5 hours after sunset, collected frogs using a dip net and gathered the data as described. We identified the species

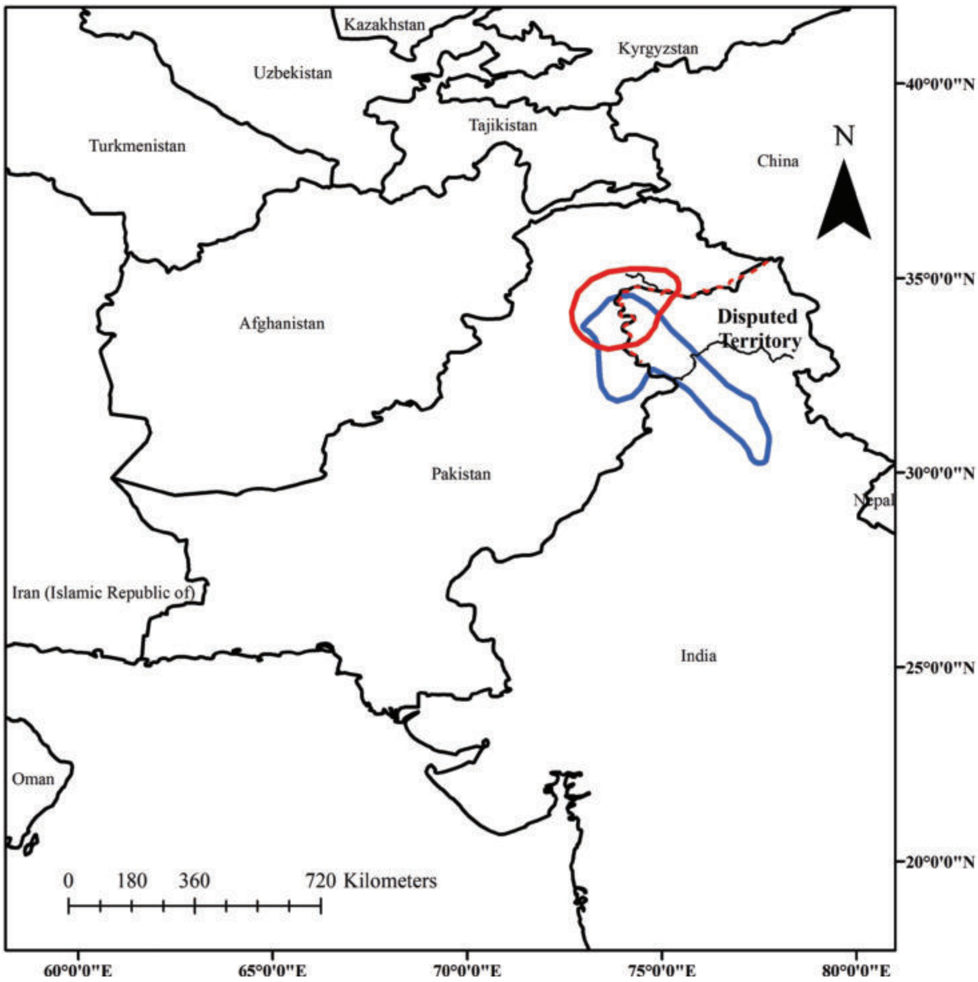

Figure 1. Global distribution range of Murree Hills Frog (*Nanorana vicina*) (blue polygon) and Hazara Frog (*Allopaa hazarensis*) (red polygon).

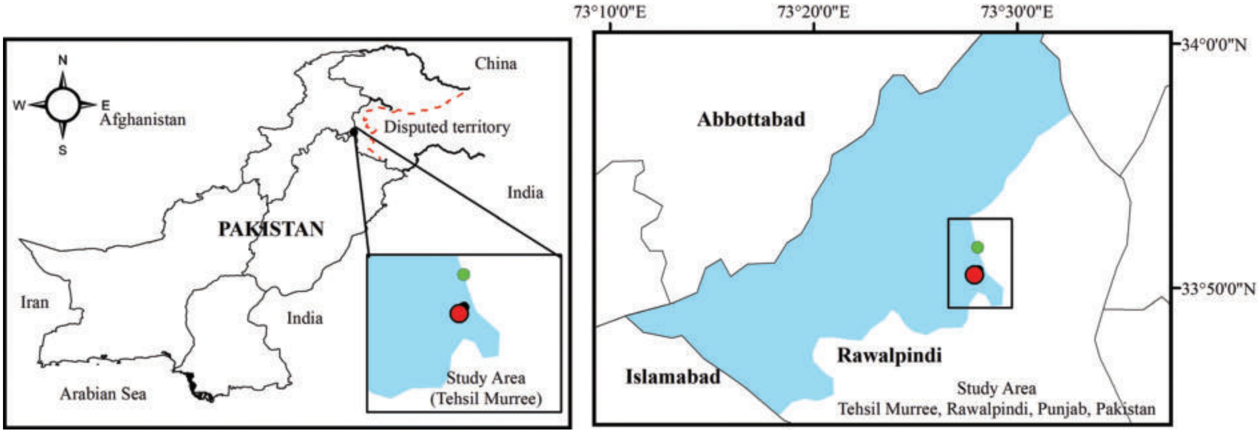

Figure 2. Map showing location of the study area and the study stream in Village Parhanna, Tehsil Rawalpindi, Province Punjab, Pakistan (left) Study stream and other nearby streams shown in red circle 370 m and green circle 1750 m from the study stream (right).

using Khan (2006), their sex (females have soft belly) and measured the snout-vent length (SVL) using a digital vernier caliper (0.01 mm, Insize). We marked the frogs using toe-clipping (Guimaraes et al. 2014; Ginnan et al. 2015), released them back and noted the point of release (pond number). We repeated the procedure for eight consecutive days, and recorded the distance (from the pond where the frog was released to where it was recaptured). We then estimated the mean daily distance (8 days) and distance moved per day. While during the second field season (August 28–September 4, 2019), we captured and marked 10 (5 ♂, SVL: 68.39 ± 30.58; 5 ♀, SVL: 61.11 ± 29.93) *N. vicina* and 30 (16 ♂, SVL: 56.96 ± 14.24; 7 ♀, SVL: 65.59 ± 17.52) *A. hazarensis* (Suppl. material 1: table 1b)

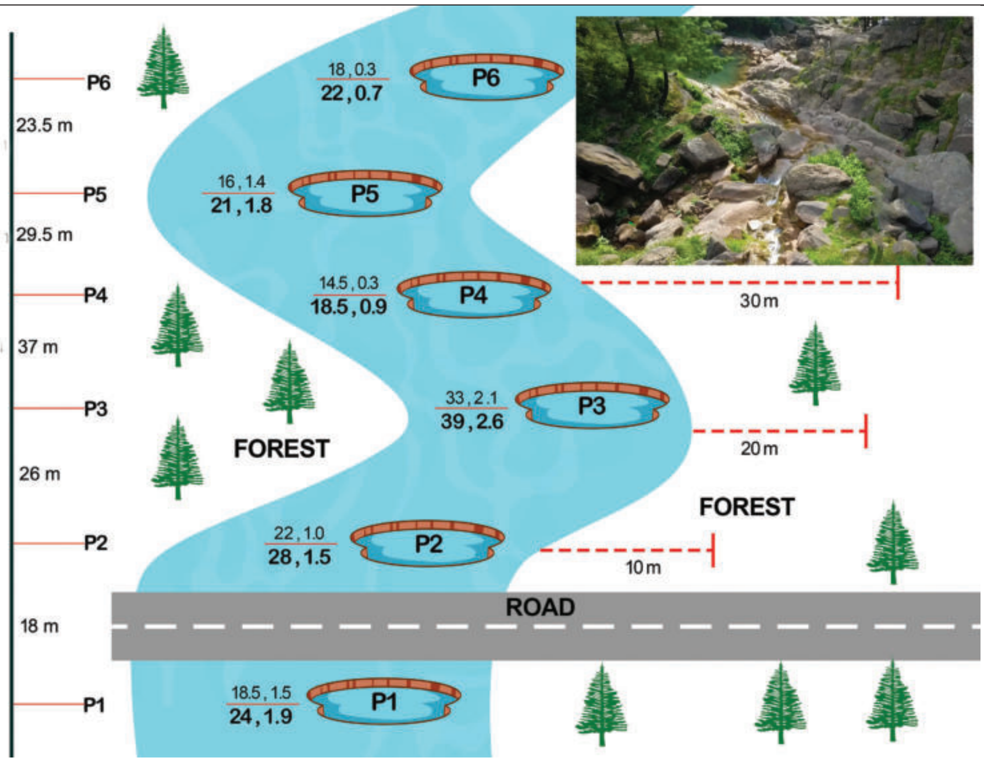

Figure 3. Structure of the study stream (inset photograph shows stream habitat) and its associated ponds. The pond 1 is separated from other ponds by a road. Numerator value shows perimeter m and depth m during pre-breeding season while denominator, in bold, show same measurements for breeding-post-breeding season. The distances (m) between ponds (measured from the center of each pond) are given on the right side. Pre-measured ropes of different lengths (10–30 m) were used to mark distances on either side of the stream.

and recorded the data as described above. The distance moved by the frogs recaptured during this season, which were marked in pre-breeding season, was used to estimate mean distance moved by the frogs for pre-breeding season. We made a two-day visit on 14–15 September, 2019, to estimate the distance moved by the frogs for the breeding-post-breeding season.

We plotted the distance data for the recaptured frogs as box plots for each sex (male and female) and season (pre-breeding, breeding-post-breeding) and ran Wilcoxon test. We also compared (median) distance between species, seasons and sex using Wilcoxon test in R. 4.3.0 ("ggstatsplot") (Patil 2021; R Core Team 2021).

We used Lincoln Index (LI) to estimate the population of each frog species: LI = (N × n) / R where n = number of individuals captured on the first occasion (May, 2019), N = number of individuals captured on the second occasion (August-September, 2019), r = recaptured those with marks (14–15 September, 2019). The standard error (SE) was calculated using formula: √ n2 X N (n-r)/ r3 while the limits of confidence interval (95%) were calculated as 1.96 (SE) ± LI. This is the simplest method based on few episodes of marking and recapturing individuals over a short period of time (Fowler et al. 1998).

Due to financial and technical constraints, we focused on one stream which was selected due to its being a permanent freshwater stream in close proximity to other streams, where both endemic species occurred. The stream is accessible all year round and is safe to carry out nocturnal surveys.

Results

Adult population estimates and sex ratio

We estimated that the number of male and female *N. vicina* was 83 (95% CI: 60–126) and 102 (95% CI: 79–151), respectively and of *A. hazarensis* was 43 (95% CI: 15–97) males and 49 (95% CI: 8–130) females. The number of individuals of *N. vicina* and *A. hazarensis* was estimated to consist of 185 (95% CI: 152–250) and 92 (95% CI: 44–181) individuals from the study area (0.79 ha.) during the study period, respectively. The sex ratio (♂: ♀) of *N. vicina* and *A. hazarensis* was 0.8 (<1):1 and 0.8 (<1):1, respectively (~ 1:1 for both species), during the breeding season.

Daily movement

Pre breeding

The mean distance (pooled for eight days) and the mean daily distance (per day) covered by *N. vicina* (n = 16, Min.–Max.= 0–92 m) during pre-breeding season was 22.71 ± 6.50 m and 2.83 m, respectively. About 25% *N. vicina* moved < 1.25 m (1st quartile) and 75% moved < 29.5 m (3rd quartile). The mean distance (pooled for eight days) and the mean daily distance (per day) covered by *A. hazarensis* (n = 7, Min.–Max.= 0–63 m) was 23.41 ±12.72 m and 2.92 m, respectively. About 25% *A. hazarensis* moved < 0 m and 75% moved < 50.87 m.

Breeding-post-breeding season

The distance (pooled for eight days) and the daily distance (per day) covered by *N. vicina* (n = 3, Min.–Max.= 22–44 m) during breeding-post-breeding season was 33.33 ± 6.35 m and 4.16 m, respectively. About 25% moved < 25 m and 75% moved < 41.5 m. The distance (for eight days) and the mean daily distance (per day) covered by *A. hazarensis* (n = 7, Min.–Max.= 18–81 m) was 51.5 ± 17.10 m and 6.43 m, respectively. About 25% moved < 20 m and 75% moved < 81 m.

Seasonal movement

Pre-breeding

The distance covered by *N. vicina* (n = 11, Min.–Max.= 0–110 m) during the pre-breeding season was 56.86 ± 10.55. About 25% moved < 20 m and 75% moved < 68.5 m. The distance covered by *A. hazarensis* (n = 7, Min.–Max.= 23.5–133.5 m) was 81.66 ± 19.27 m. About 25% moved < 26.62 m and 75% moved < 114.5 m. Four Murree Hills Frog were recorded within 20 m (7, 9, 12 and 18 m) distance outside the stream while only one Hazara Frog was recorded within 10 m (8 m) distance.

Breeding-post-breeding

The distance covered by *N. vicina* (n = 4, Min.–Max. = 22–110 m) during breeding-post-breeding season was 71.75 ± 18.32 m. About 25% moved < 35 m and 75% moved < 102.75 m. The distance covered by *A. hazarensis* (n = 5, Min.–Max.= 0–92.5 m) was 70.1 ± 17.87 m. About 25% moved < 18.5 m and 75% moved < 92 m. Two Murree Hills Frogs were recorded within 20 m (10, 12 m) distance outside the stream while only one Hazara Frog was recorded within 10 m distance. Three Hazara Frog were recorded within 30 m distance (8, 19, 22 m) during September, 2019, recapture period.

The difference between the distance (daily movement) moved by *N. vicina* (sexes pooled) during the two seasons (Fig. 4A) and two sexes (Fig. 4B) and distance (seasonal movement) during the two seasons (Fig. 4C) and two sexes (Fig. 4D) did not differ significantly. The distance (m) moved by the two endemic frogs when species and sexes pooled (Fig. 5A), species pooled (Fig. 5B) and sexes pooled did not differ (Fig. 5C).

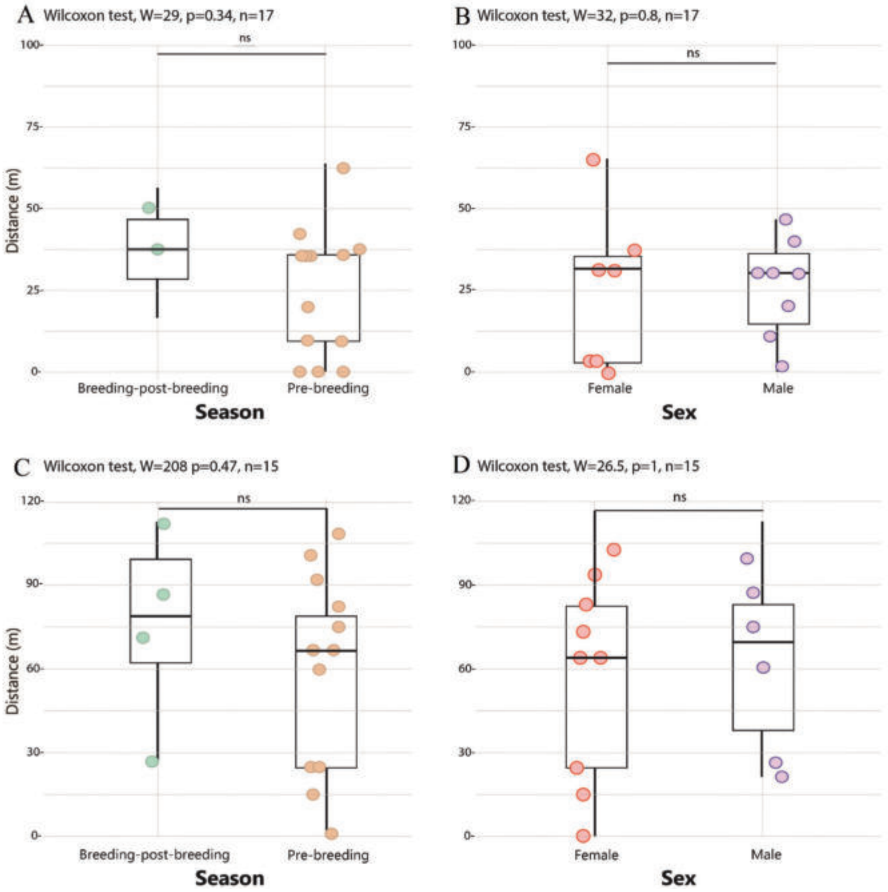

Figure 4. A. Box plot showing comparison between daily distance (m) moved by Murree Hills Frogs (*Nanorana vicina*) during the two seasons (sexes pooled, two 8 days field sessions) (ns= non-significant at α 0.05); **B.** Box plot showing comparison between daily distance (m) moved by males and females of Murree Hills Frog (*Nanorana vicina*) (seasons pooled, two 8 days field sessions) (ns= non-significant at α 0.05); **C.** Box plot showing comparison between seasonal movement (m) exhibited by Murree Hills Frogs (*Nanorana vicina*) during the two seasons (sexes pooled) (ns= non-significant at α 0.05); **D.** Box plot showing comparison between seasonal movement (m) exhibited by males and females of Murree Hills Frog (*Nanorana vicina*) (seasons pooled) (ns= non-significant at α 0.05).

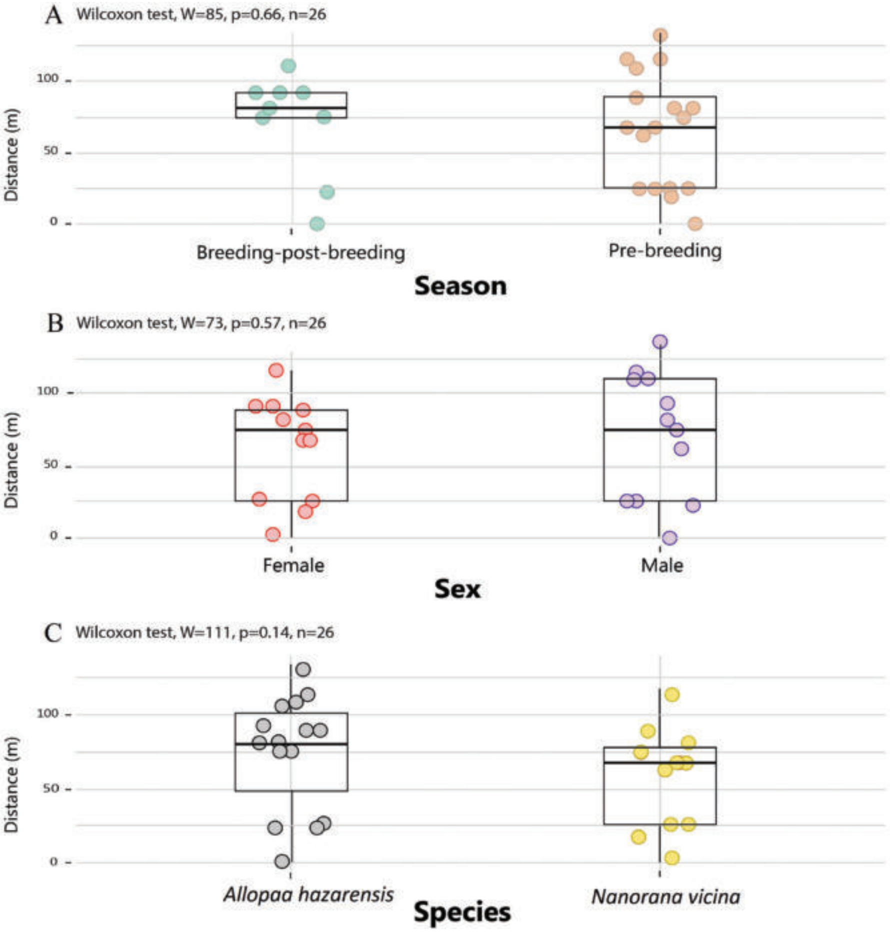

Figure 5. A. Box plot showing comparison between distance (m) moved by the two endemic frogs, Murree Hills Frogs (*Nanorana vicina*) and Hazara Frogs (*Allopaa hazarensis*) (species and sexes pooled), during the pre-breeding and breeding-post-breeding season; **B.** Box plot showing comparison between distance (m) moved by males and females of the two endemic frogs, Murree Hills Frogs (*Nanorana vicina*) and Hazara Frogs (*Allopaa hazarensis*) (species pooled); **C.** Box plot showing comparison between distance (m) moved Murree Hills Frogs (*Nanorana vicina*) and Hazara Frogs (*Allopaa hazarensis*) (sexes pooled).

Discussion

Of the many identified threats globally, habitat degradation, fragmentation, and loss have been recognized as the major factors responsible for the decline of many amphibian species (Brown et al. 2012). This has led to a great deal of research into understanding the ecology of amphibians, particularly their movement and dispersal capabilities, in a landscape undergoing these phenomena. Data from other regions (Nearctic, Palearctic, Neotropical, and Australian region) is available and has greatly increased our understanding of how to incorporate habitat management needs for amphibians into urban planning and development projects. Establishing the negative impact of habitat fragmentation and gathering data on movement and dispersal in amphibian populations is required for properly understanding the situation in the area in question (Funk et al. 2005). We, however, still lack scientific information about species from the Oriental region, particularly from under-developed countries, where resources are limited and smaller wildlife groups, such as amphibians, receive very little attention.

We provide new information about abundance and movement in Asian Spiny Frogs, *N. vicina* and *A. hazarensis*, endemic to Himalayas: Saeed et al. (2022) compared detection of *N. vicina* and *A. hazarensis* using eDNA surveys and visual encounter surveys for estimating occupancy. The former method accounted for higher occurrence probabilities. We report on the population of the two frog species for the first time. The two species were found to be fairly common at the study site. We, however, believe that factors such as open population structure as well as the short period of time that was available for autumn recaptures might affect the accuracy of our abundance estimates.

We found that *N. vicina* and *A. hazarensis* did not exhibit much movement in the two field sessions or during pre- and post-breeding seasons. Movement over short distances in amphibians is common. Of the 53 anuran species reviewed for their dispersal ability, 56% moved a distance of ≤ 1 km (Smith and Green 2005). About 30% of the marked individual of *Physalaemus pustulosus* (Tungrana Frog) moved among the ponds and covered a distance of 50 m during the period of five weeks. The majority of recorded movements were within 200 m distance (Marsh et al. 1999). Houston Toads (*Bufo houstonensis*) stayed within 75 m of the pond of initial capture, rarely dispersing to a distance of > 750 m (Vandewege et al. 2013). Akram et al. (2022) reported that radio tracked Murree Hills Frogs (6 ♂, 7 ♀) and five Hazara Frogs (8 ♂, 8 ♀) remained within 3 m distance during post-breeding (September, 2017 and 2018). The study was, however, conducted during breeding-post breeding season only. The mean daily movement and mean seasonal movement of the studied species were in accordance with the distances known from most amphibian species elsewhere in the world (Marsh et al. 1999; Vandewege et al. 2013).

The movement data, both daily and seasonal, showed that the frogs moved either between neighboring ponds or remained within an area of approximately 120 m^2, which contained a few pools on the river. The maximum linear distance from the breeding pond traveled by 11 studied female *B. americanus* (American Toad) ranged from 246 to 1,015 m (Forester et al. 2006). All males of *B. houstonensis* stayed within < 75 m of their breeding pond (Vandewege et al. 2013). Only 1% (of 10,443 individuals marked) of the adult *Rana luteiventris* (Columbia Spotted Frog) moved ≥ 2000 m among 21 ponds situated within 7 km distance (Funk et al. 2005). About 151 adult Water Frogs (*Rana lessonae*, *R. ridibunda*, and *R. esculenta*) moved among all studied ponds except the one which was separated by a road (Peter 2001). A total of 12 movements were recorded between ponds separated by 50–100 m, seven movements between 150–200 m, six movements of 150–200 m and two movements of > 200 m in *P. pustulosus* over a period of 5 weeks (Marsh et al. 1999). The small distance range in the movements we monitored is attributed to the presence of ponds at different elevations and with differing levels of disturbance. Pond 1 is separated from other ponds by the road, and ponds No. 1 and 2 experience a high level of human disturbance while Pond 5 had the least disturbance. The local community draws water from these ponds for household use, hence, frogs moved from ponds P1 and P2 to P5.

The majority of the movements were along the stream particularly in the upstream direction. The *Ascaphus truei* (Coastal Tailed Frog) is known to move upstream during low water flow in the headwater stream system whilst moving downstream to breed (Hayes et al. 2006). Our studied stream is a permanent freshwater stream. The main source is ground water, snow-melt, and drainage of rainfall from hills located in the surrounding area, especially the northern side. We suggest our species responded to disturbance level and prefer to occupy ponds in the upstream area where there are fewer disturbances.

The study area bears exceptional importance in terms of forest and biodiversity (Chawla et al. 2012). Unfortunately, the area has also been experiencing both natural and human induced changes. About 55 km^2 (24 km^2 in state-owned and 31 km^2 in private or community forest) reduction in the forest area of Tehsil Murree (Rawalpindi, Punjab, Pakistan) is reported (Shahzad et al. 2015). The natural areas have been degraded and the forest area has been transformed by buildings and other urban features. Climate change during the past two decades has affected many areas of Pakistan, including this study area. Over a dozen butterfly species have been lost due to changes in the climate of Murree (Saadat et al. 2016). The two endemic frog species are known to occur in sub-tropical chir pine forest (900–1,700 m) and other forest types such as Himalayan moist temperate forest (> 2000 m) further north of our study area, but do not exist in sub-tropical scrub (broad-leaved) forest (< 900 m) located in the south. The species are adapted to freshwater forested wetlands found at higher elevation with low air and water temperature and cool summers. The long-term survival of many amphibian populations or subpopulations depends on colonization from nearby wetlands (Petranka et al. 2004; Church 2008). There is an inverse relationship between colonization and distance to travel. If an amphibian species is not capable of moving from source pond to other nearby wetlands, the chances of recolonization are expected to decrease (Lehtinen and Galatowitsch 2001) which might cause local extinction. Hence, the presence of wetlands in close proximity helps attain long-term persistence in amphibian populations (Petranka and Holbrook 2006). The two studied species are unable to perform overland migration through the open forest to disperse or colonize nearby streams. Due to various anthropogenic threats or changes in climatic pattern, their movement and dispersal would be limited, resulting in the progressive decline of their populations which might eventually lead to local extinction.

Many researchers have proposed different mitigation approaches such as construction of artificial wetlands (created wetlands) and wetland restoration (Lehtinen and Galatowitsch 2001; Pechmann et al. 2001; Vasconcelos and Calhoun 2004). The natural wetlands, unlike created wetlands, undergo changes in hydrology, water regime, flow and persistence which might impact success of reproduction. Similarly, created wetlands are sometimes free of fish, all potentially negative factors for amphibian reproduction. We suggest inclusion of amphibian habitat requirements and ensuring stream connectivity in urban planning and development projects of the area.

We obtained low recaptures which could be due to the secretive nature of the studied species. These frogs may remain hidden most of the time and it was not possible for us to move the heavy rocks and boulders to check for their presence in these likely locations. The area has been experiencing rapid changes. We recommend carrying out more robust and long-term studies encompassing multiple streams situated within a wider area. This would help to understand the colonization of these frogs and establish if they are capable of moving through the forest to occupy other streams, thus providing a better picture of metapopulation structure and dynamics. Likewise, studies on the impact of water quality and quantity on these frogs would help associate water and amphibian conservation in the area.

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Supplementary material 1

Details of tagging of specimens of *Nanorana vicina* and *Allopaa hazarensis* for pre-breeding season and breeding-post-breeding season

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Description of the advertisement call of *Boulenophrys nanlingensis* (Anura, Megophryidae), with a case of individual identification using its dorsum pattern

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Abstract

We describe the advertisement call of the Nanling horned frog, *Boulenophrys nanlingensis* for the first time, based on recordings obtained from four individuals. One of these individuals, which was identified using its dorsum pattern, was recorded twice after nine months. Distinct shifts in the temporal parameters and call rate were observed from calls of the re-captured individual, which was suspected to be related to temperature and social context. However, due to the limited sample size, further research is needed to confirm these findings. We highlight the potential of mark-recapture method using dorsum pattern for studying and monitoring the Nanling horned frog and other megophyinid frogs.

Key Words

bioacoustics, frogs, mark-recapture, Megophryinae, southern China

Introduction

The genus *Boulenophrys* Fei, Ye & Jiang, 2016 is the largest branch of the Asian horned frog subfamily Megophryinae Bonaparte, 1950, comprising 65 species found in southern China and the Indochina peninsula (Frost 2023). During their breeding season, male *Boulenophrys* frogs emit repeated, monosyllabic calls from stream banks and often form small chorus groups (Pope 1931; Liu 1950; Wang et al. 2014). These calls are species-specific, with clear differences particularly evident amongst sympatric species (Liu et al. 2018; Cutajar et al. 2020). As a result, call parameters have become a diagnostic character in recent taxonomic studies for identifying *Boulenophrys* species (Tapley et al. 2017, 2018, 2020).

Boulenophrys nanlingensis (Lyu, Wang, Liu & Wang, 2019) is distributed throughout Nanling Mountains in southern China. According to Wang et al. (2019), the breeding season of *B. nanlingensis* was from August to December. Notably, this period contains seasonal changes with the air temperature decreasing during this period in southern China. Although the advertisement call of *B. nanlingensis* was not described, a recently published guide, “A Field Guide to the Amphibians of Eastern China” (Ding et al. 2022), has illustrated its spectrogram and oscillogram and provided an audio file for field identification. Thus, we could easily identify this species in the field from its distribution area.

Methods

Field observation

On 18 November 2021, during a night survey conducted in Mangshan (also known as Mt. Mang), Yizhang County, Hunan Province, China (24.945°N, 112.938°E, ca. 1220 m elev.), we observed a group of at least five male *B. nanlingensis* calling in chorus on rocky areas along the bank of a mountain stream. The stream was approximately 5 m wide and several individuals of *Leptobrachium liui* could also be heard calling nearby. We were able to locate four individual frogs of *B. nanlingensis*, three of which were hiding under a crevice with their feet submerged in shallow water, while one remained hidden under fallen leaves. Each individual was positioned at least 1 m apart from the others. We recorded their advertisement calls individually between 20:30 and 22:00 h and captured and photographed two of them next to an improvised scale bar (the shotgun microphone). Both were released immediately after photographing.

After nearly nine months, when we revisited this site during a night survey on 13 August 2022, a single calling male *B. nanlingensis* was located under a crevice about 5 m from the rocky areas which we visited in November 2021. After recording its advertisement call between 20:00 and 20:30 h, we captured the frog and held it in captivity for a few days before releasing it back to the collection site on 18 August 2022. During this period, we took measurements and photographs of the frog. Upon comparing photos taken during both surveys, we confirmed that this frog belongs to one of the photographed individuals we had encountered in November 2021 (see Results).

Data collection

During our initial survey, the calls were recorded by using a Zoom F6 digital sound recorder with a Boya BM6060L shotgun microphone, held approximately 0.2–1 m from each frog. Two recordings from four individuals (vocally marked as No.1–4 in the recordings) were made at a sample rate of 192 kHz and a resolution of 24-bit. The ambient air temperature was recorded as 12.3 °C by using a digital thermometer (0.1 °C, AZ Instrument 8918). For the second survey, we used a Zoom F3 digital sound recorder with a Sennheiser ME66/K6 shotgun microphone held approximately 0.5 m from the frog. A single recording was made at 192 kHz sampling rate and 32-bit float resolution. The air ambient temperature was recorded as 19.7 °C.

The snout-vent length (SVL) of the re-captured individual was measured by using a digital caliper (0.01 mm, to the nearest 0.1 mm). We also estimated the SVL of the two photographed frogs in November 2021 by measuring the columns on the shotgun microphone.

Acoustic analysis

All recordings obtained from the field were resampled to 44.1 kHz and 16-bit by using Adobe Audition 2023 and were then analysed with Raven Pro v.1.6.4 (K. Lisa Yang Center for Conservation Bioacoustics 2023). Spectrogram measurements were taken as follows: Hann window, DFT = 512 samples, overlap = 50%, Hop Size = 256 samples. We used "call-centred" terminology as summarised by Köhler et al. (2017), in which the fundamental unit was defined as a "call" and the continuous units were defined as a "call group". The following acoustic parameters were measured: call duration (ms); call interval (ms); number of calls per call group; call repetition rate (calls/s), measured by counting the total number of calls (*k*) within a call group and dividing *k*-1 by the duration between the onset of the first call and the onset of the last call of the call group (modified from Bee et al. (2013)); number of pulses per call; dominant frequency (Hz), measured using the function "Peak Frequency" in Raven Pro. We also reported the frequency bandwidth (Hz) by measuring frequencies 5% and 95%. The spectrogram and oscillogram figures 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%.

Results

The photos from the re-captured individual that were taken in both surveys are shown in Fig. 1. Multiple colour patterns and morphological characters (enlarged tubercles) indicate that the two photos belong to a same frog.

Measurements of acoustic parameters of the four individuals are shown in Table 1, the recaptured individual being marked as "No. 3". The advertisement call of

Table 1. Call parameters of *Boulenophrys nanlingensis*. *N* = number of call groups/calls analysed, NM = not measured.

Individuals	**No. 1 (*N* = 18/138)**	**No. 2 (*N* = 15/171)**	**No. 4 (*N* = 4/35)**	**No. 3 (*N* = 10/116)**	**No. 3 (*N* = 7/135)**
Recording date	18 Nov 2021	18 Nov 2021	18 Nov 2021	18 Nov 2021	13 Aug 2022
Air temperature (°C)	12.3	12.3	12.3	12.3	19.7
SVL (mm)	NM	NM	ca. 34	ca. 33	37.3
Condition	Chorus	Chorus	Chorus	Chorus	Solo
Call duration (ms)	169.3±18.3(73.5–196.8)	196.9±21.2(74.0–268.0)	199.2±26.7 (94.7–298.6)	176.9±21.6 (96.4–248.6)	101.6±8.1 (60.2–113.6)
Call interval (ms)	584.5±206.9 (295.9–1313.1)	513.9±150.3 (185.1–1030.2)	526.3±138.0 (376.1–819.0)	548.6±165.2 (321.9–1129.5)	195.0±34.8 (137.8–348.3)
Dominant frequency (Hz)	3448±237 (2412–4307)	3366±91 (3187–3704)	3411±92 (3359–3618)	3285±86 (3101–3445)	3276±96 (3187–3618)
Frequency 5% (Hz)	3008±213 (2067–3273)	3123±106 (2153–3187)	3102±186 (2326–3187)	2978±275 (2067–3187)	2712±282 (1378–3015)
Frequency 95% (Hz)	4284±145 (3445–4479)	3969±123 (3618–4393)	4243±79 (4048–4393)	3968±232 (3618–4737)	4179±100 (3790–4393)
No. of pulses per call	21.7±1.7 (14–28), *N*=133	22.5±1.9 (15–26), *N*=166	23.1±1.3 (20–26), *N*=32	20.5±1.7 (15–25), *N*=116	22.6±1.9 (13–28), *N*=124
No. of calls per call group	7.7±1.9 (4–11)	11.4±6.4 (5–32)	8.8±4.3 (5–15)	11.6±5.2 (4–18)	19.3±5.2 (14–29)
Call repetition rate (calls/s)	1.4±0.2 (1.0–1.8)	1.4±0.2 (1.0–1.6)	1.3±0.2 (1.2–1.7)	1.4±0.1 (1.1–1.6)	3.4±0.1 (3.3–3.6)

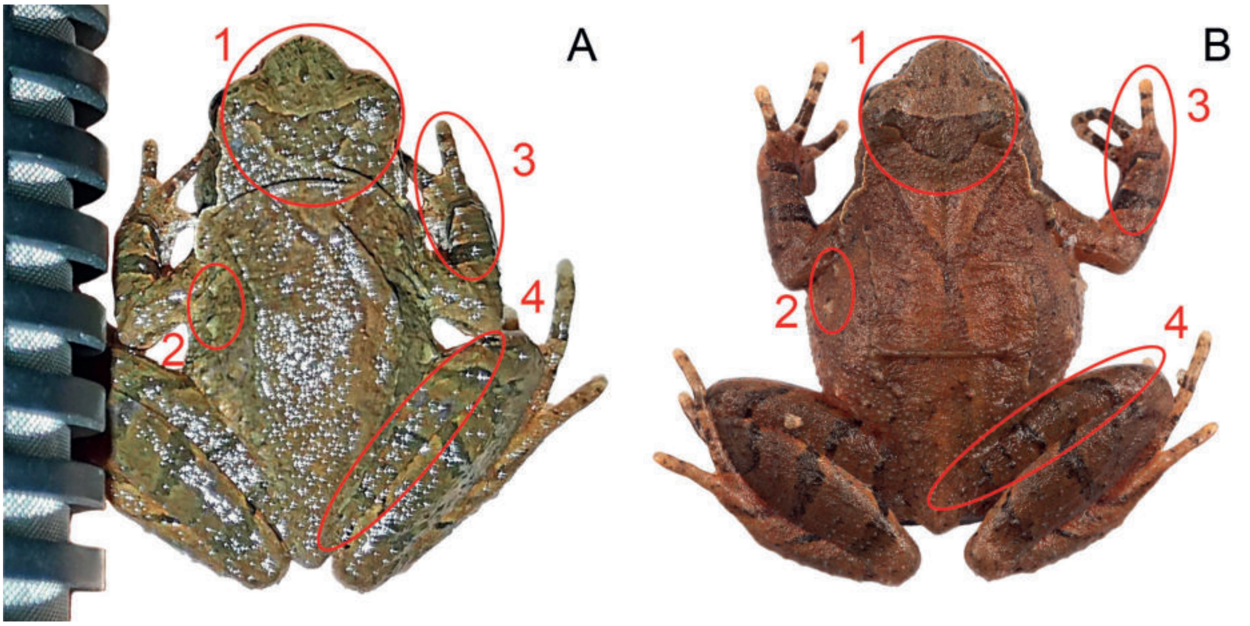

Figure 1. Comparison of the photos taken in November 2021 (**A**) and August 2022 (**B**) from the re-captured individual of *B. nanlingensis*. Red circles showing characters used for individual identification: (1) triangle pattern between upper eyelids, (2) prominent tubercles on upper left flank, (3) black bands on lower right arm and right fourth finger and (4) five black bands on right outer thigh. Images not to scale.

B. nanlingensis is a group of repeated pulsative calls. Call amplitude was consistent within each call group, except for the first 1–2 calls which had a lower amplitude. Within each call, the first pulse begins with a moderate amplitude, followed by a distinct interval. The second pulse experiences a sudden increased amplitude modulation, which then gradually increases to reach its peak amplitude at approximately 1/3 of the way through the call. The amplitude then gradually decreases to the end of the call.

The mean value of call duration amongst individuals recorded in November 2021 varied from 169.3 ms to 199.2 ms and the mean value of call interval varied from 513.9 to 584.5 ms. However, these values obtained from the re-captured individual recorded in August 2022 were considerably shorter (101.6 ms and 195.0 ms, respectively). As a result, this individual exhibited a much higher mean call repetition rate of 3.4 calls/s, compared to the calls recorded in November 2021 (1.3–1.4 calls/s). Fig. 2 demonstrates the differences in calls recorded from the re-captured individual "No. 3" between different seasons.

Discussion

According to Qian et al. (2023), *Boulenophrys nanlingensis* is in sympatry with *B. shimentaina* and *B. ombrophila* in Mangshan, while the dominant frequency of the advertisement call of *B. nanlingensis* (2.3–4.1 kHz) is lower than that of *B. shimentaina* (vs. 4.7–5.2 kHz; Lyu et al. (2020)), but overlapped with that of *B. ombrophila* (3.5–3.6 kHz; Messenger et al. (2019)). However, during our surveys in 2021 and 2022, we consistently observed *B. ombrophila* ceased its calling activities by mid-June. Thus, since *B. nanlingensis* breeds from August to December, it is unlikely that these two species with overlapping dominant frequencies will be present at the same time of the year.

Compared to the published calls of the other species in *Boulenophrys*, the advertisement call of *B. nanlingensis* differs considerably, reinforcing the specific identity of this taxon. For example, the call duration of *B. nanlingensis* (60.2–298.6 ms) is longer than that of *B. fansipanensis* (34.0–49.0 ms; Tapley et al. (2018)), *B. frigida* (43.0–50.0 ms; Tapley et al. (2021)) and *B. boettgeri* (54.0 ms, mean value; Wang et al. (2014)). The number of pulses per call of *B. nanlingensis* (13–28) is larger than that of *B. frigida* (10–11; Tapley et al. (2021)). The call repetition rate of *B. nanlingensis* (1.0–3.6 calls/s) is lower than that of *B. boettgeri* (5.0 calls/s, mean value; Wang et al. (2014)), *B. huangshanensis* (4.1 calls/s, mean value; Wang et al. (2014)), *B. jinggangensis* (5.7 calls/s, mean value; Wang et al. (2014)) and *B. minor* (4.0 calls/s, mean value; Jiang et al. (2002)).

Temperature has been reported to affect temporal parameters and call rates in most anurans (reviewed in Gerhardt (1994)). In this study, we observed distinct shifts in temporal parameter (i.e. call duration and call interval) and call rate from the recaptured individual, whose calls were recorded twice during different seasons. A similar result was reported from another megophryinid frog *Ophryophryne elfina*, whose calls were recorded at different temperatures (11.3 °C and 17.5 °C, Poyarkov et al. (2017)). However, the social context was also related to call rate shift (Capshaw et al. 2020). During our first encounter, the frogs were calling in a chorus with several conspecific males. We have noticed that these individuals would adjust their call rhythm by lengthening the call interval to avoid overlapping when other males attempted

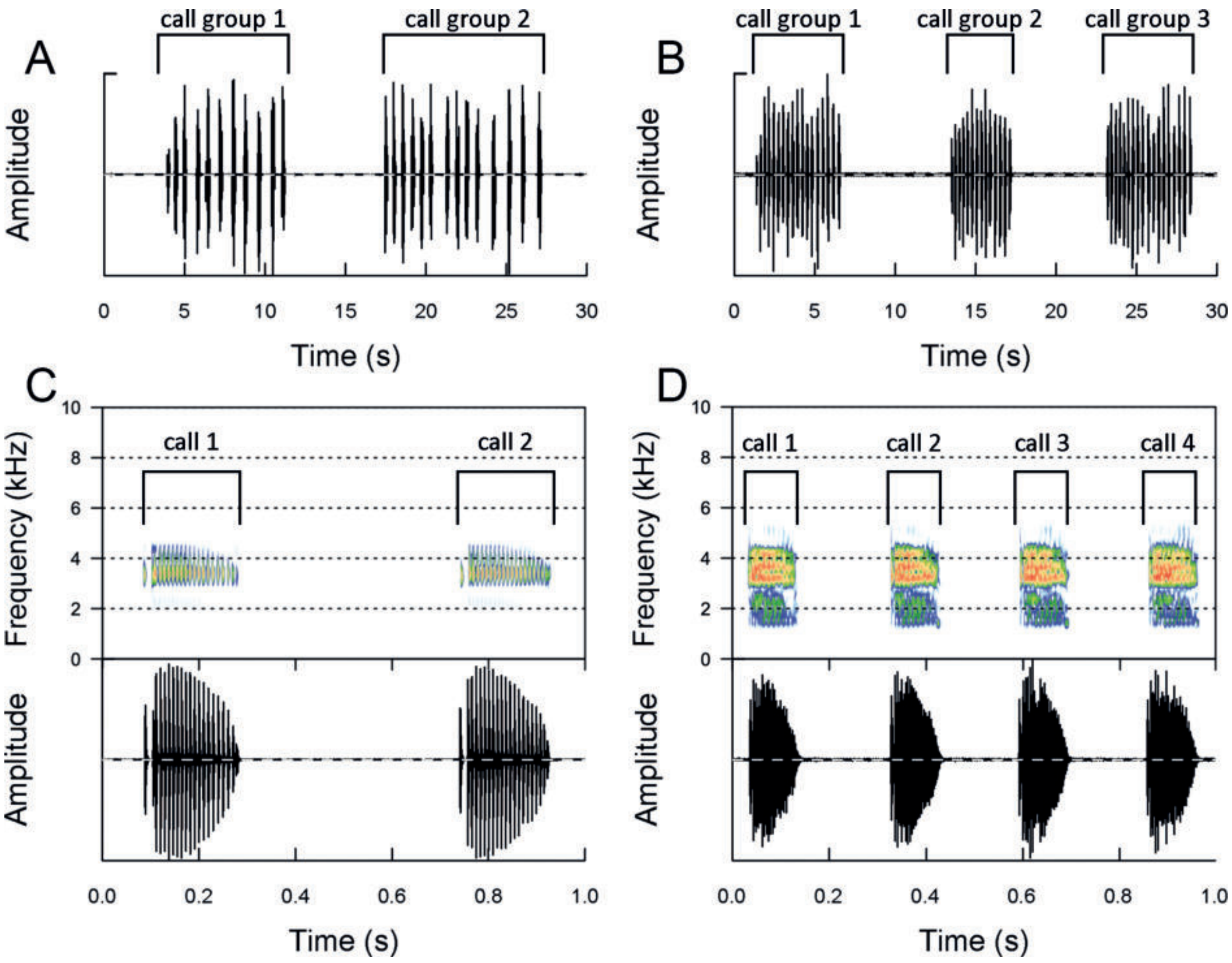

Figure 2. Comparison of the advertisement call of a recaptured individual of *B. nanlingensis* recorded from November 2021 (**A, C**) and August 2022 (**B, D**). 30 s oscillograms showing two call groups recorded in November (**A**) and three call groups recorded in August (**B**). 1 s oscillograms and corresponding spectrograms showing two calls recorded in November (**C**) and four calls recorded in August (**D**).

to join the chorus. However, during the second encounter, the frog was calling alone.

Several megophryinid frogs are known to be exhibit loyalty to their breeding habitats or calling sites. For instance, Liu (1950) observed that *B. omeimontis* "*This frog has the special habit of appearing on the same stone near the margin of the water every night*", while Mulkmus et al. (2002) reported that *Pelobatrachus baluensis* frogs "*live in very stationary calling communities*" and *P. kobayashii* frogs "*live in permanent colonies*", with five colonies of *P. baluensis* traced by Mulkmus et al. (2002) from same spots over five years. In our study, we observed a potentially similar pattern of loyalty to breeding habitats in the recaptured individual of *B. nanlingensis*, which did not move its calling site more than 5 m over a nine-month period.

The mark-recapture method has been long and widely used in amphibians in demographic, home range, behaviour and other aspects of studies (e.g. Martof (1953); Zweifel (1968); Nelson and Graves (2004); Pettitt et al. (2013)). Toe-clipping was previously the most frequently used method to mark an individual, but was reported to be harmful to individuals or influence their behaviour (reviewed in Wells (2007)). To avoid such unexpected consequences, non-invasive methods, such as individual recognition, based on colour patterns, were proposed (Bradfield 2004; Kenyon et al. 2009; Zheng et al. 2011; Caorsi et al. 2012; Morrison et al. 2016; Patel and Das 2020). Although only one individual from *B. nanlingensis* was tested in this study, we have found that its dorsal pattern and prominent skin tubercles did not show obvious changes over a nine-month period. We propose that this method could potentially be a useful non-invasive tool for studying or monitoring Nanling horned frogs and other megophryinid species which having distinct dorsum patterns (as well as ventral patterns, which were not recorded in this study) that varied between individuals.

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New localities and lineages of the Atlas dwarf lizard *Atlantolacerta andreanskyi* identified using mitochondrial DNA markers

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Abstract

Atlantolacerta andreanskyi (Werner, 1929) is an endemic lizard from the High Atlas Mountains region of Morocco. A previous molecular assessment of this species using mitochondrial and nuclear DNA markers uncovered extensive genetic diversity with seven lineages indicative of a species complex. A morphological assessment of six of these lineages did not establish simple diagnostic features, and proposed these should be considered as a cryptic species, while highlighting the need for greater sampling across the range. In this study, we sampled 8 individuals from 5 previously unsampled localities and carried out genetic analyses to compare these populations to the known variation. Phylogenetic reconstruction based on mitochondrial DNA markers (12S rRNA and ND4) corroborates the previously described lineages and identified a new one. Interestingly, the two samples that account for this newly identified lineage have been collected from distinct localities – M'goun and Toumliline – that form a sister taxon to the population of Jbel Azourki.

Key Words

12S rRNA, Atlas Mountains, evolutionary history, Lacertidae, Morocco, ND4, phylogeny

Introduction

Atlantolacerta andreanskyi (Werner, 1929), is a lacertid lizard endemic to the High Atlas Mountains in Morocco, distributed across 440 km (straight line) from the western to the central areas of the massif (Bons and Geniez 1996; Mármol et al. 2019). It can be found in areas from 2400 m a.s.l. to 3800 m a.s.l., often under small rocks near watercourses and around the base of cushion-like thorny plants that offer a buffered microclimate (Werner 1929; Bons and Geniez 1996). The species presents a patchy spatial distribution, with populations separated by regions of unsuitable, lower elevation habitats.

Despite extensive herpetological surveys across the Atlas Mountains in recent years (e.g. Avella et al. 2019; Harris et al. 2010) there have been few additional populations reported, with minimal differences between the recent field guide and ones from much earlier (e.g. distribution reported from Mármol et al. 2019, compared to Bons and Geniez 1996). A molecular assessment of 8 populations widely distributed across the range using two mitochondrial and five nuclear markers (Barata et al. 2012) revealed extreme genetic diversity among seven of the eight populations analysed, demonstrating divergence levels indicating that *A. andreanskyi* should be considered a species complex.

A later assessment of phenotypic variability of this species, employing linear measurements, pholidotic and coloration characters in six of the previously analysed populations of *A. andreanskyi* (Barata et al. 2015), indicated that despite the high genetic divergence previously detected, morphological variation among populations was low. However, with almost every population studied representing a unique lineage, distribution of these cryptic forms remains essentially unknown.

The present study is the result of multiple additional fieldtrips to the region, and the collection of samples from additional localities to compare to the known genetic lineages. The aim was to increase the knowledge of the distribution of this species complex, and to include new samples within a phylogenetic framework.

Methods

All lizards were captured under permit from the High Commissariat for Water and Forests of Morocco. Fieldwork was carried out in the Spring of 2019 and 2022, and resulted in the identification of *A. andreanskyi* from 5 new localities (Fig. 1): 1) near the previously sampled population of Outabati (32°12'29.2"N, 5°27'25.2"W; circa 2660 m a.s.l.), 2) near Toumliline, a new locality over 30 km from any previously reported populations (31°54'46.1"N, 5°29'08.2"W; circa 2610 m a.s.l.), 3) near M'goun, the second highest peak of the Atlas Mountains (31°34'52.9"N, 6°16'11.8"W; circa 2710 m a.s.l.), south of the sampled populations of Jbel Azourki, 4) near Lake Tamda (31°18'59.55"N, 6°59'54.17"W), over 30 km East of the previously sampled Tizi n'Tichka population, and 5) north of the sampled populations in the furthest South, at Jbel Awlime (30°58'50.9"N, 8°45'18.7"W; circa 2540 m a.s.l.). Specimens were captured by hand and photographed for later comparison of external phenotypic differences between lineages. Tail tip samples were collected and preserved in tubes filled with ethanol 96%. All individuals were released at the place of capture.

Total genomic DNA was extracted from tail tip samples, using standard high-salt protocols (Sambrook et al. 1989). Eight new individuals were included, four from the population near Jbel Awlime, and one from each of the remaining new locations. Two mitochondrial DNA markers were amplified via polymerase chain reaction (PCR): 12S rRNA (12S) and partial NADH dehydrogenase 4 (ND4) and flanking tRNAs (tRNA-His), using previously published primers from Kocher et al. (1989) and Arévalo et al. (1994) respectively. The PCR thermocycler conditions used started with 95 °C for 10 min, followed by thirty-five cycles of 30 sec at 95 °C, 30 sec at 50 °C (12S) or 52 °C (ND4), and 30 sec at 72 °C with a final extension at 72 °C for 10 min. PCR success was assessed through electrophoresis, and single-band samples showing PCR product of the intended length were sent to GENEWIZ (Germany) for purification and standard Sanger sequencing. New sequences have been submitted to GenBank (Accession numbers OQ731434 to OQ731441 and OQ724509 to OQ724516).

The ND4 fragment was translated into amino acids in order to assess the reading frame and confirm the sequences corresponded to the expected protein. Available sequences from Barata et al. (2012) were included in the dataset, and sequences of *Podarcis tiliguerta* were included for outgroup purposes. All sequences were aligned using Muscle v5.0 (Edgar 2004). Estimates of phylogeny were produced by Bayesian Inference (BI) using MrBayes v3.2.6 (Ronquist and Huelsenbeck 2012), and Maximum Likelihood (MI) using MEGAX (Kumar et al. 2018). Best-fit nucleotide models and partitions schemes for the BI were selected using PartitionFinder2 (Lanfear et al. 2012) and were as follows: GTR+G for 12S, HKY+G+I, HKY+I, GTR+G respectively for each of the ND4 codon positions, and HKY+G+I for tRNAs. BI was run for 8×10^6 generations, with a sampling frequency of 1000 and all other parameters left as default. Best-fit nucleotide model for the concatenated ML analysis was selected using MEGAX built-in tool, selecting the HKY+G+I. Nodal support was assessed by bootstrapping with 8000 replicates.

Results

Both Bayesian Inference and Maximum Likelihood analysis produced almost identical topologies, mostly differing slightly in the shallow nodes within populations (Fig. 2). The exception is the Jebel Awlime clade, appearing as a sister clade to Outabati, Jbel Ayache, Jbel Azourki, M'goun, Tizi n'Tichka, and Lake Tamda for the BI (posterior probability = 0.83) as seen in Fig. 2, or as sister taxon to the Outabati and Jbel Azourki clade for the ML, albeit with less support (bootstrap value = 53). As expected, the major lineages identified by Barata et al. (2012) were again recovered. The individual from locality (1), near Outabati, was most closely related to individuals from Barata et al. (2012) from nearby, although divergence was notable (3.00 ± 0.62% (SE) with the ND4 marker, 0.33 ± 0.30% (SE) with the 12S rRNA marker). Likewise, the individual from Lake Tamda (4) was most closely related to the known population from Tizi n' Tichka, but again divergence was notable (5.80 ± 0.69% (SE) with the ND4 marker, 1.54 ± 0.69% (SE) with the 12S rRNA marker). Unexpectedly, the individuals from Toumliline (2) and M'goun (3), despite being geographically separated by more than 60 km formed a well-supported clade, distinct from the sister taxa population of Jbel Azourki (8.15 ± 0.95% (SE) with the ND4 marker, 3.34 ± 0.98% (SE) with the 12S rRNA marker). The four newly sequenced individuals from north of Jbel Awlime

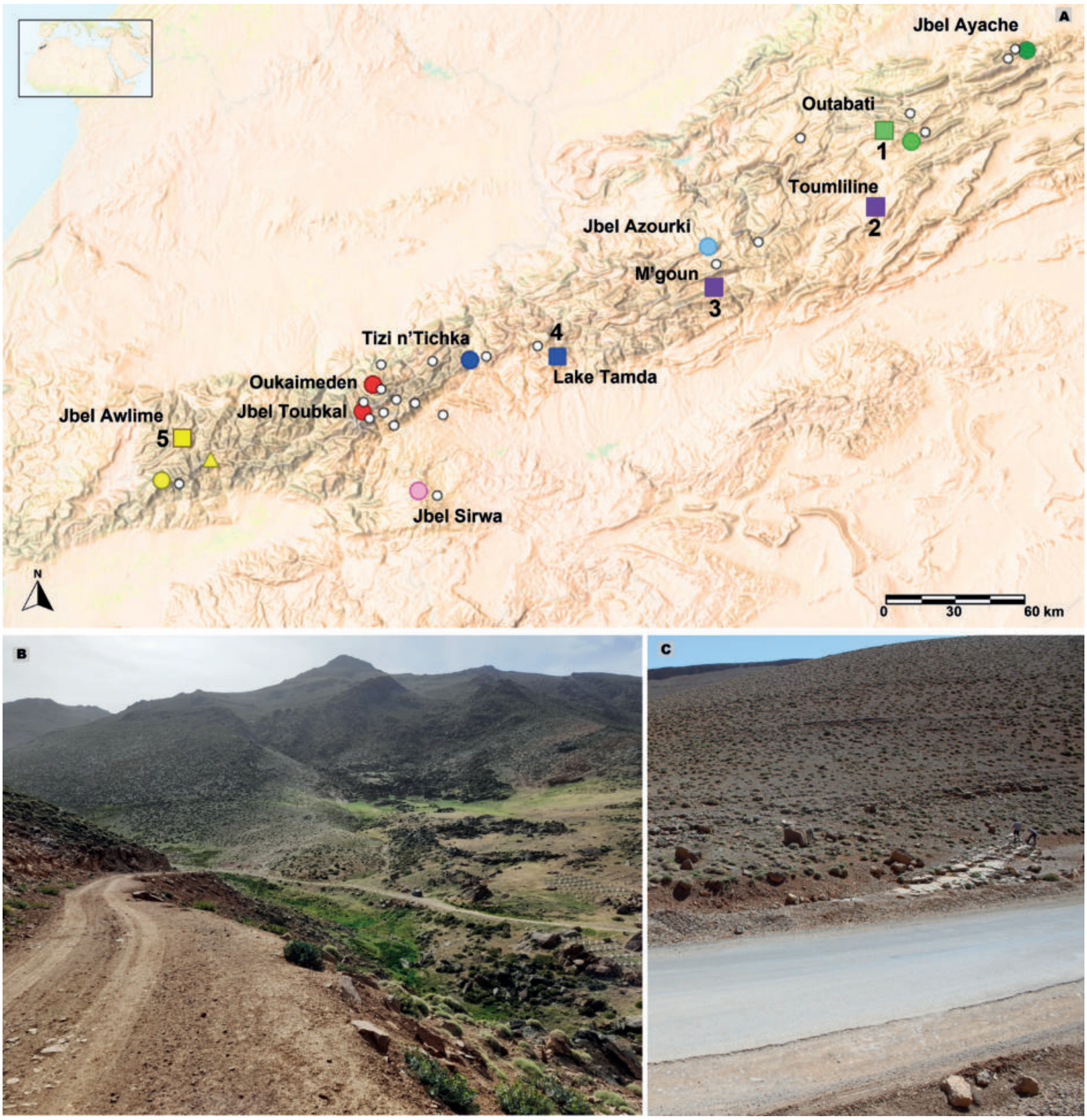

Figure 1. **A.** Distribution map of *Atlantolacerta andreanskyi*. Coloured dots are based on the distribution map populations of Barata et al. (2012). White dots represent the distribution points of Bons and Geniez (1996). The yellow triangle represents a specimen sampled in the Tichka plateau (Avella et al. 2019). The localities with newly sampled individuals are identified with numbers (1–5): Outabati (1), Toumliline (2), M'goun (3), Lake Tamda (4) and Jbel Awlime (5); **B, C.** Typical habitat of *Atlantolacerta andreanskyi* in Jbel Awlime and in Toumliline, respectively.

(5) were strongly supported as a clade along with samples from Barata et al. (2012) from nearby, despite the equivocal placement of the lineage in the overall phylogeny. The overall estimate of relationships between lineages is concordant with Barata et al. (2012): the northern Outabati and Jbel Ayache populations are sister taxa with strong support; populations from Jbel Sirwa are sister taxa to those from Oukaimeden and Jbel Toubkal; and populations from the central region of Tizi n' Tichka and Jbel Azourki form a clade along with the newly identified lineage from M'goun and Toumliline.

Discussion

The earlier molecular study of Barata et al. (2012) clearly indicated that *A. andreanskyi* could be considered a potential species complex, with important conservation implications since six lineages were identified using both mitochondrial and nuclear markers. However, these authors highlighted that the mountainous habitat was difficult to sample, and that the potential for additional cryptic forms to exist was high. In this study, by including four additional localities, we have demonstrated that this is indeed the case,

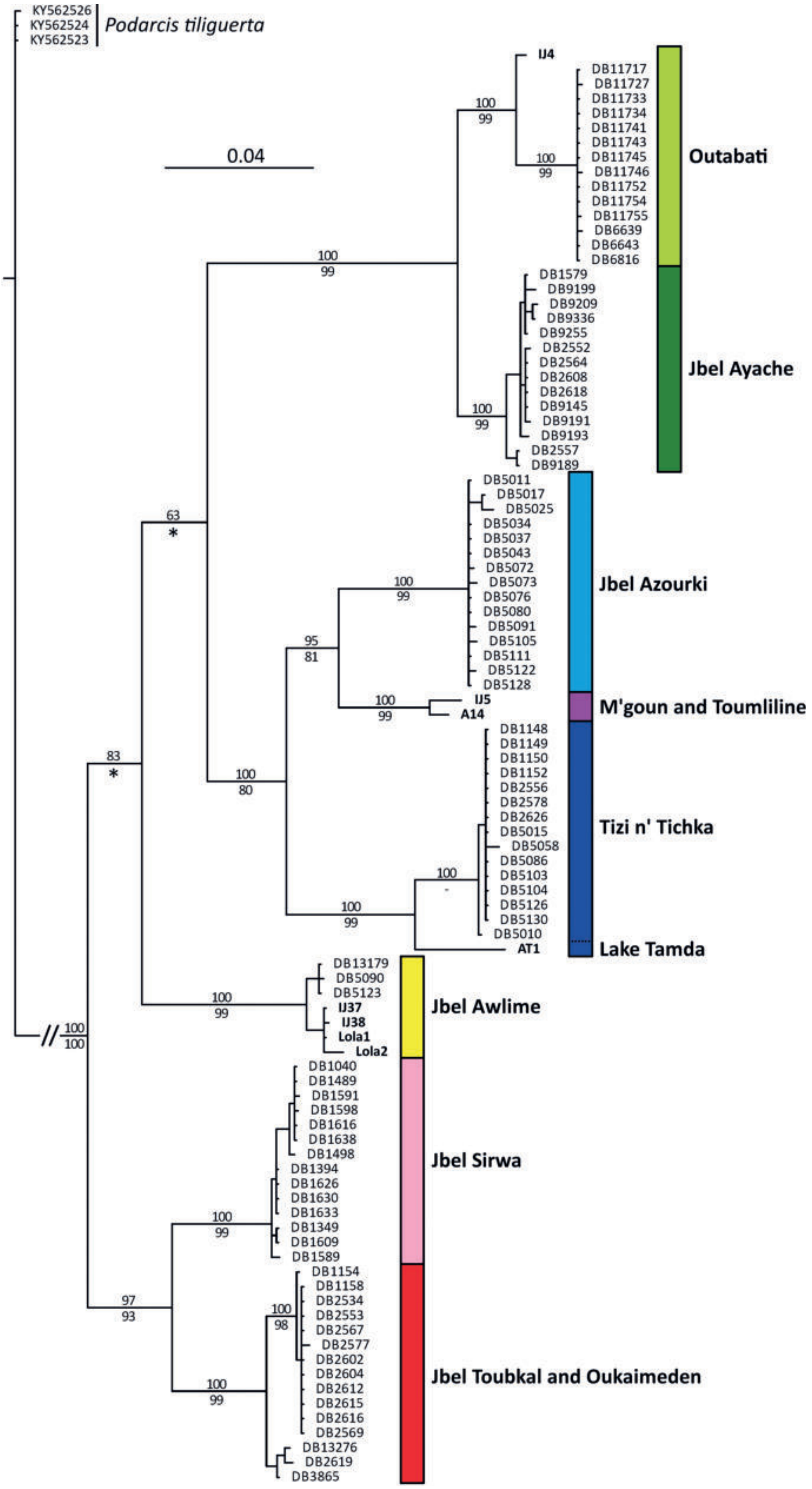

Figure 2. Bayesian tree estimate based on mitochondrial DNA sequences (12S and ND4 + tRNA-His) of *Atlantolacerta andreanskyi*. Bayesian posterior probabilities are shown above branches; Maximum Likelihood bootstrap values are shown below branches; asterisk indicate nodes not present on the ML tree estimate due to a different placement of the Jbel Awlime clade. The main lineages within the species are represented by distinct colours, corresponding to Figure 1. New samples used are named as IJ4 (1 – Outabati), IJ5 and A14 (2, 3 – Toumliline and M'goun), AT1 (4 – Lake Tamda), and IJ37, IJ38, Lola1 and Lola2 (5 – Jbel Awlime). Codes beginning "DB" are from Barata et al. (2012), in other cases GenBank numbers are indicated.

with a divergent lineage occurring in M'goun and Toumliline. The level of divergence, over 8% with the ND4 marker, is similar or higher than the variation between mountain species of the lacertid lizard genus *Iberolacerta* in the Pyrenees (Garcia-Porta et al. 2019), indicating another potential cryptic species within the *A. andreanskyi* complex.

Although Barata et al. (2015) reported some morphological differences between lineages, they also found that these could only be determined by examining multiple individuals and characters – simple diagnostic characters that could be used to classify specimens in the field were not identified. Colour pattern variation tended to match phylogenetic relationships, with the populations from Azourki and Tizi n'Tichka having a tendency towards more dark spots on the ventral head region, and a more intense ventral spotted pattern in general (Barata et al. 2015). The male specimen from Toumliline shows a similarly heavily spotted ventral pattern (Fig. 3), although with a single specimen observed, this clearly needs further assessment to see if this pattern variation is maintained within the new lineage.

In the far south of the range of *A. andreanskyi*, additional populations have recently been identified both in this study (locality 5) and in the Tichka plateau (Avella et al. 2019). Genetically, these seem to belong to the same lineage as those first identified from Jbel Awlime by Barata et al. (2012). This population has never been included in a morphological assessment, but based on the few specimens observed show a greatly reduced pigmentation pattern on the ventral (Fig. 3), similar to specimens in the occidental lineage (Oukaimeden and Jbel Sirwa) of Barata et al. (2015). The specimen collected from about 10 km northeast of the known Outabati population (locality 1), while clearly related to these, was also notably genetically distinct. The Outabati population shows a trend towards an absence of dorso-lateral lines in males (Barata et al. 2015), however, again with a single specimen observed it is not possible to assess if this feature is maintained in this new, nearby population. In much the same way, the specimen from Lake Tamda, while clearly related to the nearest assessed population from Tizi n'Tichka, was also genetically distinct using these mtDNA markers, highlighting just how much variation remains unknown.

In terms of comparative phylogeography, data is available for two other high mountain species of reptiles, the day gecko *Quedenfeldtia trachyblepharus*, and the atlas dwarf viper *Vipera monticola*. Both of these species, like *L. andreanskyi*, demonstrate divergent lineages in the Jbel Awlime region, with an endemic subspecies, *V. m. atlantica* recently described from there (Martínez-Freiría et al. 2021). While the divergences between *Q. trachyblepharus* and *L. andreanskyi* lineages are similar (both up to 9% for ND4 – this study and Harris et al. 2017), divergences between *V. monticola* lineages are much shallower. Furthermore, the number of identified lineages – 3 in *V. monticola*, 4 in

Figure 3. Male specimens of *Atlantolacerta andreanskyi* sampled in Toumliline (**A, B**) and Jbel Awlime (**C, D**).

Q. trachyblepharus and 8 in *L. andreanskyi* – also differs greatly, underlining the complexity not only within each species, but in elucidating general phylogeographic patterns.

Conclusion

Overall, our additional fieldwork within the range of *A. andreanskyi* confirms the expectation of Barata et al. (2012) that additional lineages occur, but also that single lineages can occur across larger areas. The identification of the population at Toumliline shows that the range of the *A. andreanskyi* species complex is greater than previously considered, and combined with the considerable diversity, and already known highlights, also shows how important it is to continue surveys across the region. Complete morphological assessments of unsampled lineages are needed to better determine morphological variation, even if this is slight, so that an integrated taxonomic revision can be performed. A recent assessment noted that the ecophysiological conservativeness of *A. andreanskyi* demonstrates its vulnerability to climate change (S'khifa et al. 2020, 2022), while also highlighting that low elevation populations are the most vulnerable. Detailed assessments of the range of lineages, including altitudinal ranges, will also therefore be essential to develop an appropriate conservation management plan for this cryptic species complex.

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The secret life of a rock-dweller: arboreal acrobatics observed in the European leaf-toed gecko *Euleptes europaea*

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Abstract

The European leaf toed Gecko, *Euleptes europaea*, is a strictly nocturnal species endemic to the western Mediterranean and has long been considered a rock-specialist as it is associated with this habitat during its entire daily and life cycle. In this study, we report observations of arboreal behaviour in *E. europaea*, collected during field research over a 40-year period from across the entire species range. We provide a review of the available information on this topic that contributes to a refined view of the habitat uses and arboreal abilities of this species. Arboreal behaviour in *E. europaea* was observed throughout the year, across different macrohabitats, on a wide variety of tree, shrub, and bush species, on various parts of the plant (trunk, branches, fronds, twigs, leaves), and at different height from the ground. Remarkably, *E. europaea* shows an extraordinarily agile arboreal locomotion associated with striking morphological adaptations to an arboreal lifestyle, namely a prehensile tail bearing a terminal adhesive pad that supplements grasping force, an equilibrium asset, and scansor adhesion both in static condition and during escape. We conclude that *E. europaea* is a climbing gecko (opposed to ground dwelling), occupying both rocky and arboreal microhabitats. While the evolutionary origin and ecological drivers of the arboreal behaviour of *E. europaea* remains to be fully investigated, this realization has important implications for designing fieldwork research and management strategies for conservation.

Key Words

arboreal behaviour, Gekkota, microhabitat use, prehensile tail, rock crevices, tail pads, vegetation cover

Introduction

Understanding the habitat use of species is crucial for ecologists, biogeographers, and conservationists (MacArthur and Pianka 1966; Caughley 1994; Hanski and Gyllenberg 1997). Species differ greatly in the range of habitat they use. Some species are habitat generalists while others are specialists, with a gradient of variation between the two extremes. Habitat selection is particularly important for ectothermic lizards such as geckos, because it influences thermal ecology, physiological performance, and individual behaviour, thus having an impact upon population and community dynamics (Pulliam and Danielson 1991; Pandit et al. 2009). However, for many species, we still have a very limited knowledge of their habitat use.

Here, we report original observations and literature data on the European leaf toed Gecko *Euleptes europaea* that warrant a reconsideration of its habitat use. This strictly nocturnal gecko, endemic to the western Mediterranean (Fig. 1), belongs to a monotypic genus of the Gondwanan family Sphaerodactylidae, mainly distributed in the Neotropics (Gamble et al. 2008), thus representing an outstanding biogeographical relict within the European fauna.

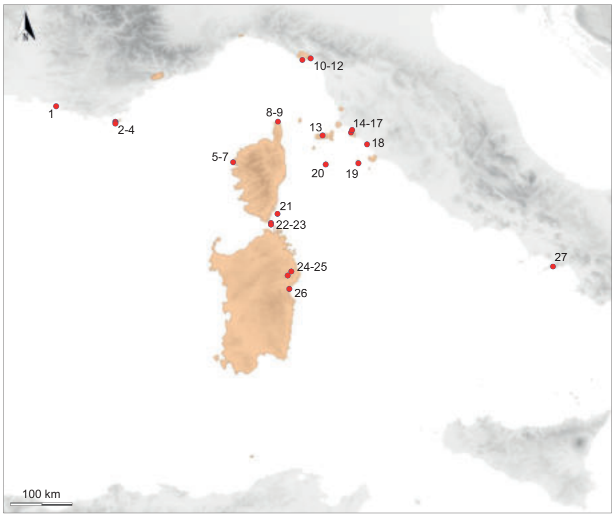

Figure 1. Distribution range the European leaf toed Gecko *Euleptes europaea* and geographic location of the observations of arboricolous behaviour reported in Table 1.

The European Leaf-toed Gecko is considered a rock-specialist and has several anatomical, behavioural, ecological adaptations to this habitat with which it is associated during its entire daily and life cycle (Salvidio et al. 2010). During the day, narrow rock crevices provide this species protection against predators and the opportunity to thermoregulate. Its small size, SVL 30–40 mm (Salvidio et al. 2010; Delaugerre and Corti 2020), its smooth (not keeled) body scales and its consequent ability to flatten itself to an extreme extent are probable morphological adaptations to crevice life. Crevices with openings as narrow as 3–5 mm permit this gecko to stay in both dorsal and ventral contact with the stone, preserving it from light and direct sun exposure and from desiccating cold winds, and providing optimal microclimatic conditions (thermal and hygrometric). Geckos are able to thermoregulate by conduction moving inside the shelter heated by the sun; whereas predators, such as birds, mammals, and other squamates, are too large to enter the crevices. In the roomiest cracks, large aggregations up to several dozen geckos may occur (Delaugerre 1981, 1992; Salvidio et al. 2010). Along with suitable climatic conditions, the availability and the quality of retreat sites and egg laying sites are likely the key factor governing the presence and the abundance of this gecko (Salvidio and Oneto 2008).

The nocturnal life of this species is also linked to the rocks. At night, *E. europaea* forages mostly on rocky surfaces (from horizontal to vertical to overhanging) that it climbs easily thanks to its distal toe pads and claws that allow attachment to dusty rock surfaces where other geckos with more powerful basal pads cannot do so -such as *Tarentola mauritanica*- (Russell and Delaugerre 2017). The heat stored by the rocks during the day is slowly released overnight making the rocks warmer than the air, wood, and other substrates, enabling this gecko to efficiently thermoregulate by thigmothermy during the night (Delaugerre 1984; Salvidio and Oneto 2008).

However, while the association of *E. europaea* to rocky habitats is well established, little is known about its ability to utilize vegetation near or far from rocks. For example, in the original description of this species it was reported that the species could be found under the bark of trees (Gené 1839), and recent observations suggest that, when rats are present, *E. europaea* shifts its spatial behaviour and feeds beneath vegetations (Delaugerre et al. 2019). Delaugerre (1992) also hypothesized that the animal may disperse in the vegetation during the hottest days of the year when the thermal supplement of the rocky substratum is no longer essential. Nevertheless, the arboreal aptitude of this gecko is still to be explored in order to have a more accurate view of its ecological niche and microhabitat use. Here we provided a comprehensive description of new field observations and a review of the available information on this topic that contribute to a refined view on the habitat use and arboreal abilities of *Euleptes europaea*.

Materials and methods

We collected observations of arboreal behaviour of *E. europaea* during field research in an opportunistic way. That is, vegetation was not primarily searched for the presence of the species but rather the species was accidentally spotted on vegetation while searching on rocks or for other species. Additional observations were gathered from colleagues and from the literature. We use the term "arboreal behaviour" in a broad meaning, including individuals climbing on trees, bushes, or low shrubs either dwelling on the bark, the branches or the leaves. For this tiny gecko, the main difference is likely dwelling on rock faces or on vegetation of any kind. On the other hand, we did not consider as 'arboreal behaviour' many observations we gathered during the years of *E. europaea* escape jumps (from heights of up to 3 m) toward other rock and into the vegetation, usually at the base of rock boulders, where they quickly disappear.

For each observation, we described the presence of the geckos on vegetation and their behaviour, and we reported the type of vegetation, the year and locality of the observations and the observers.

Results and discussion

A total of 32 observations of one or more individuals of *E. europaea* on vegetation were collected during 27 surveys. Observations cover 40 years (1982–2022) and 21 locations scattered across the species range (Fig. 1). Detailed information for each observation is reported in Table 1 and Suppl. material 2.

Eighteen different plant/tree species were used by *E. europaea*, mostly maquis species but also alien species such as *Eucalyptus* and *Asparagus aethiopicus* (Table 2). Although the opportunistic sampling does not allow proper statistical testing, the absence of large differences in observation frequency distribution across plant species (Table 2), suggests that the wide range of trees, bushes, and shrubs used by *E. europaea* reflects their availability at the sampling sites rather than plant selection by the gecko. Likewise, although our field research was mainly biased towards rocky environments (as this was considered as the elective habitat of *E. europaea*), and thus most observations concern vegetation growing on or nearby rocky surfaces, we also observed *E. europaea* on vegetation relatively far from rocks.

Arboreal behaviour of *E. europaea* was observed throughout the year, across the entire species range, on a wide variety of tree, shrub, bush species, on various parts of the plant (trunk, branches, fronds, twigs, leaves) at different heights above the ground (up to 3 m), strongly supporting that this is not an occasional behaviour of this species. This is further supported by a paper made available at the same time of our study that shows a high occupancy probability of *E. europaea* on *Eucalyptus* trees in an insular site (Deso et al. 2023). On the two *Eucalyptus* stands studied by these authors, *E. europaea* was the only gecko occupying the less-anthropized area. It is not yet known how high *Euleptes* geckos dwell in those trees and if this arboricolous habitat does provide egg-laying sites.

Table 1. Observations of arboricolous behaviour of *Euleptes europaea* (*Ee*); see Fig. 1 for the location of the observations. Island (I); islet (is).

Observation	Locality	Year	Period	Observer
1 *Ee* found on the nape of MD's neck while passing through bush by night	Scandula[5] (W Corsica)	1982	May	M. Delaugerre
Most of the *Ee* forage by night between the base of the granite and a mattress of plants	Lavezzu I[23] (S Corsica)	1986–2022	June to October	M. Delaugerre, C. Corti, M. Biaggini and P. Lo Cascio
2 *Ee* found on a bird nest box on *Arbutus unedo* (2 m high), not far from rocky boulders	Scandula (W Corsica)	1992	June	JL Martin
1 *Ee* in a bird nest box on *Erica arbora* (2 m high), not far from rocky boulders	Scandula (W Corsica)	1993	May	JL Martin
1 *Ee* climbing on *Rosmarinus officinalis* close to a schist face (Suppl. material 1: fig. S1a)	Port-Cros I[3] (Provence,	2003	October	M. Delaugerre
1 *Ee* climbing on *Lotus cytisoides* growing at the base of the rock (Suppl. material 1: fig. S1b)	Gabinière is[4] (Provence)	2003	October	M. Delaugerre
1 *Ee* climbing on *Malva arborea* (1.30 m high)	Toro is[21] (SE Corsica)	2005	April	M. Delaugerre
4 *Ee* (out of 23 diurnal sights) found under the bark of dead *Ulmus minor* trees	Tino is[10] (Liguria)	2006	20–29 June	F. Oneto, D. Ottonello, and S. Salvidio[a]
1 *Ee* found on the vest of MD while passing by bushes of *Juniperus phoenicea* (3 m high)	Rascas is[2] (Provence)	2008	17–18 June	M. Delaugerre
1 Ee found under the bark of *Eucalyptus* (Suppl. material 1: fig. S1c)	Giglio I[19] (Tuscany)	2008	August	S. Fattorini[b]
1 *Ee* climbing on *Anthyllis barba-jovis*, 3 m high	Cala Violina[14] (Tuscany)	2009	18 October	G. Radi
1 *Ee* head spotted among *Halimione portulacoides* (Suppl. material 1: fig. S1d)	Giraglia I[8] (N Corsica)	2012	6 October	M. Delaugerre
1 *Ee* found under the bark of *Juniperus phoenicea*; and 1 *Ee* found under the bark of *Cupressus* sp.	Cavallo I[22] (S. Corsica)	2014	6 November	V. Rivière
1 Ee found hidden under the bark of *Quercus ilex*	La Paolina is[11] (Tuscany)	2016	6 May	M. Delaugerre and C. Corti[c]
1 *Ee* on *Olea europaea* (1 m high)	Collelungo[18] (Tuscany)	2017	24 August	G. Radi
1 *Ee* foraging on the bark of *Juniperus Phoenicia* (1 m high)	Punta Ala[15] (Tuscany)	2019	30 September	G. Radi
1 *Ee* on *Anthyllis barba-jovis* (1.5 m high)	Punta Ala[16] (Tuscany)	2020	21 June	G. Radi
1 *Ee* on *Anthyllis barba-jovis* (2 m high)	Punta Ala[17] (Tuscany)	2020	5 October	G. Radi
>10 *Ee*, including a mating pair, observed by night on *Cistus* sp. and *Erica arborea* (2 m high; Fig. 2c)	Monte Albo[24] (Sardinia)	2021	6–7 July	E. Berrilli, M. Garzia, D. Salvi, and V. Gomez
>10 *Ee* observed by night on *Cistus* sp. and *Erica arborea* (1.5 m high; Fig. 2d)	Monte Albo[25] (Sardinia)	2021	8 July	D. Salvi, M. Garzia, and V. Gomez
2 *Ee* found under the bark of a fallen *Pinus halepensis* tree (geckos not active)	Positano[27] (Campania).	2022	5 February	F. Russo[d]
>20 *Ee* active by night on trunks of *Quercus ilex* and *Juniperus phoenicea*	Dorgali[26] (Sardinia)	2022	11–13 June	A. Macali and C. Pardo
1 *Ee* on a *Pinus halepensis* trunk, 2.5 m high	Montecristo I[20] (Tuscany)	2022	14 June	G. Radi and M. Zuffi
1 Ee found on the nape of MD's neck while passing through *Malva arborea* (1.5 m high) 1 *Ee* on the nape of MD's neck	Giraglia I[9] (N Corsica)	2022	2 August	M. Delaugerre
2 *Ee* sighted within *Opuntia ficus indica* pads	Pomègue I[1] (Provence)	2022	11 October	V. Lara and V. Rivière
1 *Ee* on *Asparagus aethiopicus* growing on a rock (Fig. 2e)	Tellaro[11] (Liguria)	2022	12 December	G. Bruni
10 *Ee* on *Pistacia lentiscus*, close to the rock face(Fig. 2a, b, f)	Tellaro[12] (Liguria)	2022	21 December	G. Bruni

1–27: sampling locality code as shown in Fig. 1.

[a]: reported in Oneto et al.(2008, 4)

[b]: reported in Corti et al. (2021, 54).

[c]: reported in Fattorini (2010, fig. 3).

[d]: reported in Di Nicola et al. (2022).

Table 2. Frequency of observations of *Euleptes europaea* on different plants and trees.

Species	N
Anthyllis barba-jovis	3
Arbutus unedo	2
Asparagus aethiopicus	1
Cistus sp.	2
Cupressus sp.	1
Erica arbora	4
Eucalyptus sp.	1
Halimione portulacoides	1
Juniperus phoenicea	4
Lotus cytisoides	2
Malva arborea	2
Olea europaea	1
Opuntia ficus indica	1
Pinus halepensis	2
Pistacia lentiscus	1
Quercus ilex	2
Rosmarinus officinalis	1
Ulmus minor	1

On vegetation this species rests, forages, and mates (Table 1). What is more important, *E. europaea* has been observed to move across branches and twigs with great agility, using its four members and its tail, and shows striking morphological adaptation to the arboreal lifestyle.

In low and thick vegetation, locomotion of *E. europaea* involves climbing, rather than crawling (on rock face), so that it may even "swim and vanish" in a puzzling way, in contrast to its relatively slow escape speed on rocks. In an arboreal context, it may perch head down as truly arboreal lizards do (Fig. 2a, c). Compared to rock faces, arboreal habitat is a much more structurally complex 3D array of branches and surfaces (Clark et al. 2021). This species' prehensile tail has long been recognized (Fitzinger 1843 p. 95; Wiedersheim 1876; Camerano 1885 p. 501; Mourgue 1910; Eijsden 1983; Bauer et al. 1997; Fig. 2c, d). Almost all adults bear a cartilaginous regenerated tail, turnip-like, enlarged for fat storage, that is still prehensile. The prehensile tail is a functional trait associated with the arboreal environment where this organ is used to grasp cork, branches, twigs and leaves (Mertens 1964; Eijsden 1983; Alibardi and Bonfitto 2019). The prehensile tail is also flexible and is an asset for equilibrium and the guiding of escape jumps (Jusufi et al. 2008; Fleming and Bateman 2012) and this species is a good jumper. Its tail is not only prehensile, it also bears terminal adhesive pads, even when regenerated (Eijsden 1983). In the arboreal context, tail pads supplement the muscular grapping of twigs or branches with dry adhesion of the sub caudal scansors (Fig. 2c, f and Suppl. material 1: fig. S1a, e). As stressed by Higham et al. (2017), in arboreal environments, when performing escape jumps or falls from trees, geckos land on leaves or smooth tree trunks. Arresting this fall requires a high loading of the adhesive system that is enhanced by an extra padded member, the tail. But these caudal adhesive systems are not the exclusive signature of arboreal lifestyle, since they are also used by geckos for climbing steep rock surfaces (Bauer 1998; Koppetsch et al. 2020) and to prevent the animal falling backward (Bauer and Russell 1994; Jusufi et al. 2008). Therefore, it is likely that these ecomorphological adaptations allow the European Leaf-toed gecko to exploit both rocky and arboreal microhabitats. To explain the origin of such ecomorphological adaptations to arboreal lifestyle we could speculate that these have been inherited by *E. europaea* from its European ancestors living in subtropical forests environments during Miocene (Estes 1969; Müller 2001; Müller and Mödden 2001; Böhme 2003; Čerňanský and Bauer 2010; Daza et al. 2014; Čerňanský et al. 2022).

Our study opens further questions also on the use of vegetated and wooded habitat by *E. europaea*. Could there be a continuum from i) a simple nightly coming and going from rocky habitat to nearby vegetation, as suggested by some observations, ii) to more seasonal wandering movements, ultimately turning back to rocky habitat for winter; iii) to truly arboreal living all year round? The rupicolous habitat niche of *E. europaea* is primarily driven by thermal constraints (Delaugerre 1984). These are particularly severe for a strictly nocturnal ectothermic vertebrate, inhabiting a temperate region where it colonizes Alpine elevations up to 1500 m (Delaugerre 1992). Rocky substrates typically have larger thermal inertia and a greater capacity for heat storage than soil or wood (Huey et al. 1989). Of course, this gecko is an active and efficient thermoregulator that uses the expansion and retraction of melanophores to speed up the acquisition of heat and to slow down its loss (Delaugerre 1984). But being a strict thigmotherm (it never basks) it would still need the rocky substratum for the primary acquisition of heat, during periods of thermal deficit, 5 to 6 months a year. Our finding of arboreal behaviour *E. europaea* in autumn and winter with air temperatures as low as 11 °C (Table 1) is therefore surprising. A possible explanation is that this species is able to locate warm environmental microclimates that retain higher temperatures throughout the night, allowing them to loosen the bond to rocks. The ability to exploit rare microclimates is especially important for nocturnal species, as heterogeneity of environmental temperatures is reduced at night compared to the day (Nordberg and Schwarzkopf 2019). However, understanding the apparent discrepancy between the thermal requirements of the species and its arboreal habits will require dedicated investigation.

In conclusion, to answer the main question of this study: is *E. europaea* a strictly rock-dwelling species? Definitely it is not. This species efficiently uses vegetated and wooded habitats, both in hot summer and colder winter nights, and shows adaptations to climbing and clinging for arboreal locomotion. It is a climbing gecko (opposed to ground dwelling), occupying both saxicolous and arboreal microhabitats like other geckos (Pianka and Pianka 1976; Norris et al. 2021).This realization has important implications for designing fieldwork research and management strategies for conservation. The evolutionary origin and ecological drivers for such behaviour remain to be fully investigated.

Figure 2. *Euleptes europaea* on *Pistacia lentiscus* **a, b, f** locality Tellaro, Liguria; photo by G. Bruni; on *Cistus* sp. **c, d** locality M. Albo, Sardinia; photo by D. Salvi; and on *Asparagus aethiopicus* **e** locality Tellaro, Liguria; photo by G. Bruni.

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Supplementary material 1

Euleptes europaea on various plant items

Authors: Daniele Salvi, Emanuele Berrilli, Giacomo Bruni, Matteo Garzia, Veronica Gomes, Giacomo Radi, Michel-Jean Delaugerre

Data type: figure (JPG image)

Explanation note: *Euleptes europaea* on *Rosmarinus officinalis* (**a**; locality Port-Cros island, Provence; photo by M. Delaugerre); on *Eucalyptus* (**b**; locality Giglio island, Tuscany; photo by S. Fattorini); on *Lotus cytisoides* (**c**; locality Gabinière islet, Provence; photo by M. Delaugerre); on *Halimione portulacoides* (**d**; locality Giraglia island, Corsica; photo by M. Delaugerre); on *Juniperus phoenicea* (**e**; locality Punta Ala, Tuscany; photo by G. Radi).

Link: https://doi.org/10.3897/herpetozoa.36.e103465.suppl1

Supplementary material 2

Observations of arboricolous behaviour of *Euleptes europaea*

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The validity of *Pelophylax chosenicus* (Okada, 1931) and *P. hubeiensis* (Fei & Ye, 1982) (Amphibia, Ranidae)

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Abstract

Pelophylax plancyi (Lataste, 1880), *Pelophylax chosenicus* (Okada, 1931) and *Pelophylax hubeiensis* (Fei & Ye, 1982) were described chronologically from East Asia. The three species have similar morphological and molecular characteristics, but the taxonomic relationships amongst them have long been ambiguous. To deal with this taxonomic issue, we examined newly-obtained topotypic specimens of *P. plancyi*, *P. chosenicus* and *P. hubeiensis* for morphological comparison. Furthermore, we investigated the phylogeny of pond frogs in Eurasia by Bayesian Inference and Maximum Likelihood analyses of a fragment consisting of mitochondrial DNA gene 16s and provided a molecular phylogeny of the genus *Pelophylax*. There were no morphological and molecular differences between *P. plancyi* and *P. chosenicus*, but both morphological and molecular differences between *P. hubeiensis* and *P. plancyi*. Hence, we conclude that *P. chosenicus* is a junior synonym of *P. plancyi* and *P. hubeiensis* is a separate species from *P. plancyi*.

Key Words

amphibians, morphology, phylogeny, synonym, taxonomy

Introduction

The pond frogs of the genus *Pelophylax* Fitzinger, 1843 originated from the western Palearctic (Pyron 2014) and are continuously distributed across the Palearctic (Lymberakis et al. 2007; Frost 2022). Including the record of *Pelophylax chosenicus* (Okada, 1931) lately, seven of them were reported to be distributed in China. (AmphibiaChina 2022; AmphibiaWeb 2022; Zhou et al. 2022). Even though the evolutionary relationships on these frogs are settled, partial members still have questionable taxonomic status in this genus because of substantial intraspecific morphological variation and high levels of interspecific morphological similarity, such as the validities of *P. chosenicus* (Shannon 1956; Dubois and Ohler 1994; Amal et al. 2017; Dufresnes and Litvinchuk 2022) and *P. hubeiensis* (Fei & Ye, 1982) (Mou and Zhao 1992; Zhao and Adler 1993; Dufresnes and Litvinchuk 2022).

Based on specimens collected from Seoul, South Korea, Okada (1927) described a subspecies *Rana nigromaculata coreana* (Okada 1927, 1928) and subsequently changed the scientific name to *R. n. chosenica* (Okada 1931). In 1956, Shannon modified *R. n. chosenica* to *R. plancyi chosenica* as a subspecies of *R. plancyi* (*sensu P. plancyi* (Lataste, 1880) in this study) (Shannon 1956). Kuramoto (1983) identified it as a separate species, *R. chosenica* (sensu *P. chosenicus* in this study), which was later endorsed by Dubois (1986, 1992), but Dubois and Ohler (1994) further identified *R. chosenica* as a subspecies of *R. plancyi*. Amphibian Species of the World (Frost 2022) placed *P. chosenicus* as a valid species in the genus *Pelophylax*. Fei and Ye (1982) described *Rana hubeiensis* (sensu *P. hubeiensis* in this study), based on specimens from Hubei and Anhui Provinces, but Mou and Zhao (1992) suggested, based on identical call parameters, that *P. hubeiensis* is a junior synonym of *P. plancyi*. Based on the obvious morphological differences (e.g. male with silent sac), Fei et al. (2009) still regarded it as a valid species. Dufresnes and Litvinchuk (2022) placed both *P. chosenicus* and *P. hubeiensis* into the synonymy of *P. plancyi*, based on low genetic distances. The two species are pending a more detailed review of the taxonomic issue (Frost 2022).

From 2021 to 2022, we collected a series of topotypic specimens of *P. plancyi*, *P. chosenicus* and *P. hubeiensis* (Fig. 1). The combined evidence from morphology and molecular phylogeny of these specimens demonstrated the distinct specific level of *P. hubeiensis* that is distant from *P. plancyi*, while indicating the homogeneity between *P. chosenicus* and *P. plancyi*. Thus, we suggest elevating *P. hubeiensis* as a separate species from *P. plancyi* and placing *P. chosenicus* to be a secondary synonym of *P. plancyi*.

Materials and methods

Specimens and sampling

We collected six specimens (three adult males and three adult females) of *Pelophylax chosenicus* from multiple localities of Seoul, South Korea and Shenyang, Dandong, China, six specimens (three adult males and three adult females) of *P. plancyi* were collected from four localities in China (Fig. 1) and six specimens (three adult males and three adult females) of *P. hubeiensis* were collected from Lichuan and Wuhan, China, including specimens from the vicinity of the type locality of the three species (detailed collection information presented in the Taxonomic account below). The specimens collected from South Korea were released after morphological data measuring and Non-Disruptive DNA Sampling. The DNA- sampling came from oral throat swab sampling. The specimens collected from China were fixed in 10% formalin and then stored in 75% ethanol. Liver tissue samples were collected from individual specimens before formalin fixation and preserved in 95% ethanol for subsequent molecular analyses. Specimens and tissue samples are preserved at Shenyang Agricultural University (**SYAU**).

Morphological comparison and analysis

Linear measurements were performed on all the adult specimens, using a Vernier caliper with a precision of 0.1 mm, with the following abbreviations: **SVL** (snout-vent length, from the tip of snout to vent); **HL** (head length, from posterior corner of the mandible to tip of snout); **HW** (head width, the greatest cranial width); **SL** (snout length, from the tip of snout to the anterior corner of the eye); **INS** (internasal space, the distance between the two nostrils); **IOS** (interorbital distance, the minimal distance between upper eyelids); **ED** (horizontal eye diameter); **LAHL** (length of lower arm and hand, from the tip of finger III to the elbow joint); **TD** (horizontal tympanic diameter); **LAD** (diameter of the lower arm); **HLL** (hind limb length, from the tip

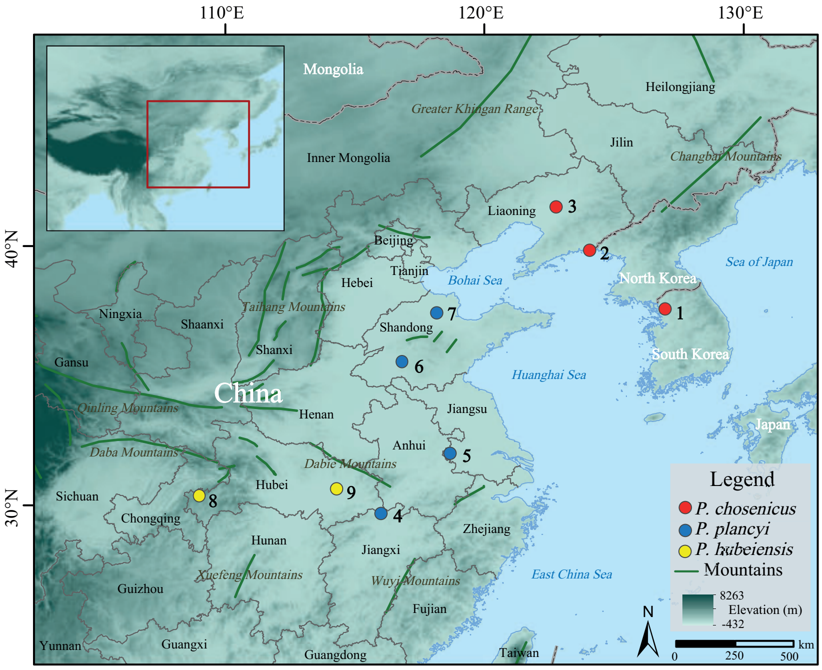

Figure 1. The collection localities of *Pelophylax chosenicus* (marked with red circles), *P. plancyi* (marked with blue circles) and *P. hubeiensis* (marked with yellow circles) in this study. (1) Liulin Wetland, Seoul, South Korea; (type locality of *P. chosenicus*); (2) Donggang District, Dandong, China; (3) Puhe Park, Shenyang, China; (4) Nanshan Park, Jiujiang, China (type locality of *P. plancyi*); (5) Yuzui Park, Nanjing, China; (6) Si River, Jining, China; (7) Bailuhu Park, Binzhou, China; (8) Qing River, Lichuan, China (type locality of *P. hubeiensis*); (9) Jiefang Park, Wuhan, China.

of toe IV to groin); **TL** (tibia length); **TW** (tibia width, the greatest width of tibia); **FL** (foot length, from the proximal end of the inner metatarsal tubercle to the tip of toe IV); and **IMT** (internal metatarsal tubercle, the length of internal metatarsal tubercle). The morphology comparisons are according to Zhou et al. (2022) for *Pelophylax mongolius* (Schmidt 1925) and Fei et al. (2009) for *P. nigromaculatus* (Hallowell, 1861), *P. fukienensis* (Pope, 1929) and *P. terentievi* (Mezhzherin, 1992). The toe webbing formula followed the protocol described by Savage (1975).

Measurement data were used for principal component analysis (PCA) on the morphometric differences amongst *P. plancyi*, *P. chosenicus* and *P. hubeiensis*. Statistical analyses were carried out by using the "prcomp" package in R 4.1.1 (Zhang et al. 2022).

Phylogenetic analyses

Genomic DNA was extracted by Qiaprep Spin Miniprep kits (QiaGen) and a 508 bp mitochondrial genome fragment of *16S* ribosomal RNA (*16S*) using primers L3975 and H4551 (Simon et al. 1994) was specifically amplified for this study. The standard PCR protocol was performed in a 20 µl reaction with at least 20 ng of template DNA and 10 pmol of primers. PCR conditions consisted of an initial denaturation for 5 min at 95 °C, followed by 25 cycles: denaturation at 95 °C for 30 sec, annealing at 54 °C for 60 sec, elongation at 72 °C for 90 sec and then finalised with an extension step of 10 min at 72 °C. Sequencing was conducted by Nanchang Kechang Biotech Co., Ltd. New sequences are uploaded to GenBank (Table 1).

For phylogenetic comparisons, corresponding sequences of 11 recognised species of the genus *Pelophylax* and one outgroup (*Rana taihangensis* Chen, 2022) were obtained from GenBank (Table 1). Sequences were assembled and aligned using MEGA7 (Tamura et al. 2013) with default settings and were further revised manually when necessary. Phylogenetic trees were estimated with Maximum Likelihood (ML) using RAxML NG v.0.9.0. (Kozlov et al. 2019) and Bayesian Inference (BI) using MrBayes 3.1.2 (Ronquist et al. 2012). We used JModelTest 2 (Guindon and Gascuel 2003; Darriba et al. 2012) to infer the model of sequence evolution that best fits our

Table 1. Species, voucher museum numbers, sample localities and GenBank accession numbers for 16S rRNA of *Pelophylax* species used in the phylogenetic analyses.

Taxa	Voucher	Country: localities	16S rRNA accession number	Reference
Pelophylax chosenicus	HGSE 01	South Korea: Seoul	OQ708390	This study
	HGSE 02		OQ708391	
	SYAU BAA000061	China: Liaoning: Dandong	OQ708385	
	SYAU BAA000062		OQ708386	
	SYAU BAA000046	China: Liaoning: Shenyang	OQ708387	
	SYAU BAA000016		OL752662	Zhou et al. (2022)
	SYAU BAA000017		OL752663	
	SYAU BAA000018		OL752664	
	NIBRAM0000000038	South Korea: Chungcheongbuk-do: Goesan-gun	JQ815307	Jeong et al. (2013)
	NIBRAM0000100371	South Korea: Chungcheongnam-do: Taean-gun	JQ815308	
	MMS176	South Korea	EU386945	Direct submission by Min et al. (2016)
	MMS179		EU386932	
	MMS533		EU386947	
	MMS431		EU386935	
	MMS446		EU386958	
	MMS524		EU386959	
	MMS531		EU386943	
	MMS189		EU386944	
	MMS171		EU386946	
	MMS102		EU386914	
	MMS510		EU386908	
	MMS523		EU386941	
	–		JF730436	Ryu and Hwang (2011)
P. plancyi	SYAU BAA000035	China: Jiangxi: Jiujiang	OQ708392	This study
	SYAU BAA000072		OQ708393	
	SYAU BAA000073		OQ708394	
	SYAU BAA000074	China: Jiangsu: Nanjing	OQ708395	
	SYAU BAA000075	China: Shandong: Binzhou	OQ708396	
	SYAU BAA000077	China: Shandong: Jining	OQ708397	
P. hubeiensis	SYAU BAA000052	China: Hubei: Lichuan	OQ708388	
	SYAU BAA000051	China: Hubei: Wuhan	OQ708389	
	–	China: Anhui: Huoqiu	AF315137	Direct submission by Jiang and Zhao (2005)
P. nigromaculatus	–	Japan	AB043889	Masayuki et al. (2001)
P. mongolius	SYAU BAA000001	China: Ningxia: Guyuan	OL752643	Zhou et al. (2022)
	SYAU BAA000030	China: Inner mongolia: Baotou	ON693246	
P. porosus	Pp2	Japan: Aichi	LC389210	Tokumoto et al. (2019)
P. cretensis	NHMC 80.2.46.18	Greece	DQ474204	Lymberakis et al. (2007)
P. epeiroticus	NHMC 80.2.109.4		DQ474207	
P. kurtmuelleri	NHMC 80.2.11.12		DQ474228	
P. bedriagae	NHMC.80.2.99.24		DQ474193	
P. cerigensis	NHMC.80.2.110.5		DQ474196	
Rana taihangensis	SYAU BAA000025	China: Ningxia: Guyuan	OQ708398	This study

16S sequences. The model HKY + I + G was selected as the best-fitted model for BI testing and HKY + F + R2 was selected as the best-fitted model for ML. All searches consisted of three heated chains and a single cold chain. Three independent iterations, each comprising two runs of 100,000,000 generations, were performed, sampling every 10,000 generations and parameter estimates were plotted against generation. The first 25% of the samples were discarded as burn-in, resulting in a potential scale reduction factor (PSRF) of < 0.005. Maximum likelihood analysis is completed through 1000 rapid guided repetitions. Pairwise distances (p-distance) were calculated in MEGA 6 using the uncorrected p-distance model.

Results

Morphological comparisons

The morphological comparisons of *Pelophylax plancyi* and *P. chosenicus* revealed similarities, our newly-collected specimens of *P. plancyi* (P) and *P. chosenicus* (C) both had large body sizes (SVL: 40.7–56.1 mm, n = 6 (P) vs. 41.1–60.1 mm, n = 6 (C)), head length slightly larger than head width (HL/HW: 1.00–1.03, n = 6 (P) vs. 1.01–1.04, n = 6 (C)), relatively short snouts (SL/SVL: 0.14–0.16, n = 6 (P) vs. 0.14–0.16, n = 6 (C)), tympanum diameter slightly smaller than the eye diameter (ED/TD: 1.02–1.09, n = 6 (P) vs. 1.00–1.15, n = 6 (C)), internal subgular vocal sacs and dorsolateral fold slightly thick. Furthermore, all of the newly-collected *P. hubeiensis* specimens can be distinguished from *P. plancyi* and *P. chosenicus* by the combination of the following characteristics: (1) head width slightly larger than head length, HL/HW 0.93–0.97 (n = 6) (vs. head length slightly larger than head width in *P. plancyi* and *P. chosenicus*, HL/HW 1.00–1.04, n = 12); (2) tympanum diameter slightly larger than the eye diameter ED/TD 0.86–0.97 (n = 6) (vs. tympanum diameter slightly smaller than the eye diameter, ED/TD 1.00–1.15, n = 12); (3) foot length shorter, FL/SVL 0.49–0.58 (n = 6) (vs. FL/SVL 0.55–0.78, n = 12); (4) silent sac (vs. internal subgular vocal sacs) (Table 2). The differences between *P. plancyi*, *P. chosenicus*, *P. hubeiensis* and other congeneric species distributed in China are summarised in Table 3.

Moreover, the PCA result indicated that the *Pelophylax hubeiensis* could be distinctly separated from *P. plancyi* and *P. chosenicus*, but overlapped between *P. plancyi* and *P. chosenicus* (Fig. 2). The first two extracted principal component axes accounted for 74.11% and 15.74% of the variation (Table 4). PC1 separated the sexes and the

Table 2. Measurements of adult specimens of *P. plancyi*, *P. chosenicus* and *P. hubeiensis* (# topotype of the three species).

Species	*P. plancyi*						*P. chosenicus*						*P. hubeiensis*					
Specimen	SYAU BAA000035#	SYAU BAA000072#	SYAU BAA000077	SYAU BAA000073#	SYAU BAA000074	SYAU BAA000075	HGSE 01 #	SYAU BAA000046	SYAU BAA000061	HGSE 02 #	SYAU BAA000016	SYAU BAA000062	SYAU BAA000052 #	SYAU BAA000049	SYAU BAA000050	SYAU BAA000053 #	SYAU BAA000051	SYAU BAA000048
Sex	♂	♂	♂	♀	♀	♀	♂	♂	♂	♀	♀	♀	♂	♂	♂	♀	♀	♀
SVL	40.7	40.9	41.7	55.2	57.2	56.1	41.1	42.0	41.3	56.2	58.8	60.1	41.8	42.0	43.3	55.5	55.9	56.6
HL	16.1	15.9	16.2	23.1	20.5	20.2	15.9	16.1	16.0	21.1	20.5	24.3	16.0	16.1	16.3	18.5	18.6	19.2
HW	16.0	15.8	15.8	22.6	20.0	20.0	15.6	15.7	15.7	20.6	20.3	23.3	17.2	17.3	17.5	19.4	19.6	19.8
SL	6.0	5.9	5.8	8.9	8.0	9.1	6.5	6.0	5.9	7.8	8.5	8.4	5.6	5.8	6.0	6.6	6.9	7.0
INS	3.0	2.9	3.2	3.9	3.5	3.9	3.4	3.3	3.1	3.9	3.5	4.3	3.7	4.1	3.8	3.6	3.6	3.8
IOS	2.9	2.8	2.8	3.7	2.9	3.7	3.3	3.0	2.9	3.6	2.8	4.0	2.1	2.2	2.2	2.5	2.6	2.7
ED	4.7	4.9	4.5	6.5	5.8	6.0	4.7	4.7	4.8	5.3	6.2	6.6	5.1	5.2	5.4	6.0	5.8	6.2
LAHL	18.1	18.3	19.6	26.9	24.9	27.1	18.0	18.7	19.0	24.3	22.5	27.5	18.5	18.6	19.0	24.1	25.0	25.3
TD	4.6	4.5	4.6	6.4	5.4	5.5	4.7	4.6	4.5	5.2	5.4	6.2	5.9	5.9	6.1	6.2	6.0	6.5
LAD	4.7	4.7	4.9	5.5	5.9	5.8	4.8	5.0	4.8	5.2	4.6	5.6	3.9	4.2	4.0	4.8	5.0	5.1
HLL	61.3	62.5	62.7	88.2	84.8	85.1	62.5	64.0	62.6	85.4	86.6	89.5	66.0	66.2	66.5	77.2	78.5	78.3
TL	17.7	18.1	19.0	27.5	25.7	24.9	19.2	18.5	18.2	24.7	23.3	28.5	18.4	18.2	18.7	25.1	25.5	25.5
TW	5.5	5.7	5.6	7.5	7.2	7.6	5.9	5.8	5.3	7.2	8.4	7.7	6.2	6.3	6.6	7.5	7.8	8.2
FL	31.1	32.0	31.0	39.8	39.1	38.0	29.4	32.1	31.5	32.9	32.1	43.0	24.2	24.2	24.5	28.8	28.6	27.8
IMT	3.0	3.1	3.2	4.9	5.1	5.0	3.3	3.2	3.2	4.8	5.1	5.0	3.2	3.1	3.3	4.8	4.9	4.7

Table 3. Brief morphological comparisons amongst *Pelophylax* species distributed in China.

Species	Head style	Vocal sac	Web	Background colouration	Dorsolateral fold	Reference
P. plancyi	HL slightly larger than HW	internal subgular vocal sacs	almost full web, webbing formula I 0 -0^- II 0-⅓ III 0 – ⅓ IV I ⅓ – 0 V	green or olive green	slightly thick	Fei et al. (2009)
P. chosenicus (sensu *P. plancyi*)	HL slightly larger than HW	internal subgular vocal sacs	almost full web	green, olive green or emerald green	slightly thick	Zhou et al. (2022)
P. hubeiensis	HW slightly larger than HL	silent sac	almost full web, webbing formula I 0^- – ⅓ II 0^- -⅓ III ⅓ – I^+ IV I^+ – 0^- V	green, olive green or light brown mixed with green flecks	thick	Fei et al. (2009)
P. mongolius	HW slightly larger than HL	external lateral vocal sacs	entire web, webbing formula I 0 -1 II ⅓-1½ III 1 – 2 – + IV 2 -1 V	light green with a few black patches, Green to brown gradation or brown	slightly thick	Zhou et al. (2022)
P. nigromaculatus	HL larger than HW	external lateral vocal sacs	the fourth finger webbed up to the distal end of the first subarticular tumor, the rest up to the fingertip, gap deep	light green, chartreuse. Dark green and taupe with irregular dark spots	range from narrow to thick	Fei et al. (2009)
P. fukienensis	HL slightly larger than HW	internal subgular vocal sacs	almost entire web	green or brownish-green, a few individuals with small black spots	narrow	Fei et al. (2009)
P. terentievi	HW and HL almost isometric	external lateral vocal sacs	entire web	olive green with dark brown rounded markings	thick	Fei et al. (2009)

Table 4. Variable loadings for principal components with eigenvalue.

	PC1	PC2	PC3	PC4	PC5	PC6	PC7	PC8	PC9	PC10
SVL	-0.28	-0.06	-0.27	0.06	0.10	-0.09	0.02	-0.33	0.06	-0.03
HL	-0.28	0.08	0.15	0.15	-0.30	-0.30	-0.05	-0.14	0.22	-0.27
HW	-0.29	-0.08	0.14	0.08	-0.27	-0.30	-0.09	-0.04	-0.08	-0.19
SL	-0.28	0.15	0.00	0.43	0.13	0.27	-0.24	0.59	-0.12	0.14
INS	-0.20	-0.29	0.68	0.02	0.36	0.24	-0.23	-0.34	0.02	0.07
IOS	-0.18	0.45	0.37	0.17	0.25	-0.15	0.64	0.07	0.00	0.08
ED	-0.27	-0.22	-0.14	0.04	-0.30	0.61	0.42	-0.25	-0.16	0.08
LAHL	-0.29	0.01	-0.06	-0.33	0.14	-0.07	0.05	0.11	-0.72	-0.40
TD	-0.19	-0.46	0.24	-0.38	-0.22	-0.05	0.08	0.53	0.16	0.13
LAD	-0.21	0.39	-0.10	-0.55	0.28	0.27	-0.21	0.00	0.33	-0.18
HLL	-0.29	-0.01	-0.06	0.23	0.01	-0.12	-0.35	-0.07	-0.06	-0.04
TL	-0.29	-0.01	-0.08	-0.29	0.02	-0.32	0.15	-0.05	0.14	0.43
TW	-0.26	-0.24	-0.27	0.21	0.20	0.10	0.22	0.16	0.46	-0.40
FL	-0.20	0.45	0.13	-0.07	-0.55	0.25	-0.16	-0.04	0.06	0.11
IMT	-0.29	-0.03	-0.31	0.04	0.19	-0.11	-0.12	-0.08	-0.11	0.53
variance	0.74	0.16	0.04	0.02	0.01	0.01	0.01	0.01	0.00	0.00

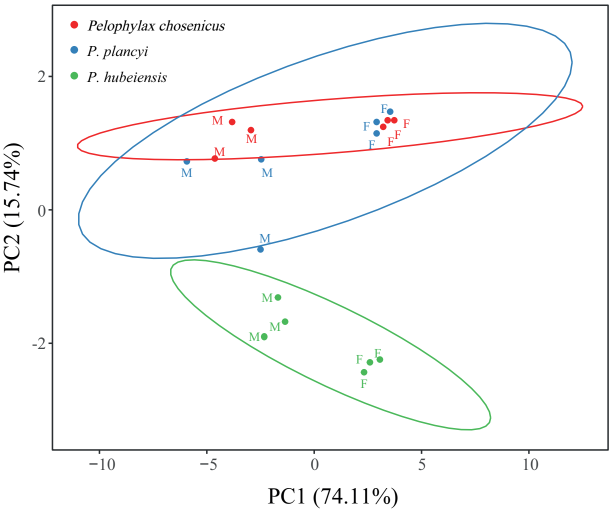

Figure 2. Plots of the first principal component (PC1) versus the second (PC2). *Pelophylax chosenicus* (red), *P. plancyi* (blue) and *P. hubeiensis* (green). M: Male; F: Female.

Interorbital distance, Horizontal tympanic diameter and Foot length are those loading strongly on PC2, were the most important morphological indexes affecting the morphological differences amongst the three species in PCA.

Molecular phylogeny

In this study, the topological structures of the Maximum Likelihood (ML) and Bayesian Inference (BI) trees are generally consistent (Fig. 3; Table 5). The genus *Pelophylax* forms a monophyletic group (Clade A), which is divided into two groups (Clades A1 and A2). In the gene tree, samples of *Pelophylax plancyi* and *P. chosenicus* (including topotype) form a supported monophyletic group (Clade B; Bayesian posterior probabilities 100, bootstrap supports 1.00) and small divergences (p-distance 0.0–0.8), which is further close to the *P. nigromaculatus* with significant support (Bayesian posterior probabilities 96, bootstrap supports 0.98). The samples of *P. hubeiensis* (including a topotype) also form a strongly-supported monophyletic group (Clade C; Bayesian posterior probabilities 100, bootstrap supports 0.99) far from clade B and with moderate divergence (p-distance 1.4–2.0).

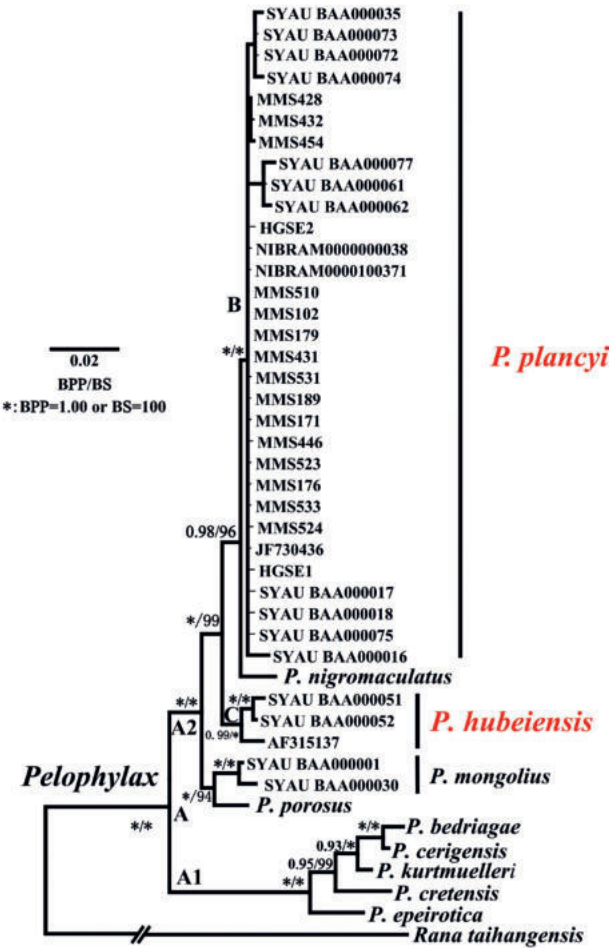

Figure 3. Bayesian-inferenced topology of *Pelophylax* species, based on *16s* rRNA sequences. BPP and BS values, respectively, occur at the nodes.

Taxonomic account

The combined evidence from morphology and molecular phylogeny suggested the specific distinction of *Pelophylax hubeiensis* that is distant from *P. plancyi*, while indicating the homogeneity between *P. plancyi* and *P. chosenicus*. Thus, we suggest that *P. chosenicus* is a junior synonym of *P. plancyi* and *P. hubeiensis* should be treated as a distinct species and we provide descriptions of these two separate species.

Table 5. Uncorrected p-distances (in %) amongst the *Pelophylax* species in this study.

ID	Species	I	II	III	IV	V	VI	VII	IIX	IX	X	XI
I	*Pelophylax plancyi*	0.0–0.5	–	–	–	–	–	–	–	–	–	–
II	*P. chosenicus*	0.0–0.7	0.0–0.5	–	–	–	–	–	–	–	–	–
III	*P. nigromaculatus*	0.9–1.1	0.8–1.1	0.0	–	–	–	–	–	–	–	–
IV	*P. hubeiensis*	1.4–2.0	1.5–1.9	1.0–1.1	0.0–0.7	–	–	–	–	–	–	–
V	*P. porosus*	2.7–3.1	2.6–3.0	2.1	2.5–2.7	0.0	–	–	–	–	–	–
VI	*P. mongolius*	2.4–3.3	2.5–3.1	2.3–2.9	2.7–3.2	2.3–2.5	0.0–0.5	–	–	–	–	–
VII	*P. kurtmuelleri*	7.1–7.3	7.1–7.4	7.3	7.2–7.4	8.2	7.3–7.4	0.0	–	–	–	–
IIX	*P. epeiroticus*	8.0–8.2	8.0–8.2	7.4	7.8–8.3	8.0	7.8–7.8	3.9	0.0	–	–	–
IX	*P. cretensis*	8.9–9.1	9.0–9.1	8.5	8.0–8.2	8.4	8.2–8.3	2.9	4.5	0.0	–	–
X	*P. cerigensis*	7.4–8.0	7.5–8.0	7.4	7.3–7.6	8.1	7.3–7.4	1.3	3.9	3.7	0.0	–
XI	*P. bedriagae*	7.3–8.0	7.3–8.0	7.3	7.4–7.6	7.7	7.3–7.5	1.9	4.3	3.9	0.8	0.0

Pelophylax plancyi (Lataste, 1880)

Rana plancyi – Lataste 1880; Boulenger (1920).

Rana nigromaculata coreana – Okada (1927, 1928).

Rana nigromaculata chosenica – Okada (1931).

Rana chosenica – Kuramoto (1983).

Rana plancyi plancyi – Kuramoto (1983).

Rana (*Rana*) *plancyi* – Dubois (1986).

Rana (*Rana*) *chosenica* – Dubois (1986).

Rana (*Pelophylax*) *plancyi* – Dubois (1992).

Rana (*Pelophyxlax*) *chosenica* – Dubois (1992).

Rana (*Pelophylax*) *plancyi chosenica* – Dubois and Ohler (1994).

Hylarana plancyi – Chen et al. (2005).

Hylarana chosenica – Chen et al. (2005).

Pelophylax chosenicus – Frost et al. (2006); Che et al. (2007); Zhou et al. (2022).

Type series. ***Syntypes*****:** Not traced, two specimens presumably originally in Lataste's personal collection and which were deposited ultimately in the BMNH; BMNH 1920.1.20.1020 is by museum records a syntype (Lataste 1880; Frost 2022). ***Neotype*****:** CIB 79I1764, adult male, collected from Yongfeng, Beijing, China (Fei et al. 2009).

Specimens examined. Twelve newly-collected specimens (six adult males and six adult females): adult males SYAU BAA000035, SYAU BAA000072 and adult female SYAU BAA000073, from Nanshan Park, Jiujiang, Jiangxi, China (29.6698°N, 116.0057°E, 28 m a.s.l.) (type locality of *Pelophylax plancyi*); adult male HGSE 01 and adult female HGSE 02 from Liulin Wetland, Seoul, South Korea (37.5663°N, 126.9779°E, 45 m a.s.l.) (type locality of "*P. chosenicus*"); adult male SYAU BAA000046 and adult female SYAU BAA000016 from Puhe Park, Shenyang, Liaoning, China (41.5114°N, 122.7579°E, 30 m a.s.l.); adult male SYAU BAA000061 and adult female SYAU BAA000062 from Donggang District, Dandong, Liaoning, China (39.8260°N, 124.0526°E, 60 m a.s.l.); adult female SYAU BAA000074 from Yuzui Park, Nanjing, Jiangsu, China (31.9801°N, 118.6723°E, 26 m a.s.l.); adult female SYAU BAA000075 from Bailuhu Park, Binzhou, Shandong, China (37.4209°N, 118.1575°E, 55 m a.s.l.); adult male SYAU BAA000077 from Si River, Jining, Shandong, China (35.5338°N, 116.8349°E, 45 m a.s.l.).

Diagnosis. (1) large body size, SVL 40.7–59.6 mm in adult males (n = 16), SVL 55.2–70.5 mm in adult females (n = 16) (Fei et al. 2009); (2) head length slightly larger than head width; (3) maxillary gland pronounced; (4) tympanum diameter large, slightly smaller than the eye diameter; (5) loreal region oblique, concave; (6) nostril located dorsally, between the eye and the tip of snout; (7) vomerine teeth two small masses; (8) tongue pyriform, free-margin notched; (9) fingers with rudimentary webs; toes almost full webs, webbing formula I 0 -0$^-$ II 0-⅓ III 0 – ⅓ IV I ⅓ – 0 V; (10) heels only meeting.

Common names. "Green Pond Frog" in English / " 金线侧褶蛙 (jīn xiàn cè zhě wā)" in Chinese.

Comparison. *Pelophylax plancyi* can be differentiated from other species in the *Pelophylax nigromaculatus* species group (*P. nigromaculatus*, *P. terentievi* and *P. mongolius*), based on the internal subgular vocal sacs in males (Fei et al. 2009). *Pelophylax plancyi* additionally differs from *P. hubeiensis*, based on its head slightly longer than wide, its tympanum slightly smaller than the eye diameter and longer foot (see Morphological Comparisons in the Results above). *Pelophylax plancyi* further differs from *P. fukienensis*, based on the dorsolateral fold wider and heels meeting (vs. dorsolateral fold narrower and heels meeting in *P. fukienensis*).

Colouration. The living specimens exhibit varying degrees of green, olive green or emerald green body colour variation, without spots on their backs, a tympanic membrane that was golden yellow or brown with a green margin and yellow eyelids. The dorsolateral fold ranged from green to golden yellow or light brown, with yellow at the ends and some minuscule irregular yellow spots. The legs were slightly lighter than the body, with brownish-yellow transverse stripes. The throat, chest and belly were light yellow. The backs of the elbows were yellow, with light brown cloudy spots behind the thighs. The ventral surface of the forelimbs and hind limbs were yellow. The nuptial pads were light grey (Fig. 4A, B).

In preservative, the dorsal surface turned dark grey without patches, while the dorsolateral fold and backline were light grey, the limbs were brown with black stripes, the ventral surface was flesh-coloured, the ventral surface of the limb was beige and the hand and toe webs were dark grey (Fig. 5).

Sexual dimorphism. Male with a pair of internal subgular vocal sacs; in the breeding season, a single, light grey nuptial pad on the dorsal surface of finger I. Males slightly smaller than females with linea masculina.

Figure 4. Colouration of *Pelophylax plancyi* **A.** ♀ SYAU BAA000073; **B.** ♂ SYAU BAA000061 and *P. hubeiensis*; **C.** ♂ SYAU BAA000052; **D.** ♀ SYAU BAA000051.

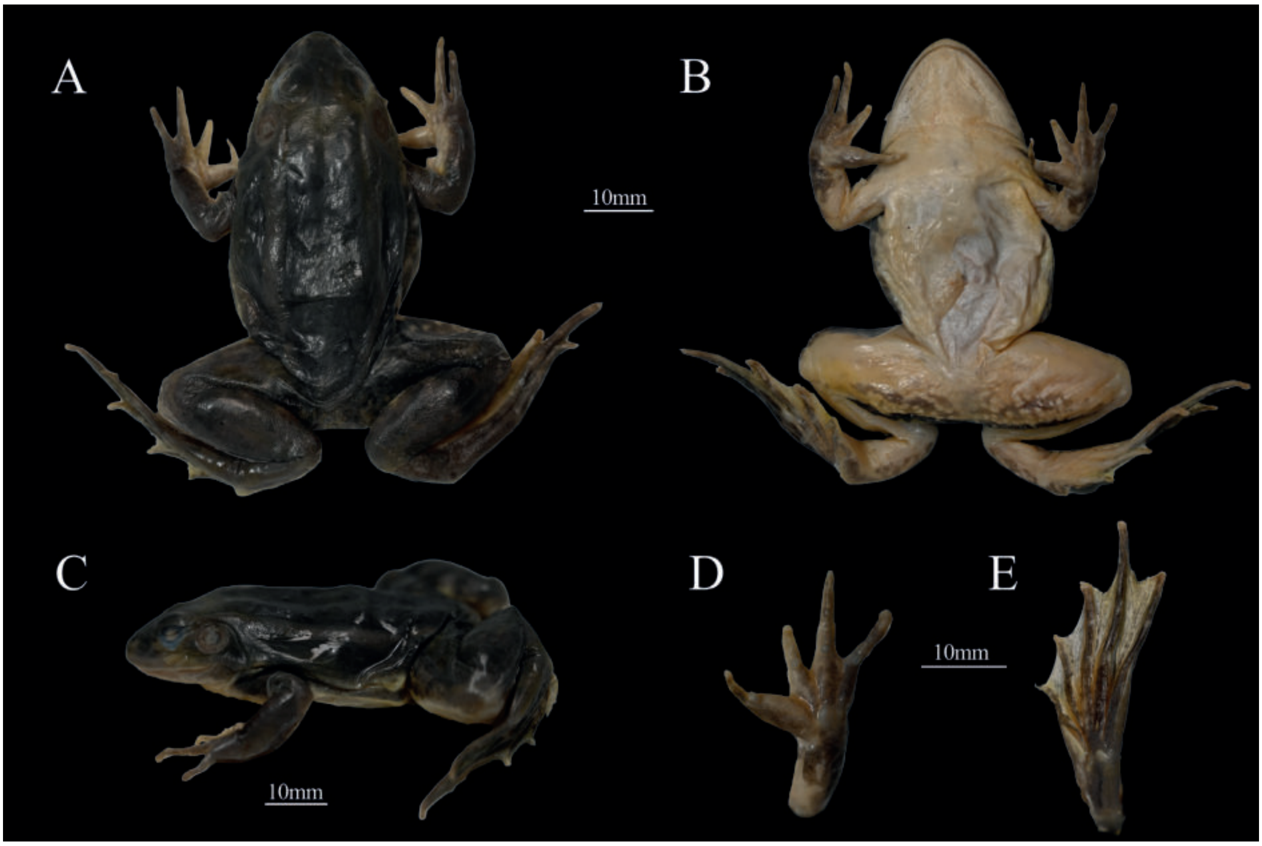

Figure 5. Specimen (SYAU BAA000073) of *Pelophylax plancyi*. **A.** Dorsal view; **B.** Ventral view; **C.** Lateral view; **D.** Ventral view of the hand; **E.** Ventral view of the foot.

Distribution and ecology. At present, specimens of *Pelophylax plancyi* have been identified in eastern China (except Hunan, Guangdong, Jilin and Heilongjiang) and the Korean Peninsula (Fei et al. 2009; Zhou et al. 2022). Specimens were collected from ponds full of aquatic plants (Fig. 7A, B), where they perch along the river bank during the night-time, although, when startled, they jump into the water.

Pelophylax hubeiensis (Fei & Ye, 1982)

Rana hubeiensis – Fei and Ye (1982).
Hylarana hubeiensis – Chen et al. (2005).
Pelophylax plancyi – Dufresnes and Litvinchuk (2022).

Type series. *Holotype*: CIB 74I0570, adult male, SVL 43.7 mm, collected from Lichuan, Hubei, China (Fei and Ye 1982).

Specimens examined. Six newly-collected specimens (three adult males and three adult females): adult male SYAU BAA000052 and adult female SYAU BAA000053, from Qing river, Lichuan, Hubei, China (30.3465°N, 108.9988°E, 780 m a.s.l.) (type locality of *Pelophylax hubeiensis*); adult males SYAU BAA000049, SYAU BAA000050 and adult females SYAU BAA000048, SYAU BAA000051 from Jiefang Paek, Wuhan, Hubei, China (30.6141°N, 114.3045°E, 40 m a.s.l.).

Diagnosis. (1) large body size, males slightly smaller SVL 38.5–47.1 mm in adult males (n = 23), SVL 41.1–61.9 mm in adult females (n = 23) (Fei et al. 2009); (2) head width slightly larger than head length; (3) maxillary gland pronounced; (4) tympanum diameter large, slightly larger than the eye diameter; (5) loreal region oblique, concave; (6) nostril located dorsally, between the eye and the tip of snout; (7) vomerine teeth two small masses; (8) tongue long pyriform, free-margin notched; (9) fingers with rudimentary webs; toes almost full webs, webbing formula I 0^- – ⅓ II 0^- -⅓ III ⅓ – I^+ IV I^+ – 0^- V; (10) heels not meeting.

Common names. “Hubei Gold-striped Pond Frog” in English / “湖北侧褶蛙 (hú běi cè zhě wā)” in Chinese.

Comparison. *Pelophylax hubeiensis* can be differentiated from all species in the *Pelophylax* genus, based on the silent sac in males (Fei et al. 2009).

Colouration. The living specimens exhibit green, olive green or light brown body colour variation, with some individuals exhibiting green flecks on their backs that were absent on other individuals, a tympanic membrane that was golden yellow or light brown and yellow eyelids. The dorsolateral fold ranged from yellow to light brown or golden yellow. The legs exhibit yellow, brown or light brown colour variation, with green or olive green markings. The throat, chest and belly were light yellow. The backs of the elbows were dark brown, with brown cloud spots behind the thighs. The ventral surface of the forelimbs and hind limbs were yellow. The nuptial pads were grey (Fig. 4C, D).

In preservative, the dorsal surface turned dark olive green with light black patches, while the dorsolateral fold and backline were brownish-yellow, the limbs were light brown with dark brown patches, the ventral surface was beige with black stripe patterns, the ventral surface of the limb was light yellow and the hand and toe webs were beige (Fig. 6).

Sexual dimorphism. Males with a single, grey nuptial pad on the dorsal surface of finger I in the breeding season. Males slightly smaller than females with linea masculina.

Distribution and ecology. At present, specimens of *Pelophylax hubeiensis* have been identified in Henan, Hubei, Anhui, Hunan, Chongqing and Jiangxi, China (Fei et al. 2009). Specimens were collected from ponds with lotus flowers or aquatic plants (Fig. 7C, D), where they perch on the leaves of the aquatic plants during the night-time, although, when startled, they plunge underwater.

Discussion

Given the fact that the *Pelophylax plancyi* species group (*P. plancyi*, *P. fukienensis*, *P. hubeiensis* and *P. chosenicus*) is widely distributed in eastern China and the Korean Peninsula, there are high levels of interspecific morphological and molecular similarity that make the division of species more challenging (Fei et al. 2009). In this study, following the integrative concepts of “morphospecies” and “phylospecies” (Zhou et al. 2022), both molecular and morphological data suggest that *P. plancyi* and *P. chosenicus* are the same species, but *P. hubeiensis* is a distinct species. Based on the priority of names designated by the International Code of Zoological Nomenclature (ICZN), *P. chosenicus* should be considered a junior synonym of *P. plancyi*.

Some researchers have found introgression amongst species within the genus *Pelophylax*, especially between *P. plancyi* and *P. nigromaculatus* (Zhang et al. 2008; Komaki et al. 2015). Therefore, mitochondrial introgression may render such evidence inconclusive, but comprehensive analysis of other aspects of evidence can supplement this deficiency. Although the sequencing of *Pelophyllax* nuclear genes was very difficult, some researchers had overcome these difficulties to obtain nuclear genes sequences. The nuclear analyses of E-Asian *Pelophylax* evidence have shown the homogeneity of *P. plancyi* and *P. chosenicus* (Komaki et al. 2015), which was consistent with the viewpoint of this article. Although there was introgression between *P. plancyi* and *P. hubeiensis*, the genetic distance between them was still far enough to support their separation into two species. The above viewpoints were supported by morphological data. The morphological differences between females and males are greater than those between species.

The acquisition of nuclear genes sequencing in *Pelophylax* genus will provide favourable evidence for further verification of species relationships and researchers look forward to breaking this barrier as soon as possible. We will also continue to search for new evidence (such as ecological characteristics, behavioural characteristics and developmental characteristics) as a supplement to molecular and morphological data.

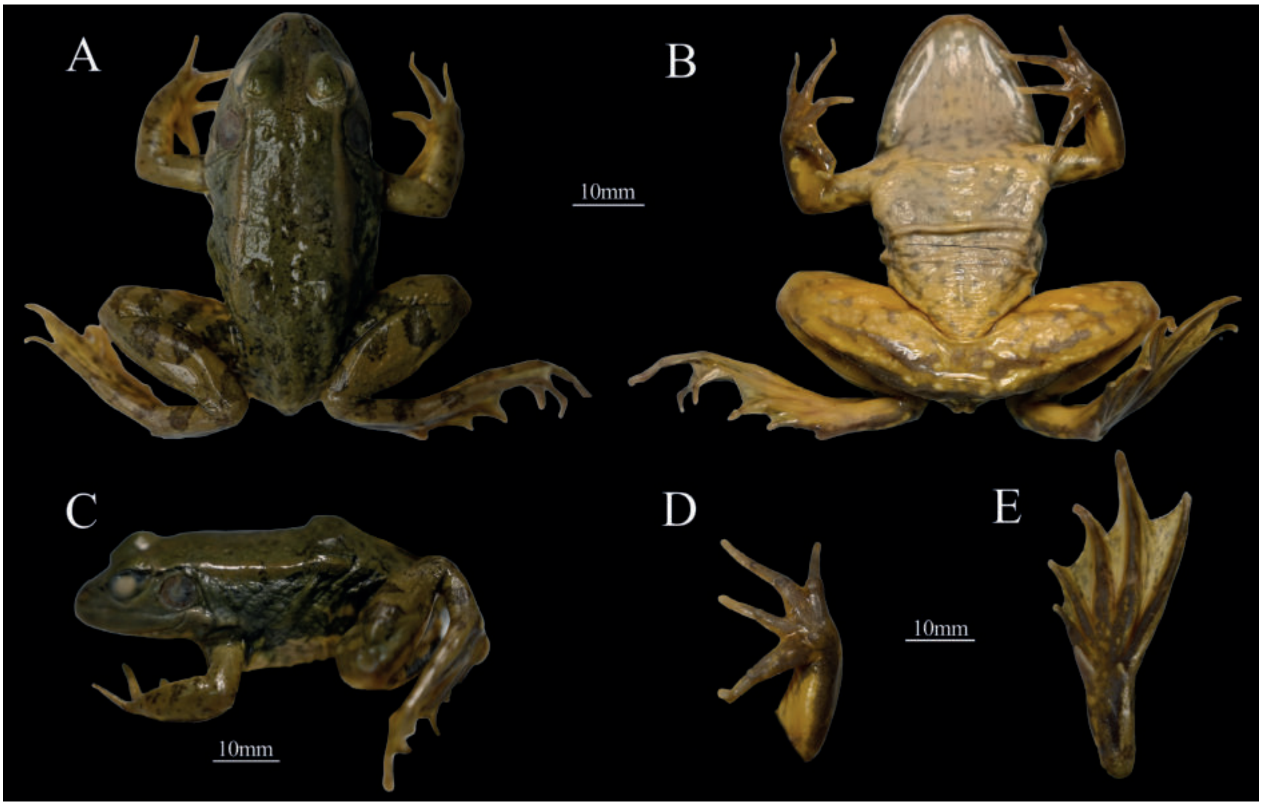

Figure 6. Specimen (SYAU BAA000053) of *Pelophylax hubeiensis*. **A.** Dorsal view; **B.** Ventral view; **C.** Lateral view; **D.** Ventral view of the hand; **E.** Ventral view of the foot.

Figure 7. The habitat of *Pelophylax plancyi* (**A**) macrohabitats, (**B**) microhabitats and *P. hubeiensis* (**C, D**).

The shortcomings of taxonomy also exist in the species of *Pelophylax* genus. Given the fact that *P. plancyi* and *P. hubeiensis* are sympatric species in eastern-central China (Zhao et al. 2009), further studies and molecular data are needed to determine whether there is introgression or if they produce filial generations between the two species. The direction and timing of mitochondrial gene infiltration had not been elucidated in existing studies and research on this issue is not yet sufficiently in-depth. More species need to be applied for detailed elucidation.

Acknowledgements

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Selection of substrate type, substrate color, and vegetation by tadpoles of *Dryophytes plicatus*

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Abstract

The characteristics of microhabitats in streams can drive the distribution of tadpoles. We experimentally examined microhabitat selection of tadpoles of *Dryophytes plicatus*. We used a series of choice experiments to test if tadpoles had preferences for particular substrate types, substrate colors, and vegetation. Tadpoles of *D. plicatus* had a strong preference for mud substrates over sand, gravel, and rock substrates and preferred darker substrates over lighter substrates. *Dryophytes plicatus* tadpoles used the non-vegetated side of an aquarium more than the vegetated side. Our experimental results matched previous field observations in the case of the preference for mud substrates but differed from the field observations for substrate color and vegetation, suggesting that basic underlying preferences may be modified by various factors in nature or by learning or conditioning.

Key Words

Arroyo los Axolotes, behavior, Mexico, microhabitat selection, stream, tadpoles

Introduction

In order to assess the consequences of potential degradation of stream habitats (Carpenter et al. 2011; Piñon-Flores et al. 2021) on aquatic species, as well as to develop appropriate habitat management plans for such habitats, greater understanding of how species use or select habitats or microhabitats in streams is needed. The distributions of tadpoles may be a function of a variety of microhabitat characteristics ranging from substrate type to vegetation or habitat structure (Hoff et al. 1999), as well as the result of oviposition site choice by adults (Buxton and Sperry 2017). Experimental investigations in the laboratory may help determine what specific attributes of the environment drive tadpole distributions in nature. For example, some tadpole species prefer vegetated microhabitats or habitats with structure over non-vegetated or simple habitats in the laboratory (e.g., Smith and Doupnik 2005; Chuang et al. 2019), whereas others show a preference for non-vegetated habitats, at least during some stages of development (Smith 1999). Other experimental studies have found tadpoles prefer specific types of substrate, such as rock or gravel (Odendaal et al. 1982; Smith 1999) or have no preferences (Smith and Doupnik 2005).

Several studies have examined a selection of substrates based on color in tadpoles. Some tadpoles show no preference among differently colored substrates (Bishop et al. 2012; Espanha et al. 2016; Eterovick et al. 2018; Melo et al. 2021; Rodríguez-Rodríguez et al. 2021). However, other species of tadpoles show a preference for particularly colored substrates. Some prefer white or light substrates over black or dark substrates (Guimarães et al. 2021; Melo et al. 2021), whereas others prefer black or dark substrates over white or light substrates (Ximenez et al. 2012; Eterovick et al. 2018). The selection of a color

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of substrate often increases crypsis, especially in the presence of predators or predator cues (Eterovick et al. 2010, 2018, 2020); although some tadpoles select backgrounds that create high contrast between the tadpole and its background (Guimarães et al. 2021). It appears color preference of tadpoles, at least in some species, may be innate (Hunt et al. 2020).

Dryophytes plicatus (formerly *Hyla plicata*; Duellman et al. 2016) is an endemic frog found in the mountains of central Mexico (Wilson and Johnson 2010). The Mexican government lists *D. plicatus* as Threatened (SEMARNAT 2019). Adult *D. plicatus* occur more often in sites with longer hydroperiods, and the tadpoles are found more often in sites with less livestock, slower water, and that were wider (Gómez Franco et al. 2023). *Dryophytes plicatus* can be scavengers (Villarreal Hernández et al. 2019). Predators of *D. plicatus* tadpoles include Giant Water Bugs (*Belostoma* spp.) (Villarreal Hernández et al. 2019). *Dryophytes plicatus* co-exist with the snake predator *Thamnophis scaliger* (Villarreal-Hernández et al. 2019); but are excluded from sections of streams where non-native Rainbow Trout (*Oncorhynchus mykiss*) are found (Estrella-Zamora et al. 2018).

We examined microhabitat selection of *D. plicatus* tadpoles using a series of laboratory choice experiments to determine if they prefer specific types of substrates (mud, sand, gravel, and rock), substrate color (dark brown, brown, light brown, and gray), and the presence and absence of vegetation. These experiments were informed by previous field observations of habitat and microhabitat use by *D. plicatus* tadpoles in nature. Visual surveys supplemented by physical probing of substrates (i.e., with a snake hook) indicated that *D. plicatus* tadpoles are often found in sites with mud (Lemos-Espinal et al. 2016; Estrella Zamora et al. 2018). *Dryophytes plicatus* are also found more often in sites with white-yellow and tan-brown substrates rather than those with black substrates (Lemos-Espinal et al. 2016), and use sites with and without vegetation at the same rate (Lemos-Espinal et al. 2016; Estrella Zamora et al. 2018). Based on these field observations, we predicted that *D. plicatus* tadpoles would prefer mud substrates over the other substrates, prefer lighter substrates (e.g., light brown and gray), and show no preference between vegetated and non-vegetated habitats in our laboratory experiments.

Methods

We collected 135 tadpoles of *D. plicatus* from the Arroyo del Axolotes, mpio. Isidro Fabela, Mexico using a dipnet from December 2021 through November 2022. We transported tadpoles to a nearby facility for the experiments. We obtained the tadpoles from a variety of microhabitats; including pools, stream edges, shallows, and at the base of vegetation; along a 1 km stretch of the Arroyo los Axolotes. We pooled individuals into the wet (June to October; N = 100) and dry seasons (November to May; N = 35). Water temperatures are warmer and dissolved oxygen levels higher in the wet season than the dry season (Villarreal Hernández et al. 2020a). We have observed predators, such as *Thamnophis scaliger*, in both the wet and dry seasons along the Arroyo los Axolotes (J.A. Lemos-Espinal pers. observ.). In addition, water depth is greater during the wet season compared to the dry season (Gómez Franco et al. 2022). All tadpoles used in the experiments were premetamorphic and had no obvious evidence of limb buds (i.e., Gosner stage 25–26; Gosner 1960), and were all < 0.8 cm in body length. All tadpoles in our experiment were jet black in color; however, Kaplan and Ramírez-Bautista (1996) described the color of *D. plicatus* tadpoles as dark olive but indicated that some populations are “almost black”.

We conducted three choice experiments: substrate type, substrate color, and vegetation. Experiments were begun at around 1400 h, approximately one-four hours after capture. Prior to the experiments tadpoles were temporarily housed in plastic containers. For each experiment, we established multiple test arenas using 36 L glass aquaria (40 cm length × 30 cm width × 30 cm height) with each aquarium divided into sections as described below. We used water from the Arroyo los Axolotes to fill the aquaria. Experiments were run at a water temperature of between 7.3 °C and 8.1 °C; which was similar to water temperatures in the stream. At the start of each trial we placed tadpoles in the center of the aquarium and allowed to acclimate for 5 minutes prior to data collection. We recorded the location of the tadpole every minute for 15 minutes. To minimize the number of tadpoles used in the experiments, we ran each tadpole through all three experiments in the same order for all tadpoles (substrate color, substrate type, and vegetation), with 5–10 minutes between experiments.

For the substrate type experiment, we created four sections on the bottom of the aquaria: mud, sand, gravel, and solid rock, using material collected from the stream. The mud substrate was dark brown in color, the sand brown, the gravel gray, and the solid rock light brown or light gray. We used small rectangular plastic trays to contain the various substrate types and keep them separate. Since our goal was to assess potential preferences for substrate types we used actual natural substrates from the Arroyo Los Axolotes without attempting to control for their color or other aspects, such as the availability of food. For the substrate color experiment, we placed four equal-sized rectangular pieces of colored paper under the clear bottom. We chose colors to approximate the four most common colors of substrates in the Arroyo Los Axolotes using the COMEX color palette (brown color family): dark-brown, brown, light-brown, and gray (Villarreal Hernández et al. 2020a, b). For the vegetation experiment, we divided each aquarium in two, with half containing artificial vegetation to simulate the vegetation in their habitat, and half without vegetation and contained no shelter.

For each tadpole we determined which substrate type or color they used the most. In the case of a tie, we excluded those individuals from the analyses (N = 0 excluded for substrate type; N = 2 excluded for substrate color; N = 0 excluded for vegetation). We used chi-square tests

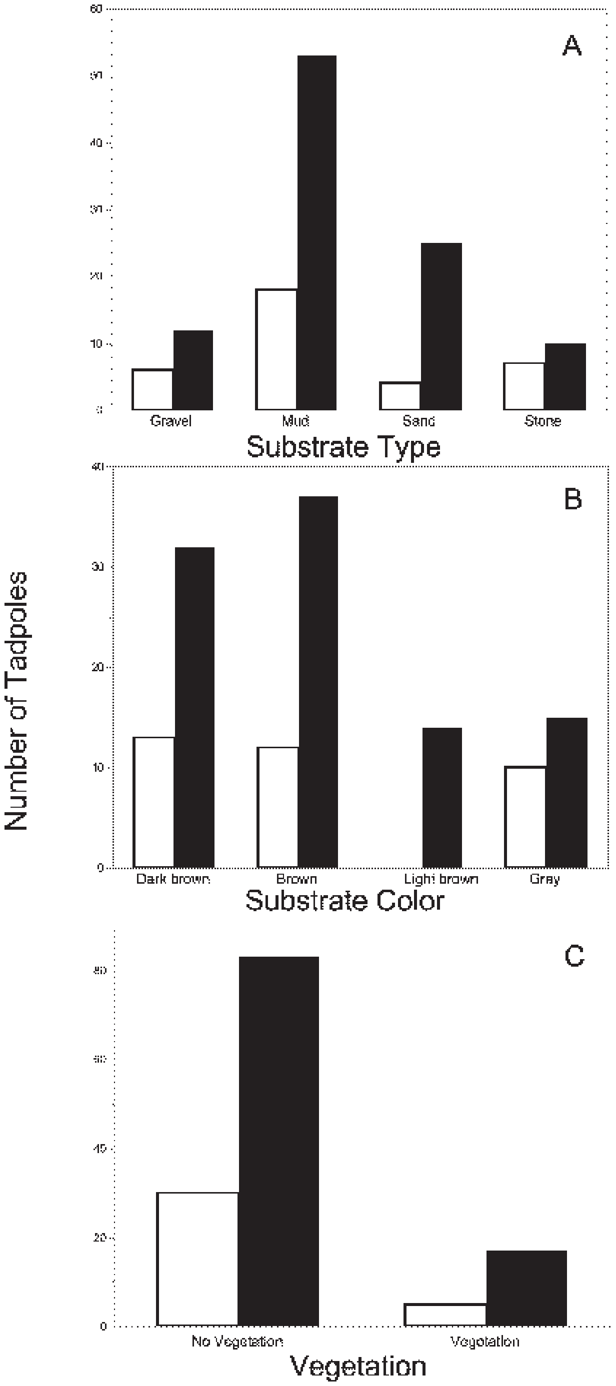

Figure 1. The number of individual *Dryophytes plicatus* tadpoles from the Arroyo los Axolotes that used each **A.** Substrate type; **B.** substrate color; **C.** vegetated or non-vegetated habitat the most in laboratory preference experiments in the dry (white bars) and wet (black bars) seasons

to determine if the numbers of individuals using each substrate type or color the most were different from that expected if the individuals chose substrate types or colors at random (i.e., 25% for each substrate type or color) or vegetated and non-vegetated sides at random (i.e., 50% on each side). We also used chi-square tests (or Fisher's exact test if expected values were < 5 in > 20% of cells) to determine if substrate type and color preferences differed between the wet and dry seasons.

Results

Tadpoles overwhelmingly chose mud substrates over the other substrates (Fig. 1A; $\chi^2_3 = 57.44$, $P < 0.0001$). The substrate type chosen by tadpoles that used a particular substrate type > 50% of the time did not differ between the wet and dry seasons (Fig. 1A; Fisher's exact test: $P = 0.17$).

Tadpoles chose dark brown and brown more than light brown and gray (Fig. 1B; $\chi^2_3 = 24.80$, $P < 0.0001$). There was a nearly significant tendency for the color chosen by tadpoles to differ between the wet and dry seasons, with light brown only used in the wet season (Fig. 1B; $\chi^2_3 = 7.65$, $P = 0.054$).

Tadpoles used the non-vegetated side of the aquarium much more than the vegetated side (Fig. 1C; $\chi^2_1 = 61.3$, $P < 0.0001$). The side of the arena, vegetated or non-vegetated, used by the tadpoles showing a choice of side did not differ between the wet and dry seasons (Fig. 1C; $\chi^2_1 = 0.14$, $P = 0.71$).

Discussion

Our experimental results suggest that tadpoles of *D. plicatus* had a strong preference for mud substrates over sand, gravel, and rock substrates. Our experimental results are similar to those from previous field studies that found mud to be the most used substrate for *D. plicatus* in the Arroyo Los Axolotes (Lemos-Espinal et al. 2016; Estrella Zamora et al. 2018). It is unclear why *D. plicatus* tadpoles prefer mud substrates over other substrates. Possible explanations include the presence of food or that mud correlates with the presence of other resources or conditions. For example, mud may be more present in slower water, a condition correlated with more *D. plicatus* tadpoles (Gómez Franco et al. 2023). Given the preference for mud substrates in both our experiment and our previous field observations (Lemos-Espinal et al. 2016; Estrella Zamora et al. 2018), further experiments or field studies designed to specifically address why mud substrates are preferred would be fruitful.

Tadpoles of *D. plicatus* preferred dark brown and brown substrates over light brown or gray substrates (i.e., they preferred the darker substrates), which is what we might expect given that the tadpoles of *D. plicatus* used in our experiment were jet black. For example, tadpoles of some species have been shown to select substrates that allow them to be more cryptic when disturbed (Eterovick et al. 2010) or in the presence of predator cues (Eterovick et al. 2020). In addition, given the strong preference for mud substrates, the choice of dark brown and brown substrates may reflect a selection for colors that typically match mud, which in the Arroyo los Axolotes is typically dark brown. Alternatively, tadpoles may select mud for its color rather than other characteristics. However, in the Arroyo los Axolotes, *D. plicatus* tadpoles were found more often in sites with lighter substrates than in sites with darker substrates (Lemos-Espinal et al. 2016) which is odd given the very dark color of the tadpoles of *D. plicatus*. Further experimentation is needed to reconcile the results of our experiment and the observations in natural streams. For example, experiments examining substrate color selection by *D. plicatus* tadpoles in the presence and absence of predators or predator cues or an experiment comparing preferences for mud, or other substrates, of different colors but similar in all other aspects would be useful.

In our experiments, *D. plicatus* tadpoles showed a strong preference for using the non-vegetated side of the aquarium over the vegetated side of the aquarium. In nature, *D. plicatus* tadpoles used sites with different types of vegetation, including no vegetation, in the same frequency as their availability along the Arroyo los Axolotes (Lemos-Espinal et al. 2016; see also Estrella Zamora et al. 2018). As with our substrate color results, it may be that the differences between our experimental vegetation selection results and the observations in nature may reflect the additional factors that are present in nature, such as predators or food. For example, some tadpoles increase the time spent hiding in simulated vegetation in the presence of predator cues (Gunzburger 2005; Gregoire and Gunzburger 2008). In addition, the avoidance of vegetation we observed in our experiment may be due to our use of artificial vegetation rather than natural vegetation. However, several experiments examining the behavior of other species of tadpoles that have used artificial vegetation have found no apparent avoidance of or alteration of behavior by using artificial vegetation (e.g., Smith 1999; Smith and Doupnik 2005; Smith et al. 2008a, b; Smith and Awan 2009; Davis et al. 2012), suggesting this is unlikely.

Conclusions

The series of experiments we conducted to examine the elements of microhabitat use in tadpoles of *D. plicatus* suggest that these tadpoles may have preferences for some characteristics of their environment (e.g., mud and darker substrates), but avoid others (e.g., vegetation). In addition, the difference between some of the results we obtained in our experiments for substrate color and vegetation and those from field studies emphasize that basic underlying preferences may be modified by various factors in nature or by learning or conditioning (see Wiens 1970, 1972; Dunlap and Satterfield 1985; Moriya et al. 1996). However, the consistency of a strong preference for mud substrates in our laboratory experiments and in our previous field observations (Lemos-Espinal et al. 2016; Estrella Zamora et al. 2018) emphasizes the potential importance of ensuring that sufficient mud substrates are present when *D. plicatus* tadpoles are found in the streams. Thus, any habitat degradation that may affect the availability of mud substrates with standing water at the appropriate time of year should be avoided or remediated (e.g., reduced water flow, increased scouring events, channelization using artificial substrates). It is our hope that the discrepancies (and similarities) in these sets of results will prompt further investigations into what those factors are.

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The current distribution and status of the Hermann's tortoise, *Testudo hermanni boettgeri* (Reptilia, Testudines, Testudinidae) in Croatia

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Abstract

Hermann's tortoise (*Testudo hermanni*) is listed as "Near threatened" in the IUCN Red list of endangered species. The importance of protecting the Hermann's tortoise populations and its habitats have led to the inclusion of the species within CITES Convention (Annex II), Annex A of EU Wildlife Trade Regulation, Annex II of the Bern Convention and Annexes II and IV of the EU Habitats Directive. To assess the distribution and status of the eastern Hermann's tortoise (*Testudo hermanni boettgeri*) in Croatia, historical and recent records were gathered and analyzed. The species was recorded in all three biogeographical regions in the country, but it's native to the Mediterranean and a small part of the Alpine region. With the increase of recent surveys and the use of citizen science platforms, the known range of the species in Croatia was increased by 35.8% and is now encompassing 123 10 × 10 km EEA reference grid cells. Most records (66%) originate from lower elevations (up to 199 m), and the highest was recorded at 570 m. Sparse forests are the most preferred habitats, followed by semi-open habitats, such as grasslands and shrubs. The most serious threat to the species is natural succession due to the increased abandonment of traditional farming and grazing. Other threats include touristic infrastructure and urban development, transportation, illegal collecting, and invasive species. The Area of Occupancy calculated using 2 × 2 km grids resulted in an AOO of 1,372.00 km^2, while Extent of Occurrence (EOO) is calculated to be 18,145.07 km^2. The current network of National protected areas includes 14% of the species' AOO while the designated Natura 2000 areas include 29.30% of its AOO. We propose to designate an additional 10 Natura 2000 areas to help with the long-term protection of the species.

Key Words

Area of Occupancy, Extent of Occurrence, habitat preferences, island populations, protected areas, range, threats

Introduction

Hermann's tortoise, *Testudo hermanni* is a western Palearctic tortoise species, distributed in southeastern Europe (Cheylan 2001). Two subspecies are recognized, *T. hermanni hermanni* Gmelin, 1789 distributed in the west, and *T. hermanni boettgeri* Mojsisovits, 1889 in the east Europe (Wermuth 1952; Cheylan 2001; Fritz et al. 2006). *T. hermanni boettgeri* has a continuous distribution in the Balkan Peninsula (Cheylan 2001; Fritz and Havaš 2007) including Croatia, Bosnia and Herzegovina, Montenegro, Serbia, Kosovo, North Macedonia, Albania, Romania, Bulgaria, Greece (Bour 1997) and Turkish Thrace (Türkozan et al. 2019). The main range of the species is in areas with a Mediterranean climate and humid continental climate. It inhabits a variety of habitats, from meadows, dry rocky pastures, macchia, forest edges, forest, thickets, and Mediterranean shrubs to rural and agricultural landscapes (Rozylowicz and Dobre 2010; Del Vecchio et al. 2011; Stojadinović et al. 2017; Nikolić et al. 2020).

The species is listed as "Near threatened" according to the IUCN Red list of endangered species (van Dijk et al.

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2004), mostly because of ongoing habitat modification and destruction, the international pet trade, and over-collecting (Cheylan 1984; Lambert 1984; Stubbs and Swingland 1985; Guyot and Clobert 1997; Gibbons et al. 2000; Mazzotti 2004; Stanford et al. 2020). The species is included in CITES Appendix II and Annex A of EU Wildlife Trade Regulation 338/97 as early as 1975 and is strictly protected under the Bern Convention and the EU Habitats Directive (92/43 EEC, Annex II, IV) (Epstein 2014). In Croatia, it is strictly protected under the Law on Nature Protection (NN 80/13, 15/18, 14/19, 127/19) and its status in the country should be monitored and reported to the European Union according to the Article 17 of the Habitats Directive.

In Croatia, the species inhabits a narrow coastal strip along the Adriatic coast, from Istria peninsula in the north to Dubrovnik city in the south, including some of the Adriatic islands (Cheylan 2001; Ljubisavljević et al; 2014; Jelić et al. 2015). Some records from Croatia are of non-native individuals. This species has a long tradition of being collected for the pet trade and transported. Accordingly, some individuals have escaped or have been released on purpose in the continental part of Croatia as well as on some Adriatic islands (Ljubisavljević et al. 2014; Jelić et al. 2015). Occurrence data in Croatia was provided by many authors but is mostly incomplete and fragmentary: Germar 1817; Kolombatović 1882, 1886; Depoli 1898; Werner 1902, 1908; Rössler 1904; Radovanović 1951; Pavletić 1964; Bruno and Maugeri 1977; Bruno 1980; Mršić 1987; Veith 1991; Schmidtler 1999; Sehnal and Schuster 1999; Cheylan 2001; Rathbauer 2002; Fritz et al. 2006; Schweiger 2006; Tóth et al. 2006; Grbac 2009; Bertolero et al. 2011; Koren 2012; Lauš 2012; Žagar et al. 2013; Ljubisavljević et al. 2014). Although the broad distribution of the species is known (Lončar 2005; Ljubisavljević et al. 2014; Jelić et al. 2015; Zadravec and Gambiroža 2019), the detailed distribution taking into considering extensive data from multiple unpublished sources has not been published so far. Moreover, a general lack of knowledge of population size and density, demographic structure, habitat preferences, species protection, and main threats make it difficult to carry out better science-based conservation management in Croatia.

The aim of this paper is to accumulate data to aid in the reevaluation of the conservation status based on IUCN criteria, including (1) range at a small scale (10 × 10 km), (2) Area of occupancy and Extent of occurrence, (3) habitat preferences, (4) coverage through the existing Natura 2000 network and nationally designated protected areas (5) national threat status.

Materials and methods

Study area

Croatia is located at the western part of the Balkan Peninsula and covers an area of 56.594 km², including a number of islands. There are three biogeographical regions in the territory of Croatia: Continental, Alpine, and Mediterranean (European Environment Agency 2017). The Mediterranean region covers coastal areas and islands in the Adriatic Sea (Ostrogović Sever et al. 2021) and can be divided into three areas: Istria peninsula, Kvarner, and Dalmatia (Cvitanović 1976).

Distribution analysis

To assess the occurrence of *T. h. boettgeri* in Croatia, records until 2021 were gathered from literature, open-access databases: Biologer.hr (Živanović et al. 2018), GBIF.org, Observation.org, Balcanica.info (Balej and Jablonski 2006–2021), authors' and colleagues unpublished data. For the usage of open access databases, we followed the terms and conditions of the websites and, where necessary, asked the authors for permission. The occurrence data were preprocessed before any further analyses. In the first step, we removed literature records containing wider localities, e.g., "Dalmatia" or "Brač island" and duplicate observations.

Records were classified into two groups: native populations and introduced individuals. Areas within the main distribution range as well as islands and parts of the marginal distribution range (e.g., north-western edge of distribution), containing at least three valid literature and/or recent records were considered as belonging to the native populations. Sporadic reports of animals found in or near urban areas or gardens on the Adriatic islands or other localities outside the main distribution range of the species were considered to be introduced. In addition, every data was assigned to be from the "mainland" or an "island".

All the processed data were then used to create a distribution map of the species in Croatia on the 10 × 10 km EEA reference grid squares in ArcMap (v. 10.7.1, ESRI) according to the standard of the European Environment Agency (DG Environment 2017), denoting introduced and native observations. We used 10 × 10 km squares labeled in order to be consistent with previous publications. To compare the previous and our research, we count the number of 10 × 10 km EEA reference grid squares. Within the distributional data, special attention was focused on island populations, to tentatively define which island populations are native and which are introduced.

The records of native populations were further divided according to coordinate accuracy into three groups: high accuracy (exact locality), medium accuracy (within 200 m), and low accuracy (more than 200 m). Only the records with high and medium accuracy were used for the analyses of the altitude, habitat selection, coverage of the protected areas, the major threats, and estimation of the national threatened status. The elevation data were obtained by using a Digital elevation model in ArcMap (v. 10.7.1, ESRI).

Spearman's rank correlation was used to test if there is a significant correlation between the number of records of *T. h. boettgeri* and altitude using cor.test function from base R (R Core Team 2020). The visualization was made with ggscatter function from ggpubr package (Kassambara 2020).

The extent of Occurrence and Area of Occupancy according to the Red List Criteria

To estimate the spatial distribution of the species, the specie's geographical range is presented in two ways, (i) Extent of Occurrence (EOO) and (ii) Area of Occupancy (AOO) according to the IUCN Red List Categories and Criteria assessments. EOO represents the area contained within the shortest continuous imaginary boundary of the present occurrence of a taxon (IUCN 2012). As eastern Hermann's tortoise is terrestrial species, we additionally removed the marine area and estimated the 'true' EOO based just on the land territory. EOO was measured by a minimum convex polygon in which no internal angle exceeds 180 degrees and which encompasses all the current known localities (IUCN 2012). Once the localities were downloaded into the geographic information system (GIS) domain, EOO was determined by joining the distant points in all directions and then calculating the area of the resulting polygon. AOO represents the area of suitable habitat currently occupied by the taxon within its Extent of Occurrence. We estimated AOO using the 2 × 2 km grid cells, as recommended by the IUCN (2012). EOO and AOO where calculated only for the presumably native populations.

Habitat preferences assessment

This assessment was based on the high and medium accuracy data without segregation based on the seasonal habitat use, or sex/age-specific habitat requirements. Using spatial selection, we have combined points of records and maps of natural and seminatural non-forest and freshwater habitats of Croatia (Bardi et al. 2016) in the ArcGIS program (v. 10.7.1 ESRI), and for each record extracted one of the possible habitat types following the National Habitat Classification (NHC): (A) inland surface waters and wetland habitats, (B) inland non and poorly covered land surface, (C) grassland habitats, (D) shrub, (E) forest habitats, (I) cultivated non-forested land and habitats with weeds and ruderal vegetation, (J) constructed and industrial habitats. Accordingly, we calculated the percentage of records falling within each habitat type.

Threats on a national scale

For each assessed threat, its impact on the conservation status of the species at the national level was ranked by coding the threat as high, medium, or low according to IUCN 3.2. Criteria (IUCN 2012). The threat impact is generally regarded as high if it directly affects the long-term viability of the species and/or its habitat causing a significant decline at the biogeographic scale. Medium impact mainly includes threats acting only regionally causing significant declines. Low impact least affects the conservation status of the species but still potentially causes population decline.

Three independent threats are known to influence the survival of tortoise populations across their range (Stanford et al. 2020):

1. Habitat loss and fragmentation.

This threat is based on three subcategories: i) Agricultural activity – which was assessed using data from the Croatian Bureau of Statistics and Environmental Protection Agency; ii) Tourist infrastructure and urban development – conclusions have been mainly reached from literature and by observations during the period 1997–2021; iii) Transportation – conclusions have been mainly reached by authors unsystematic observation of dead animals in a car traveling at low speed (40 km/h) during the research in the period 1997–2021.

2. Hunting and collecting of the species.

All the available CITES data sets were used (http://trade.cites.org/en/cites_trade/) from which all the declared records of legal import/export available for *T. hermanni* between 1975 and 2021 were extracted. In our study, the records of legally exported individuals are incomplete because many of the individuals were traded internationally before their inclusion into CITES. Also, there are no records of tortoises in the CITES database from the period 1990–1995 for Croatia, probably due to the Croatian War of Independence (1991–1995). For illegal actions, e.g. sales and export attempts, State Inspectorate, Sector for Environmental Protection Supervision, Nature Protection, and Water Law Supervision was contacted but no data set was available. Accordingly, the media articles and stories that contained reports of the seizures of the species from 2000 to 2021 were reviewed. The resulting dataset was analyzed by the number of traded individuals regulated and not regulated under CITES. The legal exports were additionally categorized by country and purpose of trade (P-personal; T-commercial; E-educational) per year.

3. Invasive species.

This threat was derived from literature sources and limited observations by the authors.

Protected areas important for the species conservation

To assess the coverage and importance of existing protected areas for the long-term survival of the target species, two types of protected areas were used: nationally designated protected areas (NPA's) and Natura 2000 network. NPA's included protected areas designated under national legislation for Nature Protection of the Republic of Croatia and comprised six categories: National Park, Nature Park, Significant landscape, Special reserve, Horticultural monument, and Park Forest. The Natura 2000 network includes sites designated under the Habitats Directive (Sites of Community Importance and Special Areas of Conservation-SCI).

To calculate the total surface of areas under current legal protection where the species is present, we initially wanted to use the national habitat map of the Republic of Croatia to determine the surface area of suitable habitats occupied by the species within the protected areas. However, it is not accurate enough for this type of analysis, since many habitats where the species was found are incorrectly classified (e.g., grasslands are classified as forests) due to low precision of habitat polygons borders and the results were not satisfactory. Accordingly, we used AOO, which is based on actual records and therefore shows much more accurately where the species is present. Hence, for this analysis, 2 × 2 km squares calculated for the Area of Occupancy analysis were used and overlaid with both types of protected areas (available from BIOPORTAL, https://www.bioportal.hr/gis/). Only the protected areas that overlap with AOO were used for consequent analysis. From the overlap, the surface (km^2) was calculated for AOO and NPA's, as well as for AOO and Natura 2000. In the end, a joined analysis was performed for AOO and the joined protected areas with the exclusion of the overlapping areas between NPA's and the Natura 2000 network.

In Croatia, *T. h. boettgeri* is a conservation priority species in 15 Natura 2000 sites (Anonymous 2019). To assess if the designated Natura 2000 areas comply with the minimum required 20–60% coverage of the species range in the country the additional analysis was performed for these 15 Natura 2000 sites by overlapping with AOO (Anonymous 2014).

Results

Distribution

In total, 980 individual records of *T. h. boettgeri* during the period 1853–2021 were found. Concerning the origin of the records, 269 are literature data and 711 are new and previously unpublished records of the species in the country (Suppl. material 1). Most records, 926 (94.3%) were attributed to native population. Within this survey, the species is for the first time reported from the Alpine biogeographical region with six records.

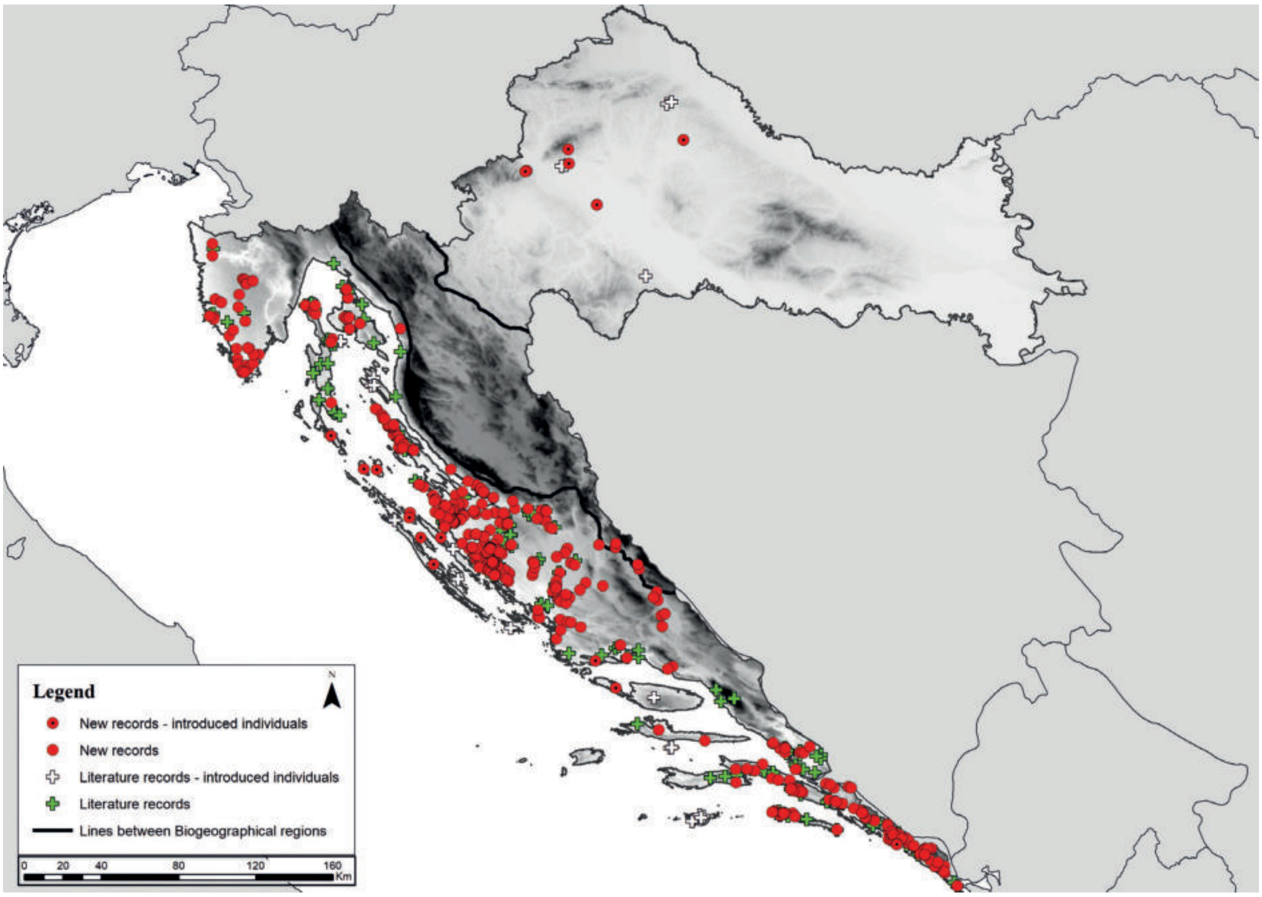

Figure 1. The current distribution of *T. h. boettgeri* in Croatia. Solid red circles without dots represent new, unpublished data ascribed to the native populations, whereas red circles with a dot represent unpublished data ascribed to anthropogenic introductions. Green crosses represent literature data ascribed to the native populations, whereas white crosses represent literature data ascribed to anthropogenic introductions.

Out of 926 records relating to the native populations, 820 of them had high or medium coordinate accuracy, while 106 were imprecisely georeferenced records that were excluded from additional analyses. Out of the records of the native populations with high and medium accuracy, most belong to Dalmatia, 727 records (88.7%), followed by Kvarner, 57 records (7%), and Istria, 36 records (4.3%).

In total, the native populations of the species were recorded within 123 10 × 10 km EEA reference grid squares (Fig. 3), of which it was for the first time recorded in 44 of them, which is an increase of 35.8%.

According to the records, the species is present in all three biogeographical regions in Croatia: the Continental, Mediterranean and Alpine (Fig. 1) but native populations are present only in the latter two. Within the Mediterranean biogeographical region, *T. h. boettgeri* is present both on the mainland, from the Istria peninsula in the north, along the Adriatic cost to Dubrovnik city in the south, and at 27 islands, eight of which are inhabited by presumably native populations: Krk, Cres, Pag, Vir, Zlarin, Hvar, Korčula, and Mljet (Suppl. material 2). The presence of the species on islands was ascertained with 28–51 records for the islands Krk, Cres, Pag, and Mljet, and with 5–9 records for islands Vir, Hvar, and Korčula. For island Zlarin, three valid records are confirmed in 2019 (Suppl. material 2). In the Alpine region, species presence was confirmed through six records in the borderline area with Mediterranean region, in the vicinity of Knin. The northernmost and southernmost localities where the species is recorded in Croatia were, respectively, Buje (Istria peninsula), and Vitaljina (Konavle, Dalmatia).

The introduced individuals were recorded in Continental and Mediterranean biogeographical regions. Altogether, 14 records from eight localities are currently known from the Continental region: Zagreb with surroundings, Samobor (this study), Velika Gorica (this study), Turopolje (this study), Kalnik, Bjelovar (this study), Grbavac (this study) and Majur. In the Mediterranean biogeographical region, the introduced individuals are present on 19 islands with 41 records (Suppl. material 2). Three islands belong to the Kvarner area, while the rest are situated in the Dalmatian area.

In terms of altitudinal range, most records (66%) originate from lower elevations (between 0 m and 99 m), and 20% of records originated from 100–199 m. Above 200 m there were only 115 (14%) records. The locations with the highest altitude where the species was recorded are Vrataruša, Mt. Velebit: 570 m in Kvarner area and Polača, Mt. Dinara: 517 m in the Dalmatia area. Strong, significant correlation was found between the number of records of *T. h. boettgeri* and altitude (Spearman correlation test, $p < 0.05$, $R = -0.67$) (Fig. 2).

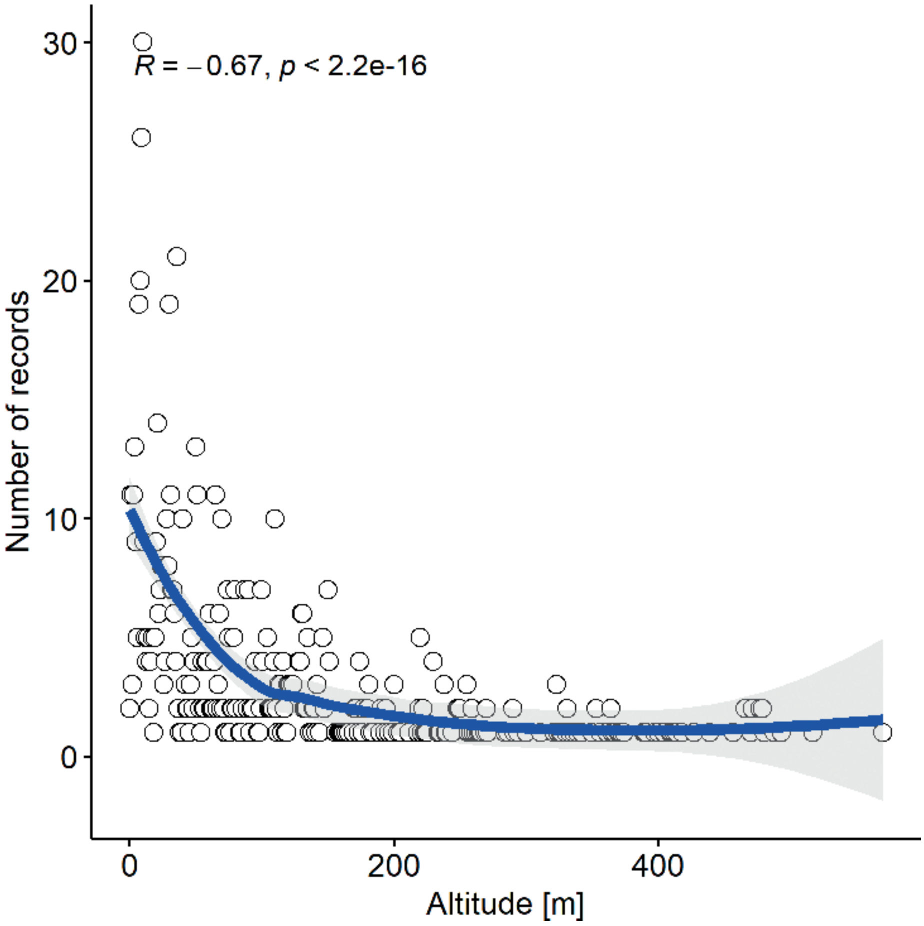

Figure 2. Relationship between the number of records of the species and the altitude (m).

The extent of Occurrence and Area of Occupancy according to the Red List Criteria

To get more precise information, the marine area was excluded and the corrected EOO was calculated to 18,145.07 km^2 (Fig. 3). Still, this is a significant overestimate as it includes unsuitable areas such as highest mountains (e.g., Učka, Velebit, Dinara, and Biokovo).

The Area of Occupancy calculated using 2 × 2 km grids resulted in an AOO of 1,372.00 km^2.

Habitat preferences assessment

Our analysis showed that the species has the highest affiliation with forests (33%), followed by cultivated non-forested land and habitats with weeds and ruderal vegetation (24.5%), and grassland habitats (20.9%). The remaining habitats included shrubs (14.7%), constructed and industrial habitats (4.7%), inland surface waters and wetland habitats (1.6%), and inland non and poorly covered land surface (0.3%).

Threats on a national scale

All three main threats: (1) habitat loss and fragmentation, (2) hunting and collecting of the species, and (3) invasive species were identified in Croatia.

Within the threat of habitat loss and fragmentation, tourist infrastructure and urban development, as well as transportation were assessed to have a medium impact while agricultural activity does not affect or minimally affects the species. Tourist infrastructure and urban development have a medium impact mostly due to the increasing development in the coastal areas, predominantly because of high touristic pressure and the increase in private accommodation and accompanying urban development. Concerning transportation, the whole species range is highly fragmented due to large numbers of local and regional roads, including highways, which impacts the population of the species. During the period 1997–2021 we observed more than 50 dead on road individuals highlighting this threat.

Hunting and collecting was assessed to have a medium impact and is reflected in the number of exported (legal or trafficked) individuals. Over the period 1997–2021, a total number of 3.291 individuals were exported from Croatia. Of those, 1.644 records were legally exported under the CITES regulation to eight countries, with the highest numbers being exported to Slovenia, Austria, and the Czech Republic for commercial and personal purposes of translocation (Suppl. material 3). The remaining 1.647 individuals were illegally trafficked from or through Croatia in the period 2000–2021. In respect to the timeframe, during the period 1997–2006, there were 33 reported legal exports and 22 illegally trafficked individuals, while in the period 2007–2016 the exports increased to amount of 3.083 individuals, of which 1.610

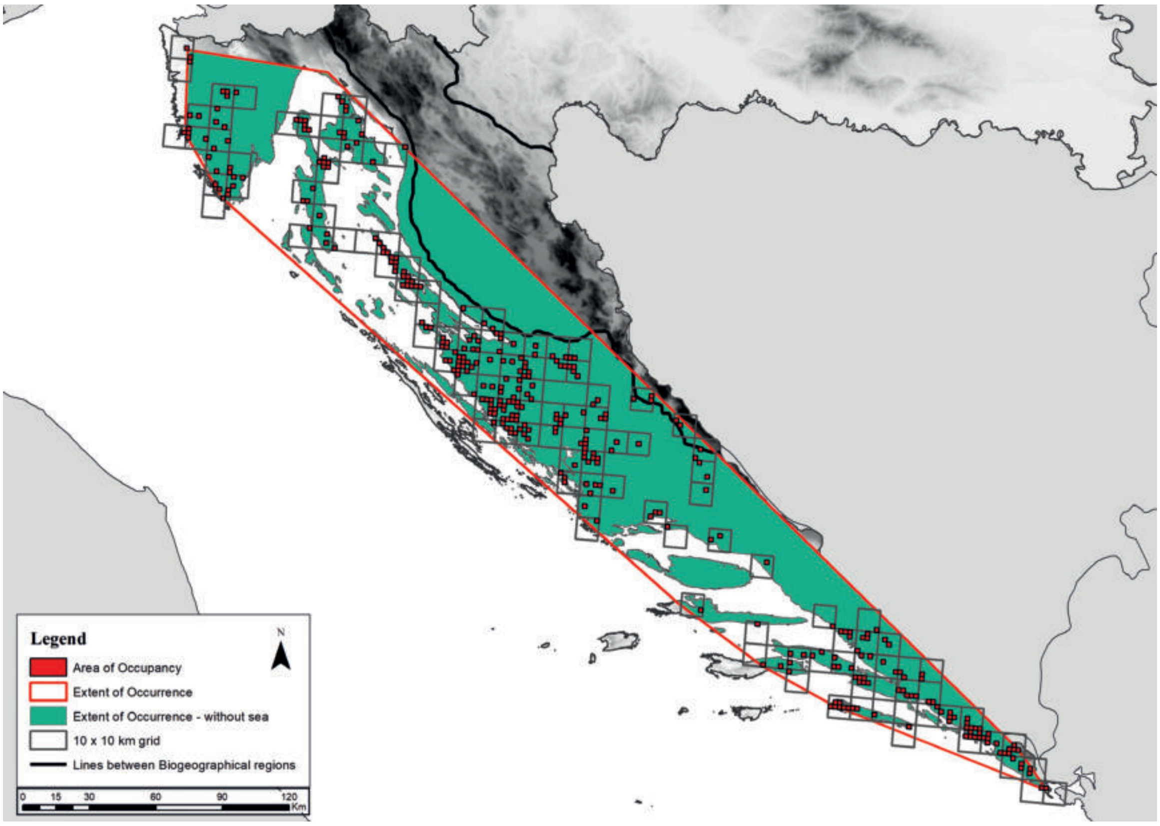

Figure 3. Geographic ranges of the species in Croatia using Extent of Occurrence (EOO), Area of Occupancy (AOO), and 10 × 10 km EEA reference grid squares.

were legally exported and 1.473 illegally trafficked. After 2017, there was a decrease in the number of exports from Croatia, counting 153 individuals respectively, of which only one was legally exported (Suppl. material 3).

The threat of invasive species affects the species with medium impact. Two main invasive species were determined as the most significant threats, wild boar, *Sus scrofa* Linnaeus, 1758, and the small Indian mongoose, *Herpestes javanicus* (É. Geoffroy Saint-Hilaire, 1818). In the area of southern Dalmatia, at least five turtle nests were observed to be destroyed by the mentioned species. Also, at least ten observed adult tortoises across the range were recorded to bear marks that could be attributed to wild boars.

Protected areas

Within Croatia, the eastern Hermann's tortoise occurs in 24 NPA's and 37 Natura 2000 areas (of which it is a target species in 15 of them). The total surface area of the species' AOO is 1,372.00 km^2, of which 191.48 km^2 (14%) is included in those 24 NPA's, while the coverage of the Natura 2000 network is 517.71 km^2 (37.73%). The 15 areas designated for the species cover only 401.92 km^2 (29.30%) of the species AOO. In addition, for two out of the 15 Natura 2000 sites designated for the species (both sites are on island Pag: HR4000018 and HR4000019**),** there is neither literature nor recent records of the species' presence. NPA's and Natura 2000 overlap on 22.9% of the territory. The coverage of both NPA's and the whole Natura 2000 network (excluding the overlaps between them) is 546.73 km^2 (40%) (Fig. 4).

Discussion

Distribution

The general distribution of *T. h. boettgeri* in Croatia is known for some time (Ljubisavljević et al. 2014; Jelić et al. 2015; Zadravec and Gambiroža 2019). Previous to our study, the species has been known from 79 10 × 10 km EEA reference grid squares (Jelić et al. 2015), while now it has increased to 123 10 × 10 km EEA reference grid squares. This increase of 35.8% is due to more intense herpetological research conducted in recent years and the development of citizen science.

The distribution of *T. h. boettgeri* in the eastern Mediterranean (including Croatia) is not homogeneous and is influenced both by the Mediterranean climate and zones of high mountains (Haxhiu 1998; Fritz et al. 2006). Our results confirmed that the natural range of the species in Croatia mostly stretches along the Adriatic coast, within the Mediterranean biogeographic region (Ljubisavljević et al. 2014). Regarding the distribution limits, the eastern boundary mostly follows the boundary between the Mediterranean and Alpine biogeographical regions and is determined by orographic parameters, e.g., Ćićarija and Učka mountain range in the north and the Dinaric Alps

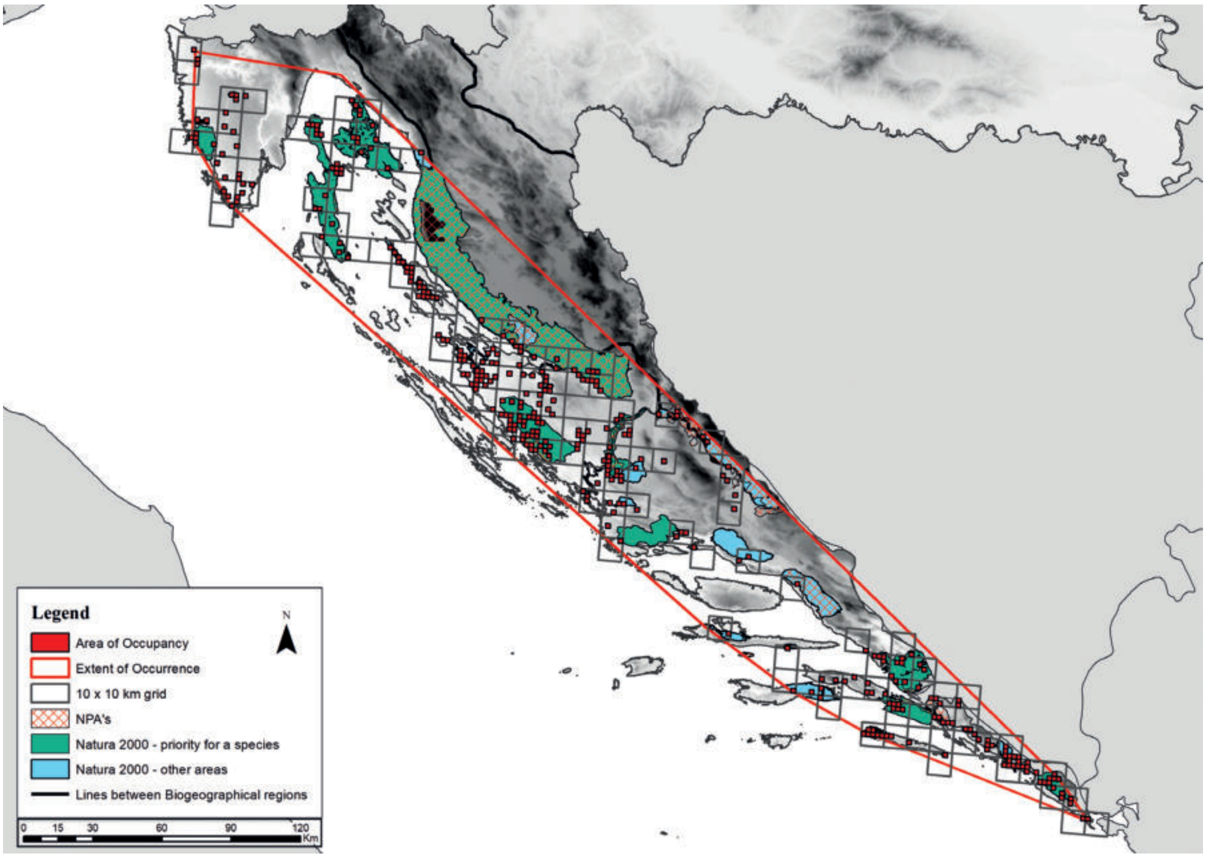

Figure 4. The original Natura 2000 sites and NPA's and Natura 2000 network priority for species.

in Kvarner and Dalmatia (Fig. 1). The inland extension of the species in Croatia, including the records from the Alpine region, are also conditioned by the Mediterranean climate. The same is true for inland regions in Bosnia and Hercegovina, Serbia, Kosovo, Montenegro, North Macedonia, Romania and Bulgaria (Ljubisavljević et al. 2014).

The records from Croatia mostly originate from an altitude lower than 500 m, the same as in Italy (Romano et al. 2013) and Slovenia (Krofel et al. 2009). In contrast, the species reaches higher altitudes, up to 1500 m, in southern European countries, due to the greater influence of the sub-Mediterranean climate on the southern mountain chains (Cheylan 2001; Mazzotti 2006; Vetter 2006; Romano et al. 2013; Couturier et al. 2014; Ljubisavljević et al. 2014; Celse et al. 2018; Duro et al. 2021). All the existing records on higher elevations are in the central and southern mountain slopes of Kvarner and Dalmatia, which are also influenced by the Mediterranean climate.

The distribution of the species in the northern part of the Adriatic coast, especially in the Istria peninsula, is still not well understood (Ljubisavljević et al. 2014; Žagar et al. 2019). Some authors refer that those populations were introduced from southern parts of the Adriatic coast by Catholic monks and gentry who breed them for consummation (Sajović 1913; Tome 1996; Krofel et al. 2009; Ljubisavljević et al. 2014) or were merely released or escaped pets (Žagar et al. 2019). The records from northern parts of Croatian Istria mostly refer to single specimen observations and can be attributed to the runaway individuals, as no historical records from this region exist. The oldest finding for the area north of Limska Draga originated from 2004 (this survey). However, records for the southern part of Croatian Istria, including the area from Limska Draga to the south, exist for the whole century (Krumbach 1918). Certainly, further genetic research will reveal the origin of Istrian populations, as also suggested for Slovenian Istria (Žagar et al. 2019). Therefore, as the Istrian population is noted as native (Jelić et al. 2015), the authors accepted it as native until proven otherwise.

Records from Dalmatia in the period from 2000 until today have greatly increased, as opposed to the Kvarner area, where most of the recent records are related to islands. Only a few recent data, mostly sporadic, are available for the mainland part of Kvarner (this study). Most likely this is the result of fewer research activities in the mainland Kvarner area, and the general trend of Mediterranean species being present only on the narrow coastline (Jelić et al. 2015).

The individuals recorded in the Continental region and individuals from 19 Adriatic islands in the Mediterranean region are considered to be introduced. In the Continental region individual specimens were mostly found in, or around larger cities, especially the capital of Zagreb, the largest city in Croatia (Bašić 2003). These individuals are most probably pets intentionally or accidentally released into nature, as almost all individuals were found in gardens or parks (Jelić et al. 2015). In Italy, reproductive populations were reported from a few urban parks (Bologna et al. 2003; Rugiero 2004; Rugiero and Luiselli 2006) but in the continental part of Croatia their reproduction has never been observed. The probable low survival of introduced individuals in the continental part of Croatia can be linked to climatic conditions which are not optimal for successful reproduction and winter hibernation. Importantly, the optimal temperature range for successful hibernation in *T. hermanni* is between 4–7 °C (McCormack 2016) and for successful breeding is between 23–34 °C (Eendebak 2001), while the mean annual temperature of the coldest months in the continental part of Croatia is between -5 °C and 5 °C and the mean annual temperatures during the nesting activities peaks are around 17 °C (Zaninović et al. 2008).

The historical presence of the species on the Adriatic islands was reported by many authors (Suppl. material 2), while the review of species distribution on Adriatic islands was never conducted in detail. Jelić et al. (2015) mentioned seven islands with native populations (Krk, Cres, Pag, Korčula, Mljet, Lastovo, and Zlarin), while other authors cite nine islands, of which some were considered introduced (Lončar 2005; Ljubisavljević et al. 2014). Within our study we found that *T. h. boettgeri* is present on 27 Adriatic islands. The native populations are present on eight islands according to the criterium of at least three valid literature and/or recent records (Suppl. material 2). In comparison with Jelić et al. (2015), we determined two more islands with native populations: Vir and Hvar due to the increased number of individuals observed in natural habitats. At the same time, we excluded Lastovo from the list because almost all individuals were found in gardens or olive groves where they were probably kept as pets.

On the other 19 islands, the populations are considered introduced. The high number of islands with introduced individuals is associated with the tendency of local inhabitants to collect and transport individuals of the species. Their survival on the islands is most probably related to typical Mediterranean warm climate which is predominant on the islands (Meliadou and Troumbis 1997; Kryštufek and Kletečki 2007).

On Rab island the species was always recorded individually and most often in settlements (Tvrtković et al. 2012). During this survey systematical research of the herpetofauna of island Rab was conducted for more than 20 days, but no individuals were recorded (Jelić et al. 2016; Sučić et al. 2018; Štih et al. 2018). If the individuals on the island still exist, they are most probably introduced ones.

The records from island Plavnik date back to the 1980s (Bruno 1980, 1988), and the same record was cited by many authors (Cheylan 2001; Tóth et al. 2006, 2017; Tóth 2018). The herpetofauna of island Plavnik was repeatedly surveyed (Tóth et al. 2006, 2017; Tóth 2018) but the species was never recorded, and the authors mentioned that the species on the island is most probably introduced.

For the islands, Ugljan, Pašman, Čiovo, Šolta, Šćedro and Brač only single records exist, and they were accordingly categorized as introduced. Although intense herpetological research on a national level was carried out on the above mentioned islands, except Šćedro, no individuals were recorded (Žagar et al. 2013; Jelić et al. 2016).

The records from the rest of the islands: Lošinj (Tóth et al. 2009), Silba (this study), Olib (this study), Veli Iž (this study), Dugi Otok (Škvarč 2000; Schmidt et al. 2020), Žirje (Lauš 2010), Lastovo (Vervust et al. 2009; Zadravec and Gambiroža 2019), Žut (Schmidt et al. 2020) and Sestrunj (Schmidt et al. 2020), as well as Lokrum (this study) mostly originate from gardens or olive groves. With fewer than three records, and the lack of historical records, the individuals from all these islands are considered introduced.

Despite the widespread distribution of the species across islands in Croatia, the genetic structure of the island and mainland population has not been studied yet. As genetic studies are recognized to be an important method for detecting the origin of island colonization by the species (Vázquez-Domínguez et al. 2012), to completely resolve the origin of the island populations in Croatia, future studies based on mitochondrial and nuclear markers are needed as was the case on some other Mediterranean islands (Giacalone et al. 2009; Perez et al. 2014; Biello et al. 2021).

The extent of Occurrence and Area of Occupancy according to the Red List Criteria

The IUCN Red List status of *T. h. boettgeri* was assessed in 1996 and the species was considered as Near Threatened (NT) globally, as well as on the national level (Jelić et al. 2015). Our results indicated that the species has a smaller AOO in comparison with previous studies (Jelić et al. 2015), but still has a higher EOO, suggesting that the species is relatively widespread, but occurs in patchy locations within the range. To compensate for a relatively high EOO value, we calculated the "true" EOO. Nonetheless, the result clearly indicates that without excluding the marine areas from the analyses, the EOO would contain a very wide range of extensive unsuitable areas and thus not estimate that the species is under threat.

Habitat preferences assessment

The habitat preferences of the eastern populations of Hermann's tortoise have been studied in more detail only in Montenegro (Meek 1985), Serbia (Stojadinović et al. 2017; Golubović et a., 2019; Nikolić et al. 2020) and Romania (Rozylowicz and Dobre 2010; Rozylowicz and Popescu 2013). As for the Croatian populations, no previous studies on habitat preferences have been carried out, outside short-term general observations (Meek 1989). Our preliminary analysis indicated that the dominant habitat types used in Croatia are characterized by semi-open areas, such as sparse forests with herbaceous vegetation, cultivated non-forested land, shrubs, grasslands, and pastures. A similar pattern has already been observed in previous studies in Serbia (Stojadinović et al. 2017; Golubović et al. 2019; Nikolić et al. 2020). Sparse forest and shrubs provide protection from predators (Pătroescu and Rozylowicz 2007; Vilardell-Bartino et al. 2015), shade during the day (Bourn and Coe 1978) and places for egg laying (Vilardell-Bartino et al. 2015). On the other hand, more open areas, cultivated non-forested land, habitats with weeds and ruderal vegetation, and grasslands are most probably preferred for efficient thermoregulation (Zug et al. 2001; Anadón et al. 2006; Falcón and Hansen 2018) or feeding requirements (Anadón et al. 2006).

Our results are based mainly on the adult tortoise, so juveniles likely prefer different habitats with more cover to avoid predators (Gaymer 1968; Walton et al. 2019). Also, we are aware that the species could have different preferences toward a specific macrohabitat type depending on the season or sex/age-specific, as indicated in other studies, e.g., (Vilardell-Bartino et al. 2015; Stojadinović et al. 2017; Nikolić et al. 2020). Regardless, our results have provided useful insights into the habitat use of *T. h. boettgeri* in Croatia which is the first important step for the conservation of the species.

Threats on a national scale

The order Testudines is among the most threatened groups of vertebrates (Rhodin et al. 2018; Stanford et al. 2020). Still, the decline of the populations of reptiles, including the population of *T. hermanni*, can be difficult to detect and long-term studies of native populations and their association with environmental and anthropogenic factors are essential for understanding the population trends and fluctuations as well as for the development of appropriate protection measures (Todd et al. 2010). Without detailed long-term studies of the threats, the only available method is to deduce them from the available literature from other countries and personal observations. While this may be biased, it still represents a good starting point for all future threat assessments.

Habitat loss and fragmentation are mediated by numerous factors, such as agricultural expansion and intensification, urbanization, tourist infrastructure development and recreational activities, the pet trade and climate change. All these threats are causing a landscape change worldwide (Forman et al. 2003; Bürgi et al. 2004; Jaeger et al. 2007) affecting numerous wildlife species (Grift 1999; Underhill and Angold 2000; Marzluff 2001), as well as the eastern populations of *T. hermanni* (Willemsen and Hailey 1989; Hailey and Willemsen 2003; Pătroescu and Rozylowicz 2007; Türkozan et al. 2008; Rozylowicz and Dobre 2010; Ljubisavljević et al. 2011, 2014; Nikolić et al. 2018). Hence, landscape use intensification is globally one of the most significant land-use changes (Ellis et al. 2021) that directly contribute to habitat loss and modification, especially in western European countries (Couturier et al. 2014). However, in eastern European countries, an opposite trend can be observed, with the increased abandonment of traditional farming and grazing which contribute to habitat overgrowth and the decline of suitable habitats and populations (Sirami et al. 2008). The traditional agricultural land use and grazing in Croatia are in decline due to massive emigration from rural to urban areas (Obad 2021), with the consequences of the dramatic reduction of grassland areas and the spreading of shrubs and dense forests. Within the period 1980–2006 the surface of agricultural areas in Croatia was reduced by 8%, while the greatest decrease was observed in meadows and/or pastures, with 37% (Kušan 2010). The trend continued also in the period 2015–2019 (Cvjetićanin et al. 2020). This is rather concerning as 46% of the land territory of Croatia before 2010 was already covered by forests and shrubs, with the trend of further increase (Kušan 2010). The habitat overgrowing is evident across the whole range of the species in Croatia but is most noticeable in the Istria peninsula and the Adriatic islands, where parts of the land are still being cultivated, while most are under significant successional process (Lauš et al. 2019). Consequently, if these negative trends continue in the future, it will cause a serious decline of the species in Croatia.

Transportation corridors with associated infrastructure, such as roads, railroads, and utility and service transport affect the tortoise through direct mortality from vehicle collisions (Ashley and Robinson 1996; Bennett 2017), as well as creating a barrier to movement and dispersal between habitats, which can potentially affect population diversity (Latch et al. 2011). Also, road surfaces act as heat traps, which many species of reptiles use as basking sites (Rosen and Lowe 1994) and are one of the main causes of amphibians' and reptiles' decline (Carr and Fahrig 2001; Hels and Buchwald 2001; Smith and Dodd 2003; Carvalho and Mira 2011). In Croatia, many urban corridors were created or renewed during the period 1980–1990, but this is still an ongoing process in many urban areas where the human population is increasing, especially during the tourist season (Kušan 2010). Such high-density residential areas with a developed network of roads can increase the chance of an animal being run over by a car. Even with the unsystematic recording of road mortality, this seems to be high, especially in the Istria peninsula and on the Dalmatian coast during the late spring and early summer months, which may be associated with the higher traffic flow that occurs during summer holidays (Erritzoe et al. 2003). On the other hand, traffic speed is reported as an important factor determining the mortality of many vertebrate groups on the road (Bradford et al. 2005; Grilo et al. 2010), which may also play important role in Croatia, but this should be investigated in the future.

Tourism is one of the main industries in Croatia, especially on the coast, with the tendency to intensify in the future (Jelinčić and Žuvela 2012). Accordingly, construction of tourist infrastructure is causing significant habitat changes including habitat loss and fragmentation through the removal of natural vegetation. This threat is also correlated with the next larger threat, hunting, and the collecting of species which is largely happening during the touristic season.

The members of the family Testudinidae belong to the most traded species worldwide (Luiselli et al. 2016), which is worrisome since the life-history characteristics of tortoises do not provide buffering against exclusion of individuals from population (Nikolić et al. 2018). Such overexploitation of the species significantly influences the decline in the populations (Türkozan et al. 2008; Bertolero et al. 2011; Ljubisavljević et al. 2011; Luiselli et al. 2016; Graciá et al. 2020; CITES 2021). In the territory of former Yugoslavia (whose member was Croatia until 1991) significant exports of tortoises lasted over half a century (Honegger 1974; Lambert 1984; Vetter 2006; Ljubisavljević et al. 2011; Nikolić et al. 2018). While *T. h. boettgeri* is a long-lived species with a long generation time and low fecundity, it is evident that this species is particularly vulnerable to over-collecting, as is the case with other tortoises and large lizards (Warwick 2014; Nikolić et al. 2018). Aside from organized trafficking, many visitors to the country, as well as residents from different regions within same country, collected tortoises during holidays and took them back to their home as presents or garden animals. This resulted in many records of the species outside its natural distribution range (Fig. 1). The current annual trafficking of the species from Croatia is largely unknown due to the lack of available databases. In this context, the data for illegal traffic presented in this paper is based on available media reports, usually confiscated during border crossings, and in many cases it is not certain whether the individuals were collected in Croatia or were just transported through Croatia. Accordingly, there is a need for establishing up to date databases that contain such data and can be used in the future for assessing the trends in the trafficking of this and other native species. Still, the observed decline shows that the national policies were partially successful in regulating the exports of the species (Suppl. material 3). Focusing on all the facts, it is challenging to assess the impact of the overexploitation in Croatia as medium or high. Indeed, the legal exports from Croatia decreased in recent years, but the illegal traffic of tortoise is still on-going and hence, its impact can be significant. In general terms, it is evident that overexploitation is more prevalent globally (Luiselli et al. 2016) than in Croatia.

Wild boar and the small Indian mongoose are the species that represent an important threat for *T. hermanni*. The wild boar is native species in Croatia but has been introduced to many Adriatic islands (Šprem et al. 2011). The mongoose has been introduced intentionally on islands Mljet, Korčula, Hvar, Čiovo, Pelješac peninsula (Tvrtković and Krystufek 1990; Barun et al. 2011) and along the Adriatic coast in Croatia at the beginning of the twentieth century (Kryštufek and Tvrtković 1992; Ćirović et al. 2011; Boršić et al. 2018) to control the populations of venomous snakes, primarily the horned viper (*Vipera ammodytes*) (Tvrtković and Krystufek 1990). The species did not survive at all the localities where it was introduced and currently inhabits the mentioned islands as well as the mainland from the southern Konavle area up to the river Neretva in the north, with several more recent observations north of the river, indicating the further spreading of the species. Both species are known predators of eggs, hatchlings (Corti and Zuffi 2003; Vilardell et al. 2008), and adults in other Mediterranean countries (Ballasina 1995; Budó et al. 2003; Corti and Zuffi 2003; Bertolero et al. 2007). The individuals with bite marks from wild boar were recorded also in Croatia, on the island of Mljet (Jelić et al. 2012). Additional observations of the excavation of *T. hermanni* nests by both mongooses and wild boars were observed by the authors in several localities in southern Dalmatia (Majkovi and Dubrovnik area). The mongoose is a target species for future insular eradication attempts in Croatia (Barun et al. 2011), as their populations tend to increase throughout the country. Both mentioned species have in many areas outside their natural distribution range in Croatia established high-density populations (Ćirović et al. 2011; Šprem et al. 2011) which can to some degree jeopardize the populations of *T. hermanni* and other native reptiles and amphibians protected on national and international levels (EU Habitats Directive, Annexes II and IV), especially in the Adriatic islands. In the future, targeted surveys should be carried out to determine the real impact of both species on the *T. hermanni* populations in Croatia.

Area important for species conservation

Although the network of protected areas is an important initiative for species and habitat conservation in Europe, some studies reveal that coverage of species and habitats by existing networks is insufficient to assure the long-term maintenance of biodiversity (Miu et al. 2020). The fact that some protected areas are or will become unsuitable for target species in the future, represents an additional concern in the preservation of species' suitable habitats (Araújo et al. 2011). In Croatia, 15 Natura 2000 sites were designated for the species, but as we stated before, for two of them (HR4000018 and HR4000019), the origin of the data on the basis of which they were designated is not clear (Jelić 2016). Although the dominant habitats present on these two Natura 2000 sites are suitable for the species (Eastern sub-Mediterranean dry grasslands and Eastern Mediterranean screes), further research is needed to confirm the species' presence.

In Croatia, the AOO area of *T. h. boettgeri* is more covered by the Natura 2000 network than NPA's, which agrees with similar studies on other European amphibians and reptiles (Sánchez-Fernández and Abellán 2015). The percentage of AOO covered by the Natura 2000 site designated for the species is 29.30%, which is more than the minimum of 20% considered in the European Union as a guideline for nature conservation, but still at the lower limit of a sufficient degree of conservation of the habitat which is important for the species at national level (proposed range 20–60%) (Anonymous 2014). Thus, we identified 10 existing Natura 2000 sites to which *T. h. boettgeri* should be added as a target species. Site HR5000028 – Dinara is the only area where the species was found within the Alpine biogeographical region and should therefore be included as an area important for the species at the national level. The additional nine existing Natura 2000 sites which we identified are: HR2001021 – Lun on island Pag, HR4000005 – Privlaka Ninski zaljev-Ljubački zaljev, HR2000132 – Područje oko špilje Škarin Samograd and HR2001010 – Paleoombla–Ombla, HR2001325 – Ninski stanovi-livade, HR2001322 – Vela Traba, HR2000629 – Limski zaljev-kopno, HR2001371 – Područje oko Dobre vode, HR2000641 – Zrmanja, which in total amount to 73.51 km^2. With the inclusion of these areas, the coverage of the species-area by the Natura 2000 network would increase to at least 34.6%. The percentage could increase even more with additional surveys of the unexplored parts of existing Natura 2000 sites.

The existing NPA's and Natura 2000 network combined cover about 40% of the species AOO while the remaining 60% of the species area remains almost completely unprotected. *T. h. boettgeri* is a side-fidelity species with low dispersal abilities (Vilardell-Bartino et al. 2015; Türkozan et al. 2018). Consequently, if the existing protected areas do not host a sufficient percentage of the total population, it may cause a decline in the population size. Thus, conducting targeted research could lead to better protection of the species at the national level.

Conclusions

This study has provided valuable information about the current distribution, habitat preferences, and conservation status of *T. h. boettgeri* in Croatia. No decrease in the distribution of the species has been recorded, but rather an increase in the known range within the country. However, there are still unresolved questions, particularly regarding the origin of certain island populations, which can only be answered through the use of DNA methods. In Croatia, *T. h. boettgeri* faces significant threats and disturbances, such as habitat overgrowth, which could pose a risk to its survival in the future. Consequently, although based on the assessment of both the Area of Occupancy (AOO) and the "true" Extent of Occurrence (EOO), *T. h. boettgeri* may currently be classified as a Near Threatened (NT) species, an up-listing to the Vulnerable category (VU) could be expected in the near future. To ensure the long-term protection of this species, it is crucial to expand its coverage within the Natura 2000 network. The study suggests that 10 areas should be considered for inclusion during the reassessment of existing Natura 2000 sites. Additionally, targeted ecological surveys are needed across the species' range in the country to better understand its ecological needs and provide a more scientifically-based approach to its protection in the future.

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Supplementary material 1

The new and previously unpublished records of *T. h. boettgeri* in Croatia

Authors: Katarina Koller Šarić, Boris Lauš, Ivona Burić, Ana Štih Koren, Toni Koren

Data type: Excel file (.xlsx)

Explanation note: The new and previously unpublished records of *T. h. boettgeri* in Croatia according to open access databases data, the authors' unpublished field records and shared unpublished field data from colleagues. For the records of introduced individuals there is no altitudinal data available because they were not use in the analysis. Reference type (TR – Technical report; PO – personal observation; OP - Bio – online platform Biologer.hr; OP - O – online platform Observado.org; OP - G – online platform GBIF; OP - B – online platform Balcanica.info).

Link: https://doi.org/10.3897/herpetozoa.36.e103510.suppl1

Supplementary material 2

List of Adriatic islands with known records of *T. h. boettgeri*

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Data type: Word file (.docx)

Explanation note: List of Adriatic islands with known records of *T. h. boettgeri*. For each island number of records and literature sources are provided. Number of records includes only the primary records from original citation, while the consecutive citations of the same records are mentioned in a literature source. The islands are arranged from the north to south.

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Supplementary material 3

The number of exported individuals regulated under CITES

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Data type: Word file (.docx)

Explanation note: The number of exported individuals regulated under CITES (DE – Germany; CZ – Czech Republic; US – United States of America; AT – Austria; DK – Denmark; SI – Slovenia; CH – Switzerland; RS – Serbia) and purpose of trade (P – personal; T – commercial; E – educational) per year and number of illegally exported individuals per year.

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Resurrection and distribution extension of *Odorrana heatwolei* (Stuart & Bain, 2005) (Anura, Ranidae)

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https://zoobank.org/FAB9FAE4-31F2-4084-ABB4-A38447A72783

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Abstract

We collected nine specimens of *Odorrana* Fei, Ye & Huang, 1990 from Xishuangbanna Prefecture, Yunnan Province, China, which is close to the type locality of *O. heatwolei* (Stuart & Bain, 2005) in Phongsaly Province, northern Laos (approximately 60 km). These specimens agree with the diagnosis of *O. heatwolei* that body size is large in females and relatively small in males, tympanum large in males and relatively small in females, first finger longer than the second, and glandular dorsolateral fold and external vocal sacs present in males, moreover, these specimens have obvious dense tiny black dots scattered on the dorsum, which is consistent with the characters of *O. heatwolei* and different from *O. tiannanensis* (Yang & Li, 1980). Phylogenetically, the sequences of these specimens clustered with the sequence of the holotype of *O. heatwolei* and formed a distinct clade together, which is sister to *O. tiannanensis*. We resurrect *O. heatwolei* and extend the distribution of this species to China.

Key Words

16S rRNA, China, Laos, new record, revalidation, taxonomy

Introduction

The odorous frogs of the genus *Odorrana* Fei, Ye & Huang, 1990 comprise a species-rich group of ranids whose range covers the Ryukyu Archipelago, southern China, northeastern India, and Indochina, and extend southwards to Sumatra and Borneo (Frost 2023). This genus contains 62 recognized species to date, more than half of which occur in southern China (AmphibiaChina 2023; Frost 2023).

Odorrana tiannanensis (Yang & Li, 1980), a species of *Odorrana*, was considered to be widely distributed in southern Yunnan, China, and northern Vietnam and northern Laos (Yang and Li 1980; Yang and Rao 2008; AmphibiaChina 2023; Frost 2023), and its type locality is in Hekou County, Yunnan Province, China (Yang and Li 1980). *Rana heatwolei* Stuart & Bain, 2005 is a species described from Phongsaly Province, northern Laos, and was placed into synonymy of *O. tiannanensis* based on morphological characteristics by Ohler (2007). Poyarkov et al. (2021) regarded it as *O. heatwolei* (Stuart & Bain, 2005) and considered it to be a valid species but did not provide any evidence.

During our field surveys in southern Yunnan Province, China, from 2019 to 2021, we collected 11 specimens of *Odorrana tiannanensis* from its type locality Hekou County, and from Gejiu City and Malipo County, and nine specimens of *O.* cf. *tiannanensis* from Mengla County, which is close to Phongsaly, the type locality of *O. heatwolei* in northern Laos (Fig. 1).

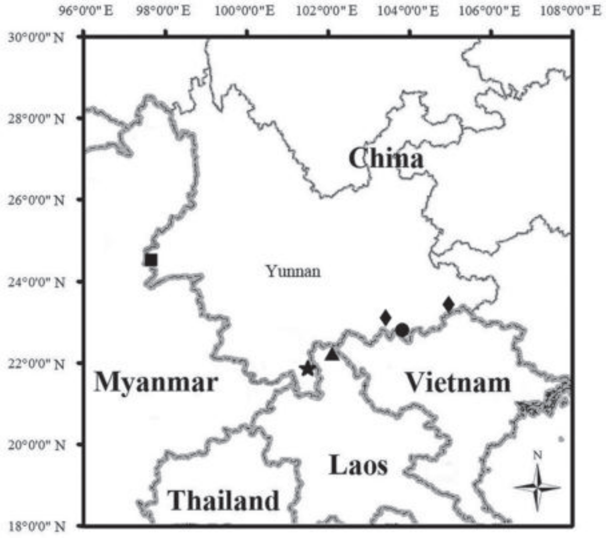

Figure 1. Map showing the type locality (black dot) of *O. tiannanensis*, the type locality (black triangle) of *O. heatwolei*, the type locality (black square) of *O. macrotympana*, the new collection site (black star) of *O. heatwolei*, and the new collection sites (black diamonds) of *O. tiannanensis*.

Molecularly, the sequences of these specimens from Mengla clustered with the sequence of the holotype (FMNH 258134) of *O. heatwolei* and the sequences which were also collected from Phongsaly but identified as *O. tiannanensis* from GenBank, and together, they formed a distinct clade sister to *O. tiannanensis*. The morphological characters of the specimens from Mengla agree with the original description (Stuart and Bain 2005) of *O. heatwolei* but differ from *O. tiannanensis*.

Herein, we resurrect *Odorrana heatwolei* and first report this species from China.

Materials and methods

Taxon sampling

Specimens were euthanised and fixed in 75% ethanol for storage. Liver tissue samples were preserved in 99% ethanol for molecular analysis. All specimens were deposited at Kunming Natural History Museum of Zoology, Kunming Institute of Zoology, Chinese Academy of Sciences (KIZ).

Molecular analysis

Total genomic DNA was extracted from liver tissues of the specimens from Yunnan, China. A fragment of the mitochondrial 16S rRNA gene was amplified via the polymerase chain reaction (PCR) using the primers L2188 (Matsui et al. 2006): 5'–AAAGTGGGCCTAAAAGCAGCCA–3' and 16H1 (Hedges 1994): 5'–CTCCGGTCTGAACTCAGATCACGTAGG–3'. Molecular experiment protocols used in this study were the same as those in Liu et al. (2022). The sequence of the holotype (FMNH 258134) of *Odorrana heatwolei* was provided by Dr. Bryan L. Stuart from North Carolina Museum of Natural Sciences, all new sequences have been deposited on GenBank, and other sequences were downloaded from GenBank (Table 1). Sequences of *Rana chensinensis* David, 1875 and *Pelophylax nigromaculatus* (Hallowell, 1861) were used as outgroups (Chen et al. 2013; Liu et al. 2021). The technical computation methods for the sequence alignment, genetic divergences calculation, best substitution model selection, Bayesian inference and Maximum likelihood phylogenetic analyses were the same as those in Liu et al. (2022).

Morphology

Measurements were taken with a digital caliper to the nearest 0.1 mm. Combining Liu et al. (2021), Luo et al. (2021), and Liu et al. (2022), we measured: eye diameter (ED), diameter of exposed portion of eyeball; finger disk width (FDW3), width at the widest part of the disk of finger III; forearm and hand length (FHL), from elbow to tip of finger III; foot length (FTL), from the base of inner metatarsal tubercle to the tip of fourth toe; internarial distance (IND), minimum distance between the inner margins of the external nares; interorbital distance (IOD), minimum distance between the inner edges of the upper eyelids; hand length (HAL), from the base of the thenar tubercle to the tip of finger III; head length (HL), from tip of snout to rear of jaw; head width (HW), width of head at widest point; total leg length (LEG), from vent to tip of toe IV; snout length (SL), distance from the tip of the snout to the anterior corner of the eye; snout-vent length (SVL), from tip of snout to vent; tympanum diameter (TD), maximum diameter of tympanum; tympanum-eye distance (TED), from anterior edge of tympanum to posterior corner of the eye; tarsus and foot length (TFL), from heel to the tip of toe IV; tibia length (TL), distance from knee to heel; and upper eyelid width (UEW), maximum width of the upper eyelids.

Results

The sequence alignment is 1090 bp in length. The Maximum likelihood and Bayesian inference phylogenetic trees were essentially consistent. The sequences of the specimens from Mengla, Yunnan, China, the sequence of the holotype (FMNH 258134) of *Odorrana heatwolei*, and the sequences identified as *O. tiannanensis* from Phongsaly, Laos, clustered together, and they formed a distinct clade sister to *O. tiannanensis* with strong supports (Bayesian posterior probability 1, Maximum likelihood bootstrap support 98) (Fig. 2). The genetic divergence (uncorrected p-distance) between the sequences of the specimens from Mengla and the sequence of the holotype (FMNH 258134) of *O. heatwolei* was 0.20%,

Figure 2. Bayesian inference tree of the genus *Odorrana* based on the mitochondrial 16S rRNA sequences. Numbers before slashes indicate Bayesian posterior probabilities (≥ 0.90) and numbers after slashes indicate bootstrap supports from Maximum likelihood analysis (≥ 90).

between the sequences identified as *O. tiannanensis* from Phongsaly and the sequence of the holotype (FMNH 258134) of *O. heatwolei* was 0.28%, between the sequences of the specimens from Mengla and the sequences of *O. tiannanensis* was 6.51%, and between the sequence of the holotype (FMNH 258134) of *O. heatwolei* and the sequences of *O. tiannanensis* was 6.47% (Table 2).

Morphologically, the specimens from Mengla differ from *Odorrana tiannanensis* by having obvious dense tiny black dots scattered on dorsum and lacking large black dots on dorsum (Figs 3A, C, 4) whereas having distinct or indistinct large black dots on dorsum and lacking dense tiny black dots scattered on dorsum in *O. tiannanensis* (Figs 3B, D, 5). However, morphological characters of the specimens from Mengla are similar to the original description and pictures of *O. heatwolei* in Stuart and Bain (2005).

Therefore, we consider the specimens from Mengla to be *Odorrana heatwolei* which were originally described as *Rana heatwolei* by Stuart and Bain (2005). We formally remove *O. heatwolei* from the synonymy of *O. tiannanensis*.

Taxonomy

Odorrana heatwolei (Stuart & Bain, 2005)

Figs 3A, C, 4, 6, 7

Rana heatwolei Stuart & Bain, 2005
Rana tiannanensis – Ohler, 2007
Odorrana heatwolei – Poyarkov et al., 2021

Type material. ***Holotype*:** FMNH 258134, adult male.

***Paratypes*.** FMNH 258279–83, 258598, six females; FMNH 258135–39, five males.

Type locality. Phou Dendin National Biodiversity Conservation Area, Phongsaly District, Phongsaly Province, Laos (22°05'38"N, 102°12'50"E; alt. 600 m).

Etymology. The specific epithet is a patronym for Dr. Harold F. Heatwole. We propose "Dense-spotted Odorous Frog" for the English name and "麻点臭蛙" (má diǎn chòu wā) for the Chinese name.

Updated diagnosis. Habitus robust; body sizes of females approximately two to three times that of males,

Table 1. Samples used for phylogenetic analyses of molecular sequence data. * = type locality.

Species	Locality	Voucher NO.	GenBank NO.
Odorrana absita	Xe Sap, Xe Kong, Laos*	FMNH258109	EU861542
Odorrana amamiensis	Tokunoshima, Ryukyu, Japan	KUHE24635	AB200947
Odorrana anlungensis	Anlong, Guizhou, China*	HNNU1008I109	KF185049
Odorrana aureola	Phu Luang, Loei, Thailand*	ZMKU AM 01137	KT002162
Odorrana bacboensis	Khe Moi, Nghe An, Vietnam*	FMNH255611	DQ650569
Odorrana banaorum	Tram Lap, Gia Lai, Vietnam	ROM7472	AF206487
Odorrana chapaensis	Lai Chau, Vietnam	AMNH A161439	DQ283372
Odorrana chloronota	Darjeeling, West Bengal, India*	BMNH 1947.2.28.6	DQ650594
Odorrana dulongensis	Dulongjiang, Yunnan, China*	KIZ035027	MW128102
Odorrana exiliversabilis	Wuyishan, Fujian, China*	HNNU0607032	KF185056
Odorrana fengkaiensis	Shiwanshan, Guangxi, China	HNNU295 7k	KF185033
Odorrana geminata	Cao Bo, Ha Giang, Vietnam	AMNH 163782	EU861546
Odorrana grahami	Kunming, Yunnan, China*	HNNU1008II016	KF185051
Odorrana graminea	Wuzhishan, Hainan, China*	HNNU0606123	KF185038
Odorrana hainanensis	Wuzhishan, Hainan, China*	HNNU0606105	KF185032
Odorrana heatwolei	Phongsaly, Phongsaly, Laos*	FMNH 258134	OR237216
Odorrana heatwolei	Mengla, Yunnan, China	KIZ2019541	OR237217
Odorrana heatwolei	Mengla, Yunnan, China	KIZ20194301	OR237218
Odorrana heatwolei	Mengla, Yunnan, China	KIZ20194302	OR237219
Odorrana heatwolei	Mengla, Yunnan, China	KIZ20194255	OR237220
Odorrana heatwolei	Mengla, Yunnan, China	KIZ20194256	OR237221
Odorrana heatwolei	Mengla, Yunnan, China	KIZ20194257	OR237222
Odorrana heatwolei	Mengla, Yunnan, China	KIZ20194258	OR237223
"*Odorrana tiannanensis*"	Houey Phihet, Phongsaly, Laos	2005.0180	KR827975
"*Odorrana tiannanensis*"	Houey Phihet, Phongsaly, Laos	2005.0183	KR827976
"*Odorrana tiannanensis*"	Long Nai Khao, Phongsaly, Laos	2004.0406	KR827977
"*Odorrana tiannanensis*"	Long Nai Khao, Phongsaly, Laos	2004.0407	KR827978
"*Odorrana tiannanensis*"	Nathen, Phongsaly, Laos	2004.0408	KR827979
"*Odorrana tiannanensis*"	Houey Phihet, Phongsaly, Laos	2005.0182	KR827980
Odorrana hejiangensis	Hejiang, Sichuan, China*	HNNU1007I202	KF185052
Odorrana hosii	Kuala Lumpur, Malaysia	No voucher	AB511284
Odorrana huanggangensis	Wuyishan, Fujian, China*	HNNU0607001	KF185059
Odorrana ishikawae	Amami Island, Ryukyu, Japan	No voucher	AB511282
Odorrana jingdongensis	Jingdong, Yunan, China*	20070711017	KF185050
Odorrana junlianensis	Junlian, Sichuan, China*	HNNU002	KF185058
Odorrana kuangwuensis	Nanjiang, Sichuan, China*	HNNU0908II185	KF185034
Odorrana kweichowensis	Shilian, Meitan, Guizhou, China	CIBGYU20130917004	MH193548

Species	Locality	Voucher NO.	GenBank NO.
Odorrana leporipes	Shaoguan, Guangdong, China*	HNNU1008I099	KF185036
Odorrana liboensis	Maolan, Libo, Guizhou, China*	GZNU20160802003	MW481352
Odorrana lipuensis	Lipu, Guilin, Guangxi, China*	NHMG1306002	KM388699
Odorrana livida	Thagata Juwa, Myanmar*	BMNH 1889.3.25.48	DQ650615
Odorrana lungshengensis	Longsheng, Guangxi, China*	HNNU70028	KF185054
Odorrana macrotympana	Yingjiang, Yunnan, China*	KIZ 2009051020	OL831010
Odorrana macrotympana	Htamanthi, Sagaing, Myanmar	SEABRI 2019120040	OL831011
Odorrana macrotympana	Htamanthi, Sagaing, Myanmar	SEABRI 2019120041	OL831012
Odorrana macrotympana	Htamanthi, Sagaing, Myanmar	SEABRI 2019120072	OL831013
Odorrana macrotympana	Htamanthi, Sagaing, Myanmar	SEABRI 2019120073	OL831014
Odorrana margaretae	Emei, Sichuan, China	HNNU20050032	KF185035
Odorrana morafkai	Tram Lap, Gia Lai, Vietnam	ROM7446	AF206484
Odorrana mutschmanni	Cao Bang, Vietnam*	IEBR 3725	KU356766
Odorrana nanjiangensis	Nanjiang, Sichuan, China*	HNNU1007I291	KF185042
Odorrana narina	Okinawa Island, Ryukyu, Japan	No voucher	AB511287
Odorrana nasica	Ha Tinh, Vietnam	AMNH A161169	DQ283345
Odorrana nasuta	Wuzhishan, Hainan, China*	HNNU051119	KF185053
Odorrana sangzhiensis	Sangzhi, Hunan, China*	CSUFT 4305220051	MW464865
Odorrana schmackeri	Yichang, Hubei, China*	HNNU0908II349	KF185047
Odorrana supranarina	Iriomotejima, Ryukyu, Japan	KUHE2898	AB200950
Odorrana swinhoana	Nantou, Taiwan, China	HNNUTW9	KF185046
Odorrana tianmuii	Tianmushan, Zhejiang, China*	NHMG1303018	KT315390
Odorrana tiannanensis	Hekou, Yunnan, China*	SCUM50510CHX	EF453751
Odorrana tiannanensis	Hekou, Yunnan, China*	KIZ20193272	OL831009
Odorrana tiannanensis	Hekou, Yunnan, China*	KIZ20193273	OL831008
Odorrana tiannanensis	Hekou, Yunnan, China*	KIZ20193274	OL831007
Odorrana tiannanensis	Hekou, Yunnan, China*	KIZ20215191	OL831006
Odorrana tiannanensis	Gejiu, Yunnan, China	KIZ20193241	OR237224
Odorrana tiannanensis	Gejiu, Yunnan, China	KIZ20193242	OR237225
Odorrana tiannanensis	Gejiu, Yunnan, China	KIZ2020411	OR237226
Odorrana tiannanensis	Malipo, Yunnan, China	KIZ20215121	OR237227
Odorrana tiannanensis	Malipo, Yunnan, China	KIZ20215122	OR237228
Odorrana tiannanensis	Malipo, Yunnan, China	KIZ20215123	OR237229
Odorrana tiannanensis	Malipo, Yunnan, China	KIZ20215124	OR237230
Odorrana tormota	Huangshan, Anhui, China*	No voucher	DQ835616
Odorrana trankieni	Vietnam	VNMN04035	KX893900
Odorrana utsunomiyaorum	Iriomotejima, Ryukyu, Japan	KUHE12896	AB200952
Odorrana versabilis	Leishan, Guizhou, China*	HNNU003 LS	KF185055
Odorrana wuchuanensis	Wuchuan, Guizhou, China*	HNNU019 L	KF185043
Odorrana yentuensis	Vietnam	IEBRA.2015.38	KX893891
Odorrana yizhangensis	Yizhang, Hunan, China*	HNNU1008I075	KF185048
Odorrana yunnanensis	Longchuan, Yunnan, China*	HNNU001YN	KF185057
Pelophylax nigromaculatus	Locality unknown	No voucher	LC389208
*Rana chensinensi*s	Ningshan, Shanxi, China	HNNU20060359	KF185061

SVL 37.1–57.3 mm in adult males, 97.3–106.7 mm in adult females; head length slightly greater than head width; dorsal skin shagreened, skin on flanks granular; supratympanic fold present; dorsolateral folds distinct in males and indistinct in females; tympanum large in males (TD/ED 0.58–0.73) and relatively small in females (TD/ED 0.45–0.58); vomerine teeth distinct; relative lengths of fingers III > IV > I > II; inner metacarpal tubercle and outer metacarpal tubercle present, outer metatarsal tubercle absent; all finger and toe tips slightly expanded; toes with entire webbing to disks; uniform light brown or dark brown on whole body, dense tiny black dots scattered on dorsum; paired external vocal sacs and nuptial pad on the base of finger I present in adult males.

Table 2. Genetic divergences (uncorrected p-distance, %) based on the mitochondrial 16S rRNA gene sequences.

	1	2	3	4
1 *Odorrana heatwolei* (Holotype)				
2 *Odorrana heatwolei* (Mengla, China)	0.20			
3 “*Odorrana tiannanensis*” (Phongsaly, Laos)	0.28	0.17		
4 *Odorrana tiannanensis*	6.47	6.51	3.75	
5 *Odorrana macrotympana*	10.10	10.25	10.78	9.70

Specimens examined. KIZ20194255, adult male, and KIZ20194256–KIZ20194258, three adult females, collected on 24 April 2019 from Mandan village, Mengla Town, Mengla County, Xishuangbanna Prefecture, Yunnan Province, China (21°28'59"N, 101°40'29"E, 780 m elevation) by Shuo Liu; KIZ20194279, adult female, collected on 27 April 2019 from Mengyuan village, Guanlei Town,

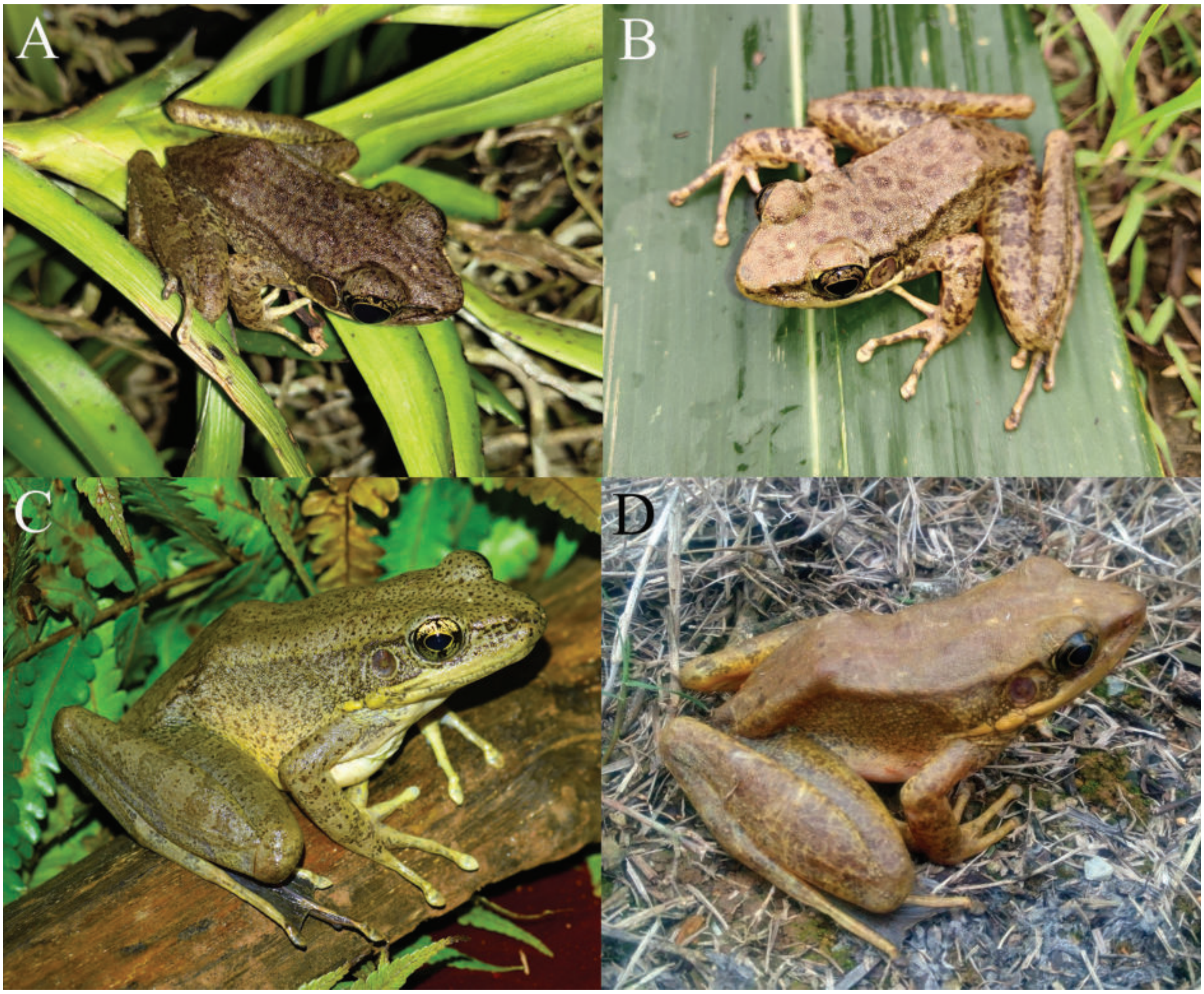

Figure 3. Comparison between *Odorrana heatwolei* and *O. tiannanensis* in life. **A.** Adult male *O. heatwolei* from Mengla, Yunnan, China; **B.** Adult male *O. tiannanensis* from Hekou, Yunnan, China; **C.** Adult female *O. heatwolei* from Mengla, Yunnan, China; **D.** Adult female *O. tiannanensis* from Hekou, Yunnan, China.

Mengla County, Xishuangbanna Prefecture, Yunnan Province, China (21°38'35"N, 101°26'54"E, 950 m elevation) by Shuo Liu; KIZ20194301–KIZ20194302, two adult males, collected on 30 April 2019 from Bubeng village, Mengla Town, Mengla County, Xishuangbanna Prefecture, Yunnan Province, China (21°36'21"N, 101°33'27"E, 800 m elevation) by Shuo Liu; KIZ2019541, adult male, and KIZ2019542, adult female, collected on 4 May 2019 from Xinshan village, Yaoqu Township, Mengla County, Xishuangbanna Prefecture, Yunnan Province, China (21°54'59"N, 101°32'16"E, 840 m elevation) by Shuo Liu.

Description of the specimens from China. Morphometric and meristic data are presented in Table 3. SVL 46.8–51.7 mm in males, 97.4–106.7 mm in females; head width slightly shorter than length (HW/HL 0.87–0.91 in males, 0.93–0.98 in females); snout obtuse, pointed in dorsal view and rounded in profile, obviously projecting beyond lower jaw; nostril closer to snout than to eye; canthus rostralis distinct; loreal region concave; internarial distance greater than interorbital distance; snout length greater than eye diameter; pineal body distinct; supratympanic fold horizontal and straight; tympanum large in males (TD/ED 0.66–0.73) and relatively small in females (TD/ED 0.48–0.58), round and transparent; vomerine teeth developed into mass on two oblique ridges between the two choanae; tongue cordiform, posterior notch enlarged and formed as U-shaped; vocal sac openings on floor of mouth in each corner and an external vocal sac behind each angle of mouth in males.

Forelimbs robust; relative lengths of fingers III > IV > I > II; all finger tips slightly expanded; lateral fringes and webbing on fingers absent; subarticular tubercle round and prominent; one oval thenar tubercle prominent on the ventral of finger I; inner metacarpal tubercle and outer metacarpal tubercle distinct; supernumerary tubercles at the base of fingers II–IV; grayish-yellow nuptial pad on finger I in adult males.

Hind limbs long, tibia longer than thigh; toes long, relative lengths IV > V > III > II > I; all toe tips slightly expanded; toes with entire webbing to disks; subarticu-

Figure 4. Dorsal view (**A**), lateral view (**B**), and ventral view (**C**) of the male (KIZ20194255) and dorsal view (**D**), lateral view (**E**), and ventral view (**F**) of an adult female (escaped) of *Odorrana heatwolei* from China in life.

lar tubercles prominent and longitudinally ovoid; inner metatarsal tubercle prominent and oval; outer metatarsal and supernumerary tubercles absent.

Dorsal skin shagreened with some very indistinct tubercles, skin on flanks granular, ventral skin smooth, and dorsolateral folds distinct in males. Skin slightly smoother and dorsolateral folds more indistinct in females.

Coloration in life. Dorsum brown scattered with dense tiny brownish black dots; upper lip yellowish white with indistinct dark spots; lower lip creamy white with distinct dark spots; lateral side of head and body brown with many irregular dark brown spots; dorsal surfaces of limbs brown with some indistinct dark brown bands; ventral surface of head and forelimbs, chest, and belly

Table 3. Morphological measurements (mm) of the specimens of *Odorrana heatwolei* from China.

	KIZ2019541 Male	KIZ20194301 Male	KIZ20194302 Male	KIZ20194255 Male	KIZ20194256 Female	KIZ20194257 Female	KIZ20194258 Female	KIZ20194279 Female	KIZ2019542 Female
SVL	51.7	48.3	48.8	46.8	97.4	102.7	103.4	106.7	104.2
HL	21.8	20.3	19.6	19.7	37.5	37.7	37.4	39.2	39.0
HW	19.1	17.7	17.9	17.2	34.9	36.1	35.7	36.9	38.4
SL	8.9	8.8	8.3	8.5	16.4	15.7	16.3	16.4	16.6
IND	5.4	5.8	5.3	5.5	10.3	10.0	9.8	10.4	11.0
IOD	4.2	4.1	4.0	4.6	8.7	9.4	8.1	9.6	9.5
ED	7.2	7.6	7.1	7.0	10.5	11.0	10.9	12.2	11.1
UEW	5.2	5.1	4.8	4.8	7.5	7.7	7.6	7.7	8.4
TD	5.2	5.3	4.7	5.1	6.1	5.9	6.0	5.8	5.7
TED	1.7	1.5	1.5	1.6	4.5	4.4	4.7	5.0	4.8
FHL	28.2	25.7	26.4	26.3	49.1	51.2	50.3	53.0	55.1
HAL	16.0	15.1	15.3	15.6	28.6	30.1	29.6	31.4	31.9
TL	34.3	31.5	32.9	31.2	65.6	66.5	65.9	70.4	71.8
TFL	44.1	40.3	42.0	40.6	83.2	86.0	82.6	90.6	94.1
FTL	29.5	27.3	28.4	26.9	55.7	59.0	56.5	60.7	65.0
FDW3	1.9	1.9	1.8	1.8	3.7	3.5	2.6	3.6	4.0

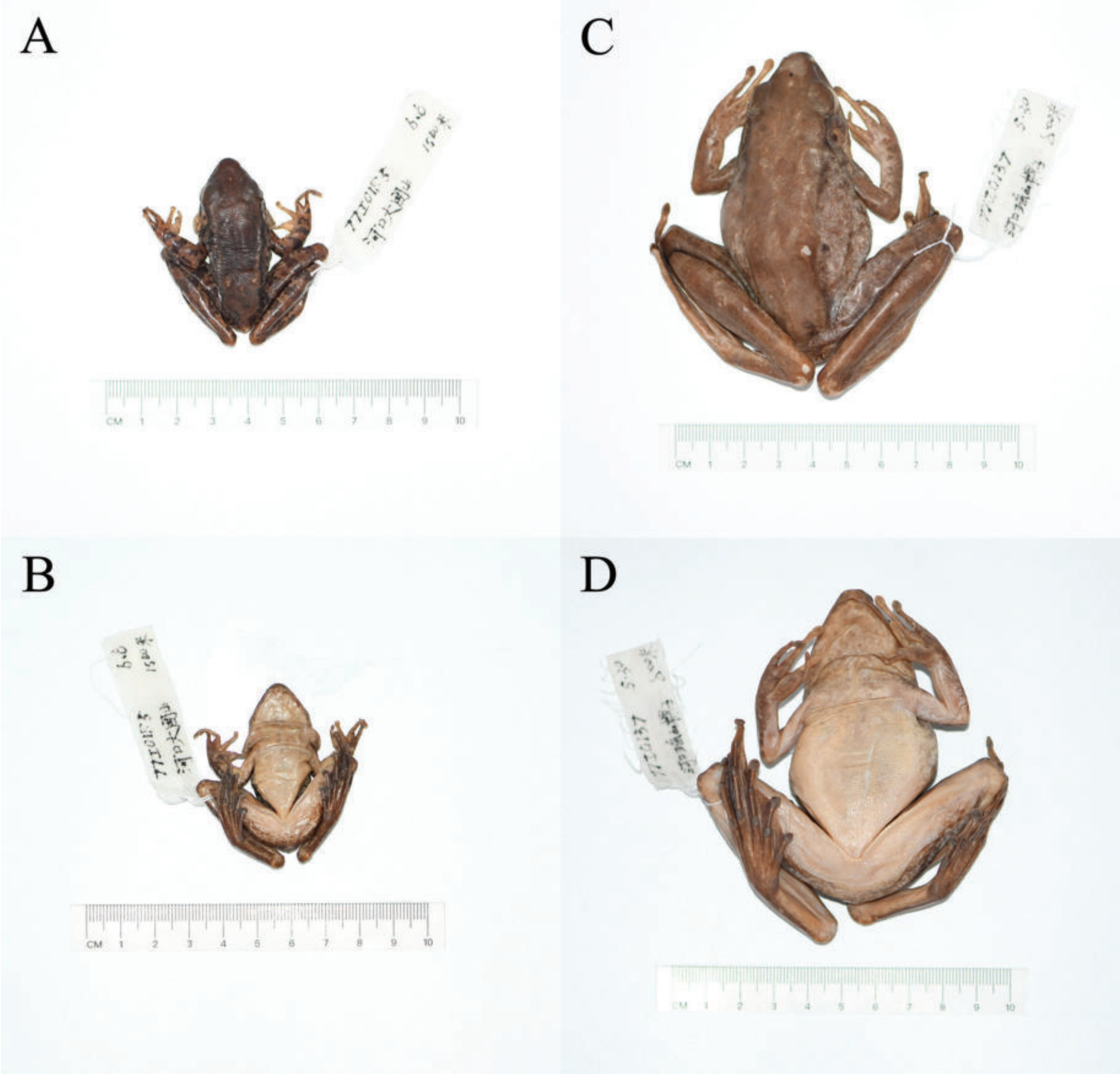

Figure 5. The type specimens of *Odorrana tiannanensis* in preservative. **A.** Dorsal; **B.** Ventral views of the holotype (KIZ 77I0185, male); **C.** Dorsal; **D.** Ventral views of the allotype (KIZ 77I0137, female).

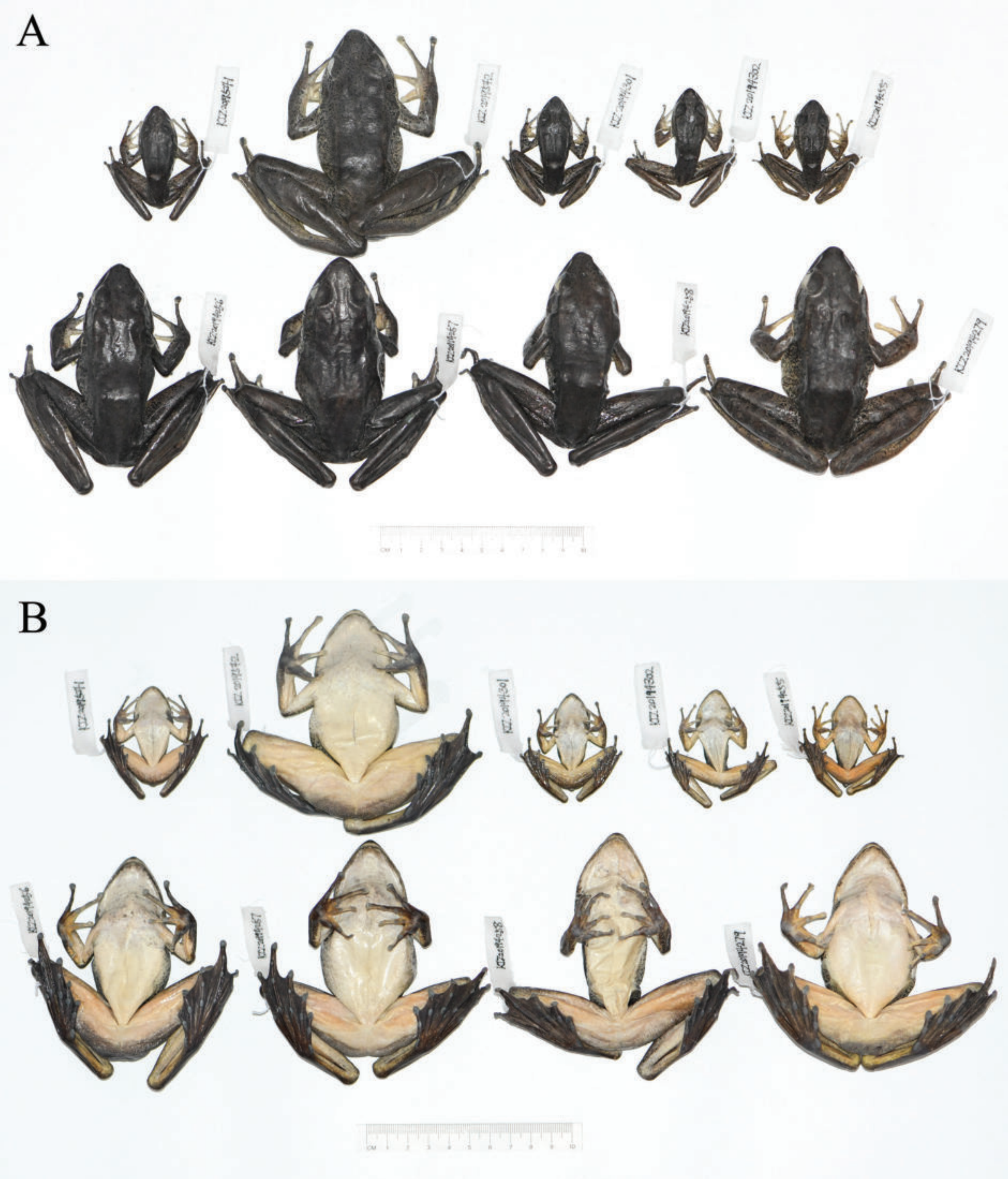

Figure 6. Dorsal view (**A**) and ventral view (**B**) of the specimens of *Odorrana heatwolei* from China in preservative.

creamy white; ventral surface of hindlimbs light yellow; buttocks slightly pink; tympanum dark brown; and iris yellow in males. Coloration in females very similar to but usually slightly lighter than that in males.

Coloration in preservative. Dorsum color turned darker to grayish black, the dense tiny black dots on dorsum still visible, dorsal color of limbs turned darker to brownish black, the bands on limbs became indistinct. Ventral color of head and forelimbs, chest, and belly faded to pale white, ventral color of hindlimbs turned to flesh color. Iris became black, pupil became white.

Natural history. The specimens from China were all found on the banks of rivers at night. No eggs or tadpoles were found from April to May. There were many stones and forests with good vegetation on the banks of the river (Fig. 8). An *Amolops* species of *Amolops* cf. *vitreus* (Bain, Stuart & Orlov, 2006) is distributed sympatric with this species.

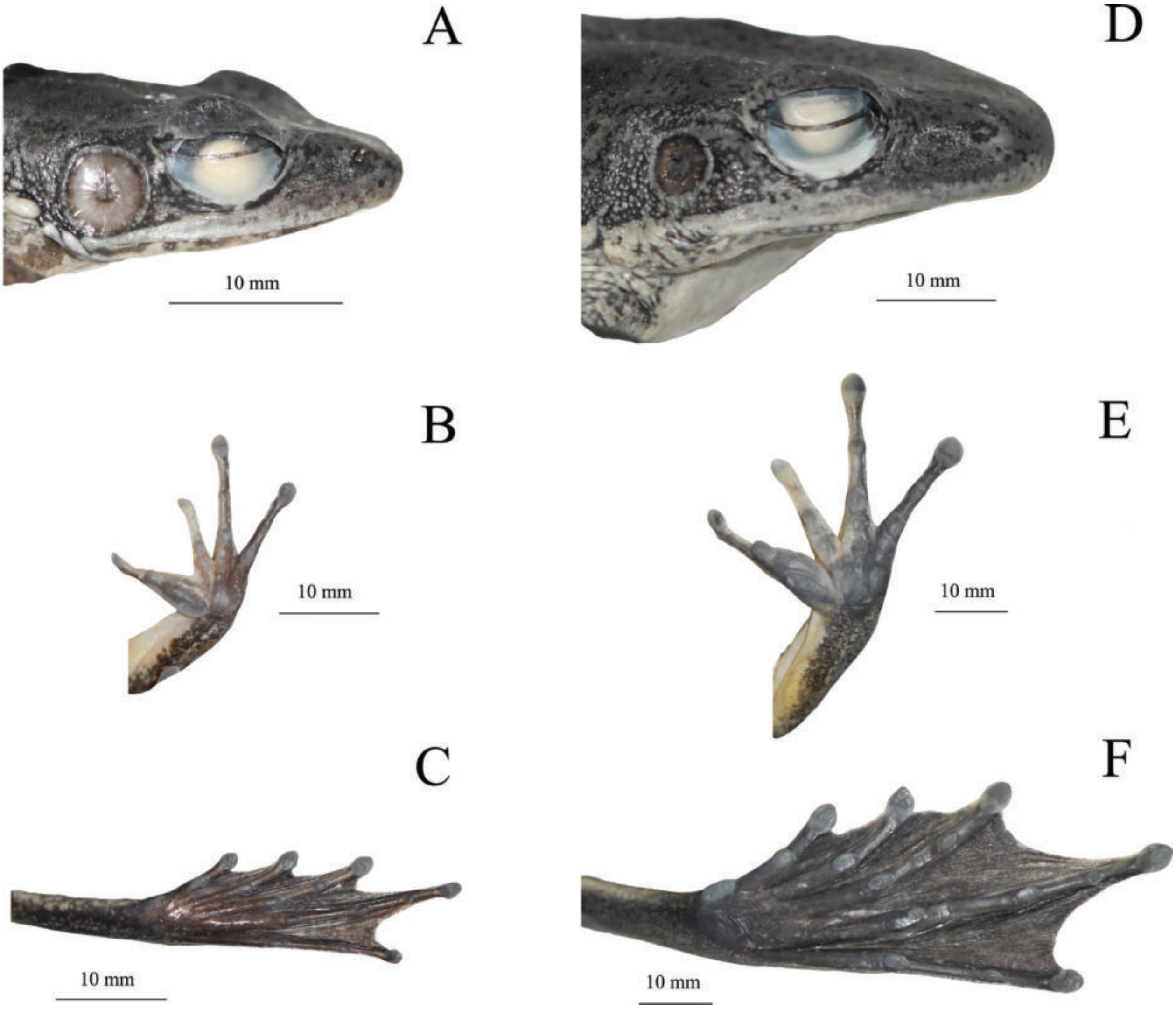

Figure 7. Close-up views of the head (**A**), vola (**B**), and planta (**C**) of the male (KIZ2019541) and close-up views of the head (**D**), vola (**E**), and planta (**F**) of the female (KIZ2019542) of *Odorrana heatwolei* from China in preservative.

Figure 8. The habitat at the collection site of the specimens of *Odorrana heatwolei* in Mengla, Yunnan, China.

Distribution. Currently known in Phongsaly Province, Laos, and Xishuangbanna Prefecture, Yunnan Province, China (Fig. 1).

Discussion

Ohler (2007) took *Odorrana heatwolei* as a synonym of *O. tiannanensis* based on only morphological characteristics. These two species are indeed very similar in morphological measurements, but their body colorations are significantly different. In addition, these two species form two separate lineages molecularly and the genetic distance between them is large.

The straight distance between the type locality of *Odorrana heatwolei* and the type locality of *O. tiannanensis* is approximately 200 km, and they are separated by the Red River (Song Hong River in Vietnam). The straight distance between the collection site of the specimens of *O. heatwolei* from Xishuangbanna, Yunnan, China, and the type locality of *O. heatwolei* is approximately 60 km, and there is no obvious geographical obstacle between them. These specimens from Xishuangbanna are the first record of *O. heatwolei* from China.

Odorrana tiannanensis was considered previously to be widely distributed in southern Yunnan, China, and northern Vietnam and northern Laos (Frost 2023). We found that this species is likely to be distributed only in the east of the Red River including southeastern Yunnan, China, and northeastern Vietnam, and *O. heatwolei* is likely to be distributed only in the west of the Red River including south-central and southwestern Yunnan, China, and northern Laos. More samples are needed to verify the geographical isolation between the two species.

It was found previously that *Odorrana macrotympana* and *O. tiannanensis* are closely related (Liu et al. 2022), however, the distributions of these two species are far apart, one in westernmost Yunnan and northern Myanmar, and the other one in southeastern Yunnan (Fig. 1), and no other species was found that can connect these two species in the large area between their distributions, which seems unlikely. *Odorrana heatwolei* just filled this gap. However, the distribution of *O. heatwolei* is close to that of *O. tiannanensis* and far apart from that of *O. macrotympana*. Therefore, we speculate that there may be species that can connect *O. heatwolei* and *O. macrotympana* in the area between their distributions. More investigations in these areas are needed to verify this speculation.

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Daily activities of water monitors (*Varanus salvator macromaculatus* Deraniyagala, 1944) in urban wetland, Bangkok, Thailand

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Abstract

From July to October 2015, a study was conducted on the water monitors (*Varanus salvator macromaculatus*) at Dusit Zoo in Bangkok, Thailand. A total of 90 monitors were captured, measured for SVL, sexed, and marked on the right axilla. The captured monitors included 70 females and 20 males, resulting in a sex ratio of 3.5:1. Body size did not differ significantly between genders. The monitors exhibited bimodal diurnal activity, with a focus on fish hunting in the morning (06:00–08:00 h) and scavenging for fish leftovers in the afternoon (15:00–17:00 h). After the monitor lizards hunted for prey in the morning, they spent 5–7 hours basking and floating in the lake during the afternoon. Sexual behaviors were also occasionally observed. The monitors displayed frequent predatory behavior towards natural prey, especially fish, in the morning, as opposed to opportunistically scavenging on tourist food and fish leftovers observed throughout the day.

Key Words

diet, ethology, sex ratio, Squamata, urban ecosystem, Varanidae

Introduction

The water monitor (*Varanus salvator macromaculatus* Deraniyagala, 1944) is the world's second-largest extant monitor species after the Komodo dragon (*V. komodoensis* Ouwens, 1912) and is widely distributed throughout Thailand, except for a large area in the north and northeast (with the northernmost record being Sukhothai Province), and southward through to Peninsular Malaysia, in various ecosystems, particularly urban wetlands, like those of Bangkok (Shine et al. 1996; Lauprasert 1999; Lauprasert and Thirakhupt 2001; Pauwels et al. 2002, 2003, 2009; Cota et al. 2009; Chan-ard and Makchai 2011; Chan-ard et al. 2011; Chuaynkern and Chuaynkern 2012; Auliya and Koch 2020). Abundant niches in Bangkok attract this species, which faces growing human populations overlapping with established territories, leading to human-wildlife conflicts over resources and habitats (Traeholt 1993; Traeholt 1994a, b; Auliya 2003; Uyeda 2009; Cota 2011; Trivalairat et al. 2016). Despite being protected under the Wild Animal Reservation and Protection Act of 1992 (B.E. 2535) (the newest version is Act of 2019 (B.E. 2562)) in Thailand, hunting of water monitors frequently occurs in overlapped areas, especially in Bangkok, for solving the human-wildlife conflict or harvesting animal products (Luxmoore and Groombridge 1990; Lauprasert and Thirakhupt 2001; Uyeda 2009). To manage conflicts effectively, studies of their population structure, natural history, and behaviors in certain habitats are necessary.

Bangkok, the capital city of Thailand, is situated in the Chao Phraya River delta on the central plain of the

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country. The region is flat and low-lying, with an average elevation of 1.5 meters above sea level (Sinsakul 2000). Historically, most of the area was swampy, but it was gradually drained and irrigated for agricultural purposes before being transformed into an urban area. Nowadays, many wetlands are fragmented and disconnected from one another. In 1938, one of the largest wetland habitats in Bangkok, covering a total area of 188,000 m^2, was converted into the first zoo in Thailand known as Dusit Zoo or Khao Din Zoo (Sae-ngo et al. 2013). The large lake and surrounding canals are primarily influenced by the Chao Phraya River (Cota 2011). This zoo features various exhibited animals, activity areas, education museums, and restaurants for visitors. However, the increasing human activities have led to an abundance of food leftovers in the environment, which are exploited by local animals such as fish, crows, and especially water monitors. More available niches make the area more attractive for colonization by different species. Therefore, this locality may be one of the most suitable areas in the central part of Thailand for investigating the relationship between humans and water monitors.

The aim of this study was to explore the population structure, daily behaviors, and dietary habits of the free-ranging water monitor population within the Dusit Zoo range. These findings could offer insights into the species' natural history and provide a better understanding of its urban ecosystem's population structure, daily activities, and behavior, aiding in effective wildlife management.

Materials and methods

Sample collection

During the period of 2 to 30 June 2015, catchable subadult and adult water monitors in the Dusit Zoo range (13°46'18.58"N, 100°30'57.98"E) were captured using a noose pole, excluding those with a snout-vent length (SVL) of less than 30 cm, which are considered juveniles (Fig. 1). The direct sampling method was used to determine the abundance of the population, and the sex ratio at a specific time was recorded for each captured individual (Skalski et al. 2005; Rodda 2012). SVL was measured ventrally, from the tip of the snout to the vent, and sex was identified via hemipenes eversion or probe insertion. In most cases, males everted their hemipenes themselves during capture. Each individual was tagged on the right axilla for future identification before being released at its capture site. The tracking tag was customized using an elastic hospital tag, cable tie, waterproof paper, and waterproof pens. A different color-coded band was used for each water monitor to identify its body size range, sex, and number, along with a unique identification number (Fig. 2). After the study was completed, each tagged individual was re-captured for a final time, and its tag was removed.

Figure 1. Study area of Dusit Zoo range, Bangkok, Thailand (Google Map 2015).

In addition to the previous data on personal hatchling from five clutches (4, 5, 8, 10, 12 individuals/clutch) in Bangkok and metropolitans (from Trivalairat et al. 2016), our research team also classified and compared the proportion of each captured individual during observation. The previous data provide information on the SVL and total length (TL) of the hatchlings, which were found to be 10.68 ± 1.43 cm (range 8.6–13.8 cm, n = 39) and 24.88 ± 1.88 cm (range 21.2–29.3 cm, n = 39) respectively. It is worth noting that the total length of hatchling longer than 30 cm was never found. Therefore, this data was used as a reference for classifying and comparing a proportion of each captured individuals during observation by defined X as a three-time proportion of average hatchling SVL (× = 30.0 cm).

The SVL was divided into four sized ranges: 2× range – less than 60.0 cm (yellow tag); 3× range – 60.1 to 90.0 cm (orange tag); 4× range – 90.1 to 120.0 cm (green tag); and 5× range – more than 120.0 cm (purple tag).

Observations

Tagged water monitors were observed between 06:00 h and 18:00 h during the rainy season (July to October 2015) in Dusit Zoo. This observation period overlapped with the breeding season of the water monitor, which occurs between March and October, particularly after the first heavy rains following the long dry period (Shine et al. 1996; Cota 2011). Observations were conducted on Monday, Wednesday, and Friday of each week, with each round of observation starting from an entrance and walking clockwise along the lake. The average time per round was one hour, totaling 12 rounds per day. The first contact activity of the tagged monitors was counted and recorded within 30 seconds of observation. Data were recorded in different time intervals and analyzed separately. Additionally, preys or items consumed by the water monitors were also counted and recorded to identify the diet types.

This study observed and described five activities: 1) basking activity– lying on or being exposed to the sunshine on both lands and trees, with some showing little

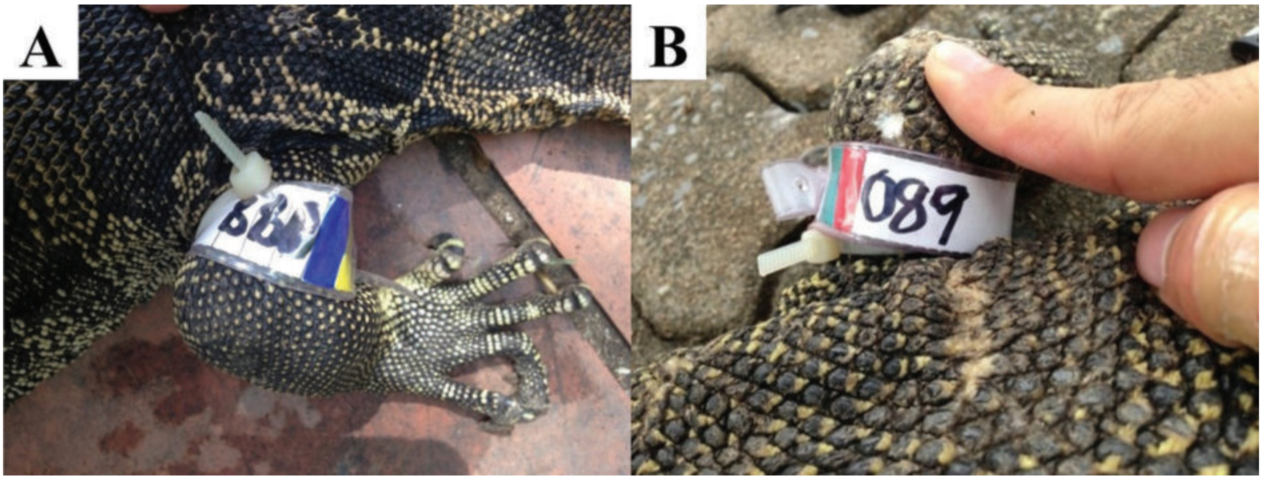


Figure 2. Captured water monitor lizards (*Varanus salvator macromaculatus*) in Dusit Zoo range with customized tag on right axilla. **A.** Male number 088 with snout-vent length ≤ 60.0 cm; and **B.** Female number 089 with snout-vent length between 90.1 and 120.0 cm.

movement for changing position; 2) foraging activity – moving towards prey, hunting, or consuming resources, including the classification of predation behavior into two types (grouped predation, which is predation performed by more than one individual, and solo predation, which is predation performed by an individual); 3) surveying activity – moving for a long distance or time without direction towards nearly attractive thing; 4) floating activity– floating or slowly swimming within a small area in water; and 5) sexual activity – a period wherein each individual displays some pre-courtship or courtship behaviors. Additionally, the calculation of disappeared individuals, referring to those that disappeared or were unable to be observed during the study, was determined for each hour to be included in the analysis of activity rates. This was done by subtracting the number of observed individuals from the total count of tagged individuals (number of disappeared individuals = total count of tagged individuals – number of observed individuals).

Furthermore, in this study, sexual activities were classified into two types: pre-courtship and courtship. Pre-courtship behavior consisted of two observed behaviors. The first behavior, known as male-male competition or wrestling behavior, involved aggression where individuals with an SVL greater than 60 cm stood and pushed each other. The second behavior, called sneaking behavior, was observed in smaller individuals with an SVL shorter than 60 cm. They would wait and satellite around the territory area of larger males, occasionally avoiding the larger male and briefly seizing opportunities to mate with females.

Regarding courtship behaviors, three distinct behaviors were observed. The first was forced mating behavior, characterized by multiple males engaging in polyandrous behavior. The second behavior was single couple mating, indicating monogamous behavior. The third behavior observed was female-harem mating, representing polygynous behavior.

Statistical analysis

The IBM SPSS Statistics 28 software package (SPSS Inc.; Chicago, IL, USA) was used to calculate the mean and standard deviation of body size (snout-vent length, SVL, excluding total length due to potential bias caused by incomplete tails in some individuals), as well as daily activity encounters (basking, foraging, surveying, floating, and engaging in sexual activities) which also accounted for disappeared individuals, and diet types. The body size, daily activities, and diet types were analyzed with a 5% types I error risk. As these parameters were not normally distributed, non-parametric tests were used to compare variables. An independent sample *t*-test was conducted to examine the differences in body size between genders (female and male) and diet types (predating and scavenging diets). The mean daily activities were analyzed using one-way ANOVA, including examining the relationships between six variables of daily activities and monthly mean environmental factors such as temperature (°C) and rainfall (mm).

Results

Sex ratio and body size

Ninety individuals of water monitor (*Varanus salvator macromaculatus*) were captured and tagged in this study, revealing a significantly higher proportion of females (n = 70) than males (n = 20), at a ratio of 3.5:1. Through observation, an additional 32 non-catchable individuals were identified, bringing the total number of individuals to 122. The captured sample had an average SVL of 72.14 ± 12.44 cm (range 46.0–94.0 cm, n = 90) for all captured individuals, with females having a higher SVL (72.89 ± 12.84 cm, range 46.0–94.0 cm) than males (69.51 ± 10.84 cm, range 48.6–81.5 cm). No catchable monitor with an SVL larger than 120 cm was caught.

Most captured females were in 3× range (76.67 ± 8.66 cm, range 62.6–90.0 cm, n = 49), followed by the 2× range (55.33 ± 4.30 cm, range: 46.0–60.0 cm, n = 16), and the 4× range (92.12 ± 1.46 cm, range 90.2–94.0 cm, n = 5) (Table 1). For the male population, the most frequent SVL was observed in the 3× range (74.01 ± 6.07 cm, range 62.9–81.5 cm, n = 16), followed by the 2× range (51.50 ± 4.40 cm, range 48.6 – 58.0 cm). Although there was a small difference in SVL between females and males, this difference was not statistically significant (*t* = 1.18, *p* = 0.12).

Table 1. Number and mean of each snout-vent length (SVL) range of captured female and male water monitor lizards (*Varanus salvator macromaculatus*) in Dusit Zoo range.

SVL range		Female		Male		Total	
		n	Mean ± SD (range) (cm)	*n*	Mean ± SD (range) (cm)	*n*	Mean ± SD (range) (cm)
≤ 60.0 cm	2×	16	55.3 ± 4.17 (46.0–60.0)	4	51.5 ± 3.81 (48.6–58.0)	20	54.6 ± 4.49 (46.0–60.0)
60.1–90.0 cm	3×	49	76.4 ± 8.57 (62.6–90.0)	16	74.0±5.88 (62.9–81.5)	76	76.0 ± 8.14 (62.6–90.0)
90.1–120.0 cm	4×	5	92.1 ± 1.31 (90.2–94.0)	–	–	5	92.1 ± 1.31 (90.2–94.0)
Total		70	72.9 ± 12.8 (46.0–94.0)	20	69.5 ± 10.8 (48.6–81.5)	90	72.1 ± 12.4 (46.0–94.0)

Daily activities

The activity patterns of water monitors ranging in Dusit Zoo were continuously observed for four months during the rainy season (July through October 2015). Observations were carried out for 14 days in July 2015 and 13 days in each month of August, September, and October 2015. The tagged monitors were observed for more than 33.25% in each hour (28.79% for females and 38.85% for males), and no significant difference was found in their activities among each month, except for surveying activity (*f* = 4.463, *p* = 0.003) (Table 2). Monthly mean temperature and rainfall did not affect the activities, except for surveying activity, which was influenced by rainfall (*r* = -0.312, *p* = 0.015) (Table 3) (Pierson and Pierson 2015a-d).

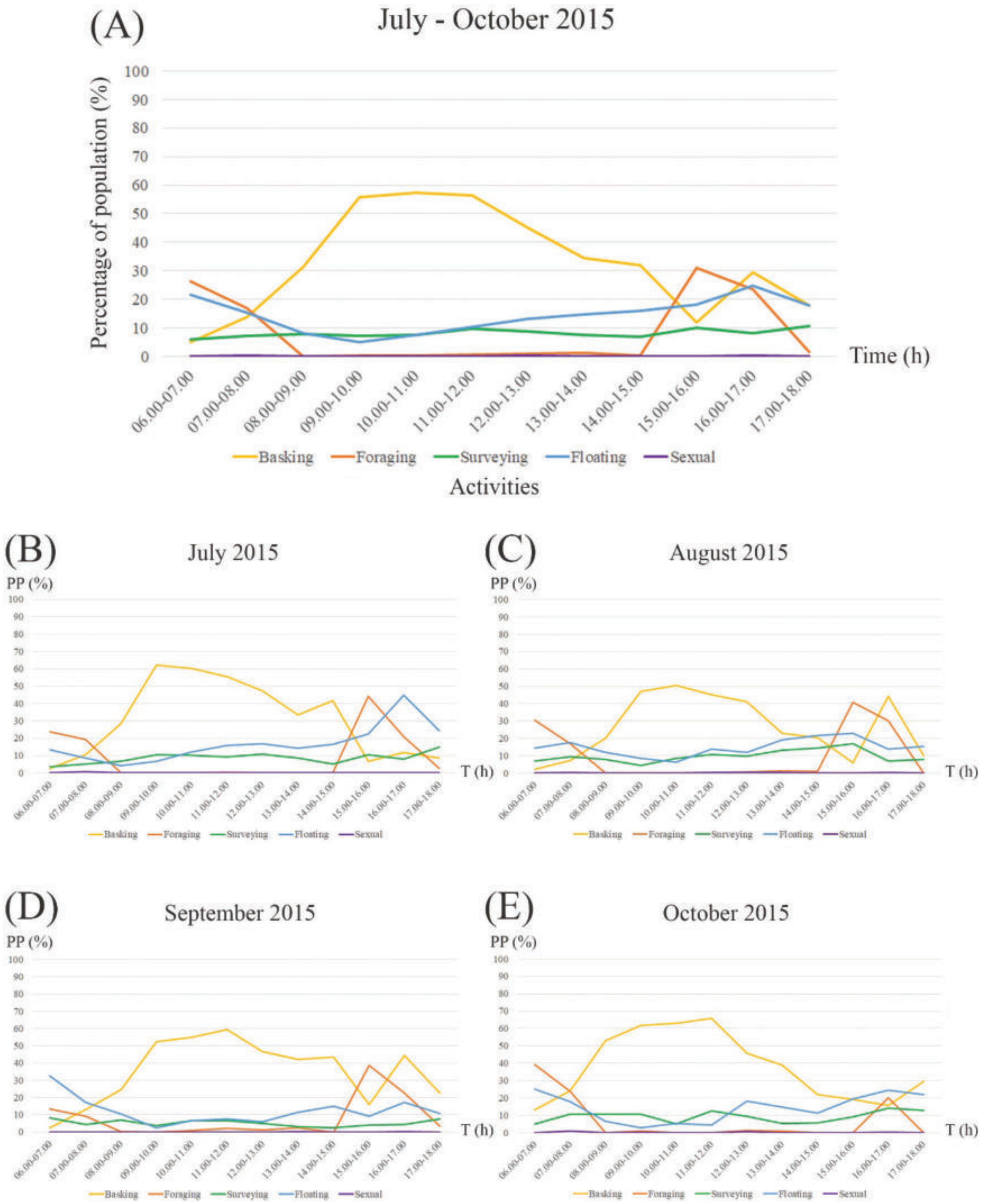
Over forty percent of the monitors (mean 32.53 ± 18.21% per hour, min-max = 2.05–65.81% per hour) mostly spent their time basking between 09:00–13:00 h. The longest average duration of basking activity, with a proportion higher than the mean, was recorded in September (7 hours), followed by October (6 hours), July (6 hours), and August (5 hours**)** (Fig. 3) (Suppl. material 1). Basking activity significantly increased with decreasing foraging (*r* = -0.591, *p* = 0.000), floating (*r* = -0.619, *p* = 0.000), and disappeared individuals (*r* = -0.440, *p* = 0.000) (Table 3).

Foraging activity peaked twice a day, with records of at least 10% of proportion (mean 8.59 ± 12.12% per hour, min-max = 0–44.02% per hour), starting with the first period in the morning (06:00–08:00 h) and then in the afternoon (15:00–17:00 h). The proportion of foraging in the afternoon (range 23.42–30.96%) was slightly higher than in the morning (17.04–26.39%) among the study. Accordingly, the foraging activity in the morning was typically group predation for large fishes in the lake, while the foraging activity in the afternoon scavenged on fish leftovers from zookeepers. Moreover, the foraging rate was positively correlated with floating (*r* = 0.445, *p* = 0.000), but negatively correlated with disappeared individuals (*r* = -0.339, *p* = 0.008).

For floating activity, at least 10% proportion (mean 14.44 ± 5.91% per hour, min-max = 2.38–44.70% per hour) was spent in water during 06:00–09:00 h and 11:00–18:00 h. Approximately 5–11% of the population in each hour performed surveying activity within the Dusit Zoo range (mean 8.13 ± 1.43% per hour, min-max = 2.46–17.01% per hour). Both floating and surveying activities were significantly correlated (*r* = 0.291, *p* = 0.024). Additionally, the analysis revealed three specific time intervals during which the tagged population exhibited a disappearance rate exceeding 40%. These intervals were observed from 06:00 to 09:00 h (with a

Table 2. Mean percentage (± SD) and One-Way ANOVA Analysis of daily activities in tagged water monitor lizards (*Varanus salvator macromaculatus*) (*n* = 90 individuals), including disappeared individuals, in the Dusit Zoo Range from July to October 2015.

Times (h)	Total mean percentage (100%)					
	Mean daily activities percentage (%)					Mean disappeared individual percentage (%)
	Basking	Foraging	Surveying	Floating	Sexual	
July to October 2015						
06:00–07:00	5.03	26.39	5.89	21.61	0.00	41.07
07:00–08:00	13.69	17.04	7.30	15.39	0.42	46.16
08:00–09:00	31.34	0.08	7.99	8.26	0.00	52.33
09:00–10:00	55.74	0.19	7.21	5.05	0.08	31.72
10:00–11:00	57.23	0.25	7.55	7.57	0.00	27.40
11:00–12:00	56.46	0.75	9.66	10.34	0.04	22.75
12:00–13:00	45.26	0.86	8.68	13.08	0.23	31.89
13:00–14:00	34.49	1.26	7.44	14.76	0.08	41.97
14:00–15:00	32.05	0.23	6.83	16.08	0.00	44.80
15:00–16:00	12.03	30.96	10.08	18.28	0.00	28.64
16:00–17:00	29.33	23.42	8.20	24.91	0.21	13.94
17:00–18:00	17.76	1.59	10.75	17.97	0.00	51.93
Mean ± SD	32.53 ± 18.21	8.59 ± 12.12	8.13 ± 1.43	14.44 ± 5.91	0.09 ± 0.13	36.22 ± 12.01
F	0.585	0.091	4.463	0.507	0.950	0.571
P	0.675	0.985	0.003	0.731	0.442	0.685



Figures 3. A–E. Mean daily activity graph of water monitor lizard (*Varanus salvator macromaculatus*) population in Dusit Zoo range in 2015 **A.** July to October; **B.** July; **C.** August; **D.** September; and **E.** October. PP = percentage of population (%), T = time (h).

disappearance rate ranging from 41.07% to 52.33%), 13:00 to 15:00 h (with a disappearance rate ranging from 41.97% to 44.80%), and 17:00 to 18:00 h (with a disappearance rate of 51.93%). The mean disappearance rate for the entire day was 36.22 ± 12.01% per hour, with a range of 4.70% to 66.75% per hour.

In addition, a total of 51 instances of sexual activities were observed, with varying durations. Two types of pre-courtship behavior were documented. The first type involved male-male competition, which was observed in four pairs, resulting in a cumulative occurrence of eight instances. The second type entailed sneaking behavior, observed in 11 smaller individuals, transpiring a total of 11 times.

Regarding courtship behaviors, three distinct patterns were discerned. Forced mating behavior manifested itself in 13 occurrences. Single couple mating behavior was observed in two pairs, amounting to a total of four instances. Additionally, female-harem mating behavior was observed, featuring a male to female ratio of 1:2, 1:2, 1:3, and 1:4, respectively, with a cumulative occurrence of 15 instances.

Table 3. Spearman's rank correlation (*r*) and *p* – value (*p*) of daily activities (basking (BAS), foraging (FOR), surveying (SUR), floating (FLO), and sexual activities (SEX), including undiscovered individual (UND)) of water monitor (*Varanus salvator macromaculatus*) and mean monthly temperature (TEMP) and rainfall (RAIN).

Activities		TEMP	RAIN	BAS	FOR	SUR	FLO	SEX
BAS	*r*	-0.178	0.193					
	p	0.174	0.139					
FOR	*r*	0.075	-0.076	-0.591*				
	p	0.569	0.562	0.000				
SUR	*r*	0.147	-0.312*	-0.083	-0.003			
	p	0.264	0.015	0.530	0.984			
FLO	*r*	0.128	-0.091	-0.619*	0.445*	0.291*		
	p	0.331	0.488	0.000	0.000	0.024		
SEX	*r*	-0.092	-0.030	-0.038	0.179	0.088	0.006	
	p	0.484	0.821	0.772	0.172	0.504	0.961	
UND	*r*	0.069	-0.070	-0.440*	-0.339*	-0.251	-0.161	-0.141
	p	0.600	0.595	0.000	0.008	0.053	0.220	0.284

* Correlation is significant at 0.05.

Diet evidence

This study identified a total of 18 prey types (5,015 observations). Leftovers showed the highest number of observation (2,857 observations, 56.97%), followed by bony fish (2,084 observations, 41.56%), turtles (68 observations, 1.36%), birds (4 observations, 0.08%), toad (1 observation, 0.02%), and cat (1 observation, 0.02%) (Fig. 4) (Table 4). There was no significant difference found between predated and scavenged prey items by the monitor (t = -0.833, p = 0.417).

During the study, two types of observed predation behaviors were documented. The first type is group predation, which was observed regularly in *V. salvator macromaculatus* at Dusit Zoo. This behavior usually occurred during the morning hours, between 6:00 and 8:00 h, at a specific location in the lake (13°46'16.7"N, 100°31'00.2"E). The target of this behavior was usually a large fish, typically striped catfish (*Pangasianodon hypophthalmus* (Sauvage, 1878)), which could be more than one meter in length. The individuals initiated the hunt by swimming and diving in a swirling pattern. Once they had caught a fish, they would drag it to the surface and other individuals would join in to help drag the fish to the bank for consumption. The individuals involved in group predation shared the meal together. Additionally, another target of this behavior was the Chinese softshell turtle (*Pelodiscus sinensis* (Wiegmann, 1835)), an allochthonous species that had been released and thrived in this lake.

Solo hunts were also recorded for other prey types, such as small fish, toads, turtles, birds, and cats. Juvenile *V. salvator macromaculatus* were observed eating Asian common toads (*Duttaphrynus melanostictus* (Schneider, 1799)). Upon sensing a toad, the juvenile monitor lizard quickly ran towards it and caught it by the head before retreating to the water to search for a safe place on the island to eat. The caught toad was swallowed whole, beginning with the head. Similar prey-swallowing behavior was observed in the case of fishes and turtles that were small enough to fit into the monitor lizard's mouth.

Typically, it is difficult for *V. salvator macromaculatus* to catch a flying bird such as the Black-crowned night heron (*Nycticorax nycticorax* (Linnaeus, 1758)). However, during the breeding period, some birds try to protect their nests by spreading their wings against climbing monitors, which proves to be ineffective. In one instance, the monitor was able to rush at the bird and catch it before dropping the body into the lake. Based on observation, heron nests are usually constructed on tree limbs that hang over the lake at a height of 2–3 meter from the ground.

The cat was an unexpected prey in this observation. It began with the cat approaching the large monitor that was basking on the bank, seemingly to play with the monitor's tail. However, when the tail was drawn back and thrashed towards the cat, it became motionless. Subsequently, the monitor proceeded to flick and tear the cat into pieces before consuming it.

Table 4. Types of diet evidence of water monitor lizard (*Varanus salvator macromaculatus*) in Dusit Zoo. P = predated diet, S = scavenged diet.

No.	Prey group	Prey type (P/S)	Frequency (times)	Proportion (%)
1	Giant gourami (*Osphronemus goramy*)	Bony fish (P)	512	10.21
2	Striped catfish (*Pangasianodon hypophthalmus*)	Bony fish (P)	1,368	27.28
3	Siamese giant carp (*Catlocarpio siamensis*)	Bony fish (P)	192	3.83
4	Unidentified fish	Bony fish (P)	12	0.24
5	Asian common toad (*Duttaphrynus melanostictus*)	Amphibians (P)	1	0.02
6	Asian box turtle (*Cuora amboinensis*)	Turtles (P)	11	0.22
7	Chinese softshell turtle (*Pelodiscus sinensis*)	Turtles (P)	38	0.76
8	Red-eared slider turtle (*Trachemys scripta*)	Turtles (P)	13	0.26
9	Unidentified turtle eggs	Turtles (P)	6	0.12
10	Black crowned night heron (*Nycticorax nycticorax*)	Birds (P)	2	0.04
11	Mynas (*Acridotheres* sp.)	Birds (P)	2	0.04
12	Cat (*Felis catus*)	Mammalia (P)	1	0.02
13	Bread	Leftover (S)	15	0.30
14	French fries	Leftover (S)	15	0.30
15	Fried Chicken	Leftover (S)	32	0.64
16	Plastic bag	Leftover (S)	9	0.18
17	Processed meat (Meatballs, sausages, or crab sticks)	Leftover (S)	16	0.32
18	Yellow-stripe scad (*Selaroides leptolepis*)	Leftover (S)	2,770	55.23
Total			5,015	100

Figures 4. Diet evidence of water monitor lizards (*Varanus salvator macromaculatus*) **A.** Asian box turtle (*Cuora amboinensis*); **B.** Chinese softshell turtle (*Pelodiscus sinensis*); **C.** Asian common toad (*Duttaphrynus melanostictus*); and **D.** Feather of mynas (*Acridotheres* sp.) in stool.

Discussion

Life history patterns

Foraging activity

Activity pattern in different spatial populations of intraspecific species, particularly water monitors (*Varanus salvator macromaculatus*), may occur convergently or divergently, depending on adaptive shifts towards food availability and ecological systems (Traeholt 1997a, b; Uyeda et al. 2013; Rahman et al. 2017; Gautam 2020). In this study, we observed that the Dusit population exhibited a routine bimodal diurnal pattern, with a preference for preying on big fish in the early morning (06.00–08.00 h) and scavenging on fish leftovers in the afternoon (15.00–17.00 h) during the rainy season, even when there were no leftovers. This circadian rhythm is similar to that of other water monitor populations, such as those found in the Philippines, Bangladesh, and Tulai Island (located east of mainland Peninsular Malaysia), where they are active foraging twice during the day (Gaulke 1992; Traeholt 1997b; Rahman et al. 2017). Bimodal diurnal patterns have also been observed in other monitors, such as Bengal monitor (*V. bengalensis* (Daudin, 1802)), sand monitor (*V. gouldii* (Gray, 1838)), desert monitor, (*V. griseus* (Daudin, 1803)), Komodo dragon (*V. komodoensis*), lace monitor (*V. varius* (Shaw, 1790)), and perentie monitor (*V. giganteus* (Gray, 1845)), during summer (Corkill 1928; King 1980; King et al. 1989; Auffenberg 1994; Trembath 2000; Guarino 2002; Ibrahim 2002; Yuni and Purwandana 2022). However, the foraging pattern of the Dusit population contrasts with those of the Tinjin island (Philippines) and Timor Oil Palm Estate (Malaysia) populations, which adapt to the food availability in nighttime and predator pressure (i.e., pythons, civets, dogs, and leopards), respectively (Traeholt 1997b; Uyeda et al. 2013). Consequently, this evidence may support the idea that food availability is one of the primary determinants in adapting their foraging patterns based on available diet (Auffenberg 1994; Gaulke 1989; Trembath 2000; Cota et al. 2008; Yong et al. 2008; Uyeda et al. 2013).

Basking activity

Monitor lizards are ectothermic organisms that rely on environmental temperature or solar radiation to generate their metabolism and regulate their body temperature through behavioral means (Pianka 1970; Pafilis et al. 2007; Mendyk et al. 2014). In the wild, the average body temperature of water monitors during activity was found to be around 29.9 °C in Sri Lanka and 30.4 °C Malay Peninsula, while captive juveniles presented an average temperature of 35–36 °C (Gleeson 1981; Wikramanayake and Green 1989; Traeholt 1995). The average activity temperature of monitor lizards varies depending on their body size, species, and the heat they receive from the sun and ambient environment. It reaches its highest in the late afternoon (32.7 °C), does not exceed 30 °C in the morning, and drops at night (Gleeson 1981; Auffenberg 1994; Traeholt 1995).

Normally, monitor lizards need to elevate their body temperature by basking in the sun after experiencing a cooling period overnight, before they can partake in activities such as foraging (Traeholt 1997a; Abayaratna and Mahaulpatha 2006; Rahman et al. 2017). Surprisingly, the Dusit population of monitor lizards exhibits an unusual pattern by initiating their first active behavior (hunting) prior to sunrise, before engaging in basking. This exceptional phenomenon might be influenced by favorable ecological factors, particularly high ambient temperatures that benefit the water monitor lizard. In Bangladesh and Tulai Island, the water monitor lizard populations exhibit basking behavior before hunting after colder nights (9.2 °C) or during rainy nights, respectively (Traeholt 1997a; Rahman et al. 2017). The wetter month also delays their activity until 09.00, resulting in a unimodal diurnal pattern. However, the Dusit population does not experience cool nights during the study period despite the occurrence of rainfall. Instead, they endure warm weather overnight (mean 27.83±1.85 °C at midnight: 28.74±2.10 °C in July; 28.03±1.56 °C in August; 27.30±1.95 °C in September; 27.23±1.36 °C in October) transitioning to warmer dawns (mean 26.76±1.32 °C at midnight: 27.45±1.59 °C in July; 27.03±1.05 °C in August; 26.37±1.27 °C in September; 26.16±0.90 °C in October) (Date and Time 2015). Consequently, the Dusit population is able to raise its body temperature sufficiently from the surrounding conditions in order to engage in active behavior without basking. Therefore, the choice between being active before basking or basking before being active may be influenced by ambient temperature and the retained body heat during the night.

After finishing their morning feeding session, a majority of tagged individuals (30–65%) spend 5–7 hours per day basking. The proportion of time spent basking continually increases and peaks at 10:00–12:00 h before dramatically falling in the afternoon. However, this basking period is representative of population activity by time and does not indicate a specific or exact individual rate. Some intra- and interspecific individuals show specific and exact individual rates, such as Malaysian water monitor population (38 minutes per day), Komodo dragons (110 minutes per day), and sand monitors (60–102.5 minutes per day) (King 1980; Auffenberg 1981; Traeholt 1995).

Two ideas could explain the length of time spent basking in these monitors. Firstly, the previous studies of King (1980), Auffenberg (1981) and Traeholt (1995) were performed in the hot season, while this study was done in the rainy season. Even though the monthly mean temperature and rainfall in Bangkok Metropolis since July through October 2015 revealed no relationship with basking activity, rainfall could directly affect the sunlight and the relatively narrow range of ambient and ground temperature (which was absent in this study) that facilitate thermoregulation in the body (King 1980; Gleeson 1981; Traeholt 1995). Accordingly, mean rainfall showed a significant quantity in September (352.4 mm), which was related to an average basking rate of 7 hours in the same month, followed by October (334.2 mm, 6 hours), July (220.8 mm, 6 hours), and August (50.5 mm, 5 hours) (Pierson and Pierson 2015a-d). Consequently, rainfall can directly influence the basking duration of the monitors by reducing sunlight exposure and increasing heat loss during rainy conditions. This may lead to an increase in basking behavior as they attempt to compensate for the decreased heat availability and ensure sufficient heat gain.

Another reason that could have influenced the prolonged basking time was the significant number and size of prey available. At Dusit Zoo, the water monitor's diet consisted mostly of large-sized fish, including iridescent shark catfish (*Pangasianodon hypophthalmus* (Sauvage, 1878)), giant gourami (*Osphronemus goramy* (Lacépède, 1801), and Siamese giant carp (*Catlocarpio siamensis* Boulenger, 1898). In contrast, the population from Malaysia primarily fed on crabs and small invertebrates (Rahman et al. 2017). Therefore, consuming a significant number and size of prey may have affected the lizard's basking time, as noted by Bontrager et al. (2006).

Floating and surveying activities

The frequency of floating activity increased twice a day, with the first peak occurring in the morning (06:00–09:00 h) as water monitors prepared to hunt for fish in the lake. The second period was observed after basking, from 11:00 h through 18:00 h, during which most water monitors spent time floating in the lake without movement, spreading their forelimbs and hindlimbs. This behavior may serve as a cool-down mechanism to regulate their body temperature, as noted by Traeholt (1995), who reported a similar behavior in the population from Tulai Island, Malaysia, where the monitors entered the sea to cool down and escape tourists. The increase in floating activity in the afternoon could also be influenced by visitors, who tend to visit the zoo more frequently in the afternoon, and the presence of leftover fish on the banks of the lake, which attracted the water monitors. Thus, the water monitors may enter the water for different reasons at different times, such as hunting, cooling down, or escaping.

Sexual behavior

Sexual behaviors were observed randomly throughout the day during the study period. Cota (2011) suggested that changes in weather conditions between the cold (November to February) and hot seasons (March to May) may trigger hormonal changes in water monitors in the Dusit

Zoo range. However, this study was conducted during the late rainy season (May to October) (Pierson and Pierson 2015a-d). Therefore, the long duration between the initial triggering of sex hormones in March and the observation period may have led to a decrease in sex hormones and a reduction in sexual behaviors, which were rarely observed in this study.

However, some sexual behaviors, including pre-courtship and courtship, were recorded. Firstly, the most well-known male-male competition behavior, wrestling behavior, occurred between large male monitors during the breeding season. This behavior was observed to compete and territorially defend to gather an area, resources, and a chance to court other females (Auffenberg 1981; Wikelski et al. 1996; Cota 2011; Uyeda et al. 2015). Male-male competition behavior also occurs in other reptiles such as the marine iguana (*Amblyrhynchus cristatus* Bell, 1828), which displays head-bobbing behavior to gather a lek area (a rich-resourced territory area) and also females (Wikelski et al. 1996). Unfortunately, many people, especially Thai people, frequently perceive this behavior as courtship behavior between males and females, but they are mistaken. Additionally, the dominant male in this competition usually attracts many females to the area, forming a harem (polygamous behavior).

Sometimes, wrestling competitions were absent, and numerous similar-sized males attempted to mate with only one female, displaying polyandrous behavior. This behavior is also observed in other reptiles, such as the keeled earless lizard (*Holbrookia propinqua* Baird & Girard, 1852), where males compete in "sperm competition" to inherit their genetics (Cooper 1984).

Surprisingly, smaller, non-territorial males, triggered by sex hormones, also develop strategies to court during the breeding season. Observations suggest that these smaller individuals swim or sneak around the females in the territory of dominant males like satellites, called sneakers, and wait for the dominant male to appear careless or drive away other males before quickly moving towards the females to court briefly before the dominator returns. The non-territorial marine iguana also displays this behavior around the lek areas of the dominant male (Wikelski et al. 1996).

Therefore, water monitors display five sexual behaviors during the breeding season: 1) single couple mating behavior; 2) wrestling behavior (male-male competition behavior) by large males; 3) harem behavior (polygyny) by dominant males; 4) forced mating behavior (polyandry) by large males; and 5) sneaking behavior (non-territorial competition behavior) by small males.

Sex ratio bias

Sex ratio is the most basic demographic parameter that involves birth, death, immigration, and emigration rates, providing a relative change in the number of females and males in particular conditions (Galliard et al. 2005; Skalski et al. 2005; Sapir et al. 2008). According to Fisher's principle, dioecious species tend to maintain a sex ratio close to 1:1, the most common evolutionary stable strategy (ESS), due to independent segregation of chromosomes during meiosis for balancing the gender ratio in the next generation (Fisher 1930; Komdeur 2012; Mitchell et al. 2013). Some monitor populations, including water monitors from Singapore, Bengal monitors from India, and spiny-tailed monitors (*V. acanthurus* Boulenger, 1885) from northern Australia, have displayed a sex ratio close to 1:1 (King and Rhodes 1982; Auffenberg 1994; Rashid 2004). However, maintaining an equal sex ratio after fertilization is more complicated when environmental factors come into play (Bulmer and Bull 1982; Janzen 1994; Janzen and Phillips 2006).

Natural selection often results in a balance between individuals in a population, which depends on the existing sex ratio and the relative costs and benefits of producing offspring of each gender (Sapir et al. 2008; Komdeur 2012). The quality and stability of the environment, both temporal and spatial, can influence the dynamic of a population by establishing a gender that is cheaper to exist under minimum environment conditions and can create a bias in the sex ratio (Skalski et al. 2005; Sapir et al. 2008; Fišer 2019). Even genetic elements, such as genetic-dependent sex determination (GSD), can contribute to the balancing of the primary sex ratio in some monitor lizards, such as spiny-tailed monitors, rock monitors (*V. albigularis* (Daudin, 1802)), Nile monitors (*V. niloticus* (Linnaeus, 1766)), and lace monitors (*V. varius*) (Valenzuela 2004; Bachtrog et al. 2014). In other reptile species, sex determination has evolved in response to environment effects, especially ambient temperature transition, known as temperature-dependent sex determination (TSD) (Viets et al. 1994; Valenzuela 2004; Bachtrog et al. 2014; Pipoly et al. 2015; Cornejo-Páramo et al. 2020). This development occurs in amniotes, including water monitors. As environments vary spatio-temporally, TSD may fluctuate in populations after fertilization, making them susceptible to experiencing maladaptive sex-ratio skews, up to 4–20 times more than GSD species due to fluctuation of ambient temperature in nests (Auffenberg 1994; Valenzuela 2004). Therefore, the TSD system associated with global warming might be the first reason for indicating bias in the secondary sex ratio of water monitors from the skew in birth rate. Furthermore, TSD species are more at risk of encountering inbreeding and genetic diversity loss than GSD from a single sex dominance due to the present global warming effect, but this thermal sensitivity might also generate an adaptive maternal nesting behavior for sex ratio selection in some individuals (Nelson et al. 2004; Mitchell et al. 2013).

The other reason why sex allocation in a population of relatively long-lived individuals might be skewed at any time is due to various effects, including food availability, maternal condition or quality, attractiveness or quality of males, social environment, sibling competition, sexual conflict, and life-history traits, which can cause changes in mortal and migration rates (Komdeur 2012; Cornejo-Páramo et al. 2020). The results suggested that the observed duration overlapping with the breeding season showed a greater number of adult female water monitors than males. Additionally, the increasing production of sex hormones during the breeding season might be the main effect that triggers sexual behaviors and biases in the sex ratio for both females and males (Cota 2011). Aggression, which is the most common male sexual emotion, triggered by testosterone, could lead to territorial behaviors by displaying male-male competition for dominance and hierarchical ranking, providing a chance to sire offspring, including guarding them from sneakers, the smaller or non-territorial individuals (Bradbury 1977, 1981, 1985; Emlen and Oring 1977; McNicol and Crews 1979; Svensson and Petersson 1988; Wiley 1991; Wikelski et al. 1996; Galliard et al. 2005). However, aggressions between male and female or among females were absent in this study. Furthermore, in the non-breeding season, aggressions and conflicts are rare or absent (Cota 2011). In summary, the skew in female monitors during the breeding season might be affected by non-conflict toward the population and the attractiveness of alpha males as a sexual selection. In contrast, male individuals might decrease due to competition and limited area. A diet resource was abundant. Thus, the bias in the tertiary sex ratio (ratio in sexually mature) of water monitors in Dusit Zoo range during breeding season might exist in females rather than males affected mainly by individual conflicts in population.

Consequently, the sex ratio at any one time or area might not reflect the true ratio in the robust population, but it can represent the ratio in some season, age range, and habitat, as well as the ratio in this study, which represented the greater female ratio in adult water monitors during the breeding season (July to October 2015) in Dusit Zoo.

Sexual dimorphism

Sexually dimorphism is typically observed in monitor lizards (Khan 1969; Auffenburg 1994; Shine 1993; Shine et al. 1996). Previously, studies have reported on morphological differences between sexes, such as male Bengal monitors being 9.2% larger than females, and male water monitors being heavier and longer in tail than non-pregnant females at the same SVL (Khan 1969; Auffenberg 1994; Shine et al. 1996). In this study, captured female individuals indicated a greater length in SVL than males, but there was no significant difference in SVL between females and males. This similarity might be influenced by dominant and territorial males driving other males out of the study area, leaving mostly females. Thus, in the non-breeding season, a comparison of body size characters might show some differences like other water monitor populations when conflict is absent, but not in this breeding season.

Diet preference

Total dietary evidence indicated no significant difference between predation and scavenger behaviors of monitors in the Dusit Zoo range. As a result, the fish prey had a strong influence on this population, as they likely learned to hunt and at the designated leaving area (13°46'23.5"N, 100°31'01.8"E). This area is where zookeepers consistently deposit the leftover food and waste materials generated by zoo animals at a specific time each day (15:00–17:00 h), even on days when no additional resources are provided. In addition, they also habituated to opportunistic feeding on leftovers from visitors such as bread, French fries, fried chicken, and processed meat (meatball, sausage, or crab stick), including food-contained plastic bags, without harming tourists. The leftover scavenging and foraging time of water monitors occurred similarly relative to the availability of humans' leftovers in various urban ecosystems, such as Indonesia, Malaysia, and Thailand) (Traeholt 1994a; Uyeda 2009; Uyeda et al. 2013, 2015; Kulabtong and Mahaprom 2015). This opportunistic scavenging provides reduced energy loss from foraging, encountering, and handling (Kane et al. 2017). Accordingly, this adaptive change in feeding habits associated with an absence of hunting or human harm might occur convergently in the maintenance and coexistence of water monitor populations in overlapping urban areas (Traeholt 1994a; Auliya 2003). However, the well-adaptive shift in diverse ecosystems and wide-ranging diet preferences of this monitor currently causes conflicts with humans in various areas due to the bad attitude perception toward this species (especially among Thai people) and commercial damage from domestic and farm animal loss (Shine et al. 1998; Lauprasert 1999; Uyeda 2009; Uyeda et al. 2012, 2013, 2015; Kulabtong and Mahaprom 2015; Rahman et al. 2017). Thus, to reduce the conflict from scavenging in urban areas, the leftover management must be done well to eliminate the attractive factor, especially food leftovers.

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Supplementary material 1

Mean percentage (± SD) of daily activities in tagged water monitors (*Varanus salvator macromaculatus*)

Authors: Poramad Trivalairat, Sompod Srikosamatara

Data type: docx

Explanation note: Mean percentage (± SD) of daily activities in tagged water monitors (*Varanus salvator macromaculatus*) (*n* = 90 individuals), including disappeared individuals, in Dusit Zoo range during July through to October 2015.

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A new cryptic species of *Tylototriton* (Amphibia, Caudata, Salamandridae) from mysterious mountain lakes in Manipur, north-eastern India

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Abstract

An integrative taxonomic analysis combining molecular and morphological lines of evidence revealed a new cryptic species in the *Tylototriton verrucosus* species group from Manipur, north-eastern India. The new species was previously confused with *T. himalayanus* and *T. verrucosus*. *Tylototriton zaimeng* **sp. nov.** can be distinguished from its congeners by medium body size, head massive and wide with rounded snout and very wide and protruding supratemporal bony ridges and a well-developed sagittal ridge, short limbs not overlapping when adpressed along body, wide and not segmented vertebral ridge distinct, 13–14 pairs of rib nodules, brown colouration with dull orange to yellowish-brown markings on head, vertebral ridge, rib nodules, palms, soles, vent and ventral tail ridge and by vomerine teeth organised in two distinctly curved bell-shaped series. Phylogenetic analysis of the ND2 and 16S rRNA mtDNA genes confirmed the placement of the new species to the Clade I of the subgenus *Tylototriton* and suggested it is a sister species of *T. panwaensis* and *T. houi* (*p*-distance 3.0% in ND2 gene). The range of the new species is restricted to the Khongtheng Mountain Range and is isolated from the range of *T. panwaensis* and *T. houi* in northern Myanmar and southern China, respectively. We suggest the new species to be considered as Vulnerable (VU) in the IUCN Red List.

Key Words

16S rRNA, endemism, Khongtheng Mountain Range, mtDNA genealogy, ND2, taxonomy, *Tylototriton zaimeng* sp. nov.

Introduction

The salamandrid genus *Tylototriton* Anderson, 1871 (colloquially known as Crocodile newts) represents an ancient salamander lineage which currently includes 39 recognised species, inhabiting montane forest areas throughout the Asian monsoon climate zone from the southern and eastern Himalaya, southern and central China, to the northern part of Indochina (Poyarkov et al. 2021a; Dufresnes and Hernandez 2022; Frost et al. 2023). The genus has been traditionally subdivided into the two subgenera: the subgenus *Tylototriton* Anderson, 1871 is characterised by the presence of bright orange or red markings on the head, dorsum, tail or sides of the body, while the subgenus *Yaotriton* Dubois & Raffaëlli, 2009 is characterised by generally much darker almost blackish colouration (Yang et al. 2014; Wang et al. 2018; Li et al. 2020; Poyarkov et al. 2021b; Luy et al. 2021;

Dufresnes and Hernandez 2022). Many new species of crocodile newts have been described recently, based on a combination of morphological and molecular investigations (e.g. Nishikawa et al. (2013, 2014); Le et al. (2015); Phimmachak et al. (2015); Khatiwada et al. (2015); Grismer et al. (2018, 2019); Zaw et al. (2019); Bernardes et al. (2020); Li et al. (2020); Pomchote et al. (2020a, 2021); Poyarkov et al. (2021b); Dufresnes and Hernandez (2022); Phung et al. (2023)).

In India, salamanders of the genus *Tylototriton* were reported from north-eastern part of the country and were traditionally identified as *Tylotriton verrucosus* Anderson, 1871 (Seglie et al. 2003; Ahmed et al. 2009; Mathew and Sen 2010; Singh and Devi 2011). Salamanders of the genus *Tylototriton* were recorded from the eastern Himalayas (from Nepal to West Bengal, Sikkim, Bhutan and the westernmost Arunachal Pradesh), but were also recorded from montane areas of Meghalaya (Das 1984) and Manipur (Selim 2001; Devi and Shamungou 2006). In the latest reviews on distribution of *Tylototriton* salamanders (Hernandez 2016; Hernandez et al. 2018), these populations were all tentatively assigned to *T. himalayanus* Khatiwada et al., 2015, a species described from the Himalaya in eastern Nepal (Khatiwada et al. 2015). Hernandez et al. (2018) noted that the region between the eastern Himalayas and the Irrawaddy River valley is still unclear and might represent a contact area between the two species of crocodile newts, *T. himalayanus* and *T. verrucosus*. They suggested that this region may be home to some cryptic, undescribed taxa. However, to date, no integrative analysis of the taxonomic status of Manipur *Tylototriton* populations has been conducted and these populations were not included in the most recent phylogenetic assessment of the genus (Dufresnes and Hernandez 2022).

In the present study, we apply an integrative taxonomic approach comparing the results of morphological analyses with those of mitochondrial DNA (mtDNA) genealogy, based on the ND2 and 16S rRNA genes to assess the taxonomic status of *Tylototriton* sp. populations from highland montane forests of Manipur, north-eastern India. Our examination of the morphological differentiation of the newly-collected material from Manipur and northern Myanmar resulted in the discovery of stable character state differences in colouration and external morphology between the two populations which are concordant with the results of the molecular phylogenetic analyses. Herein, we describe the population of *Tylototriton* sp. from Manipur State of India as a new, previously overlooked, cryptic species of *Tylototriton*.

Materials and methods

Sample collection

Specimens of *Tylototriton* sp. were collected from three localities within Manipur State of north-eastern India during several field surveys in 2022. Specimens of *Tylototriton* sp. were collected by hand in lakes or swamps in forest clearings surrounded by montane evergreen tropical forests. Geographic coordinates and altitude were obtained using a Garmin GPSMAP 60CSx GPS receiver (Garmin Ltd., USA) and recorded in datum WGS 84. Specimens were euthanised by 20% benzocaine and tissue samples (liver) for genetic analysis were taken and stored in 96% ethanol prior to preservation. Specimens were subsequently fixed in 4% formalin, transferred to 70% ethanol and deposited in the herpetological collection of the Departmental Museum of Zoology, Mizoram University (**MZMU**) in Aizawl, India. Additional specimens examined are listed in Appendix 1.

Morphological description

Specimens of *Tylototriton* sp. were photographed in life and after preservation. The sex and maturity of the specimens were checked by minor dissections. Measurements were taken using a digital caliper to the nearest 0.01 mm, subsequently rounded to 0.1 mm. We used a stereoscopic light binocular microscope when necessary. Morphometrics followed Poyarkov et al. (2021b) and included the following 23 measurements taken for the type series and used in inter-specific comparisons: (1) **SVL** (snout-vent length) from tip of snout to anterior tip of vent; (2) **HL** (head length); (3) **HW** (head width); (4) **MXHW** (maximum head width); (5) **IND** (internarial distance); (6) **AGD** (axilla-groin distance); (7) **TRL** (trunk length); (8) **TAL** (tail length) from anterior tip of vent to tail tip; (9) **VL** (vent length); (10) **FLL** (fore-limb length); (11) **HLL** (hind-limb length); (12) **VTW** (vomerine tooth series width): greatest width of vomerine tooth series; (13) **LJL** (lower jaw length from tip of lower jaw to articulation of upper and lower jaws); (14) **SL** (snout length from tip of snout to anterior tip of upper eyelid); (15) **IOD** (minimum interorbital distance); (16) **UEW** (maximum upper eyelid width); (17) **UEL** (upper eyelid length, distance between anterior and posterior angles); (18) **OL** (orbit length); (19) **BTAW** (basal tail width at level of anterior tip of cloaca); (20) **MTAW** (tail width at mid-level of tail); (21) **MXTAH** (maximum tail height); (22) **MTAH** (tail height at mid-level of tail); and (23) **ON** (orbitonarial distance). For holotype description, we additionally examined the following 12 morphometric characters following Poyarkov et al. (2012, 2021b): (24) **ICD** (intercanthal distance); (25) **CW** (chest width); (26) **NSD** (nostril-snout distance); (27) **1FL** (first finger length from base to tip); (28) **2FL** (second finger length from base to tip); (29) **3FL** (third finger length from base to tip); (30) **4FL** (fourth finger length from base to tip); (31) **1TL** (first toe length from base to tip); (32) **2TL** (second toe length from base to tip); (33) **3TL** (third toe length from base to tip); (34) **4TL** (fourth toe length from base to tip); (35) **5TL** (fifth toe length from base to tip). We also examined the following meristic characters: (36) **DLWN** (dorsolateral wart number, the number of rib nodules on right/left sides of the dorsum); (37) **UJTN** (number of teeth on the upper jaw); (38) **LJTN** (number of teeth on the lower jaw); (39) **VTN** (number of teeth on vomer).

An analysis of variance (ANOVA) was performed to ascertain if statistically significant mean differences amongst morphomeristic characters ($p < 0.05$) existed amongst the discrete populations delimited in the phylogenetic analyses. ANOVAs having a p-value less than 0.05, indicating that statistical differences existed, were subjected to a Tukey HSD test to ascertain which population pairs differed significantly ($p < 0.05$) from each other. We used the Principal Component Analysis (PCA) to determine if populations from different localities occupied unique positions in morphospace, as well as the degree to which their variation coincided with potential species boundaries as predicted by the molecular phylogeny and univariate analyses. Characters used in the PCA included continuous mensural data. In order to normalise the PCA data distribution and to transform meristic and mensural data into comparable units for analysis, we natural log-transformed all PCA data prior to analysis and scaled it to their standard deviation. To exclude possible overweighting effects, when we found a high correlation between certain pairs of characters, we omitted one of them from the analyses. Statistical analyses were carried out using Statistica 8.0 (Version 8.0; StatSoft, Tulsa, OK, USA).

The diagnosis of the genus *Tylototriton* and morphological characters for comparison were taken from original descriptions and taxonomic reviews of the genus: Anderson (1871); Unterstein (1930); Fang and Chang (1932); Liu (1950); Fei et al. (1984); Nussbaum et al. (1995); Böhme et al. (2005); Chen et al. (2010); Stuart et al. (2010); Hou et al. (2012); Shen et al. (2012); Nishikawa et al. (2013; 2014); Yang et al. (2014); Le et al. (2015); Phimmachak et al. (2015); Hernandez (2016); Khatiwada et al. (2016); Qian et al. (2017); Grismer et al. (2018, 2019); Than Zaw et al. (2019, 2020); Hernandez and Pomchote (2020); Onishi et al. (2020); Pomchote et al. (2020a, 2021); Dufresnes and Hernandez (2022); and Phung et al. (2023).

Laboratory methods

Total genomic DNA was extracted from 95% ethanol-preserved liver tissues using standard phenol-chloroform extraction protocols (Hillis et al. 1996). Total DNA concentration was estimated in 1 μl using a NanoDrop 2000 spectrophotometer (Thermo Scientific, USA) and consequently adjusted to 100 ng DNA/μl. We amplified two mtDNA fragments including the partial sequences of the ND2 and 16S rRNA mtDNA genes. These markers were chosen as they were proven to be informative in studies of *Tylototriton* phylogeny and taxonomy (Wang et al. 2018; Than Zaw et al. 2019; Poyarkov et al. 2021b). We used the 16L-1 (forward) (5’-CTGACCGTGCAAA GGTAGCGTAATCACT-3’) and 16H-1 (reverse) (5’-CTCCGG TCTGAACTCAGATCACGTAGG-3’) primers to amplify the 16S rRNA fragments following Hedges (1994). For amplification and sequencing of the ND2 gene, we used the SL-1 (forward) (5’-ATAGAGGTTCAAACCCTCTC-3’) and SL-2 (reverse) (5’-TTAAAGTGTCTGGGTTGCATTCAG-3’) primers of Wang et al. (2018). Polymerase chain reaction (PCR) conditions followed Poyarkovet al. (2021b). PCR was performed in 20 μl using 50 ng genomic DNA, 10 nmol of each primer, 15 nmol of each dNTP, 50 nmol additional $MgCl_2$, Taq PCR buffer (10 mmol/l Tris-HCl, pH 8.3, 50 mmol/l KCl, 1.1 mmol/l $MgCl_2$ and 0.01% gelatin) and 1 U of Taq DNA polymerase. PCR cycles included an initial denaturation step of 4 min at 94 °C and 35 cycles of denaturation for 30 s at 94 °C, primer annealing for 30 s at 48–58 °C and extension for 1 min 30 s at 72 °C. PCR products were visualised by agarose gel electrophoresis in the presence of ethidium bromide and consequently purified using 2 μl from a 1:4 dilution of ExoSAP-IT (Amersham, UK) per 5 μl of PCR product prior to cycle sequencing. Sequencing was performed in both directions using the same primers as used in PCR on an ABI3730xl automated sequencer (Applied Biosystems, USA) at Barcode Bioscience, Bangalore (India). The newly-obtained sequences from seven specimens of *Tylototriton* sp. from Manipur were aligned and deposited in GenBank under the accession numbers OQ996133–OQ996139 and OR039162–OR039168 (Table 1). Sequences of 39 other *Tylototriton* species used for comparisons along with two sequences of *Echinotriton* Nussbaum & Brodie, 1982 spp. which were used as outgroup taxa were obtained from GenBank (see Table 1).

Phylogenetic analyses

Sequences of partial fragments of ND2 and 16S rRNA mtDNA for 53 Salamandridae specimens, including 51 representatives of *Tylototriton* (representing 39 taxa) and two sequences of outgroup members of Salamandridae *Echinotriton raffaellii* Hernandez & Dufresnes, 2022 and *Echinotriton chinhaiensis* (Chang, 1932) were included in the final alignment with a total length of up to 1665 bp. For comparison purposes, we selectively picked one individual per each nominal species/lineage of *Tylototriton*; therefore, the resulting phylogenetic hypothesis is not representative of the entire variation of *Tylototriton* at 16S rRNA and ND2 mtDNA genes. Information on voucher specimens and GenBank accession numbers used in phylogenetic analyses are summarised in Table 1. Nucleotide sequences were initially aligned in MAFFT v.6 (Katohet al. 2002) with default parameters and then checked by eye and slightly adjusted in BioEdit 7.0.5.2 (Hall 1999). The dataset was divided into four partitions: three codon partitions for the ND2 gene and a single partition for 16S rRNA, with the optimal evolutionary models for each estimated using ModelTest v. 3.06 (Posada and Crandall 1998). According to the Akaike Information Criterion (AIC), the HKY+G model was selected for the ND2 gene as the best fit for the first and second codon partitions

Table 1. Sequences and voucher specimens of *Tylototriton* and outgroup taxa used in this study. For institutional abbreviations, see below. Numbers in brackets after the locality name correspond to those shown in Fig. 1.

No.	Species name	Voucher number	Locality	16S rRNA	ND2
	Ingroup:				
1	*T. anguliceps*	TBU PAE671	Thuan Chau, Son La, Vietnam	–	LC017833
2	*T. anhuiensis*	CIB 08042905-2	Yuexi Co. Anhui, China	KY800587	KY800853
3	*T. asperrimus*	CIB 70063	Longsheng Co., Guangxi, China	KY800549	KC147816
4	*T. broadoridgus*	CIB 200084	Sangzhi Co., Hunan, China	KY800570	KY800837
5	*T. dabienicus*	HNNU 1004-015	Shangcheng Co., Anhui, China	KY800607	KC147811
6	*T. daloushanensis*	CIB WG200600019	Suiyang Co., Zunyi, Guizhou, China	KY800544	KY800817
7	*T. hainanensis*	CIB 20081048	Mt. Diaoluo, Hainan, China	KY800553	KC147817
8	*T. gaowangjienensis*	JWS20210100	Guzhang Co., Hunan, China	ON764431	ON764431
9	*T. himalayanus*	CIB 201406246	Mai Pokhari, Illam, Mechi, Nepal (1)	KY800590	KT765173
10	*T. houi*	MZL46960	Yulong, Lijiang, Yunnan, China (13)	ON008211	–
11	*T. zaimeng* sp. nov.	MZMU-2947	Zaimeng Lake, Kangpokpi Dist., Manipur, India (2)	OQ996133	OR039162
12	*T. zaimeng* sp. nov.	MZMU-2948	Zaimeng Lake, Kangpokpi Dist., Manipur, India (2)	OQ996133	OR039162
13	*T. zaimeng* sp. nov.	MZMU-3035	Phungyar, Kamjong Dist., Manipur, India (4)	OQ996134	OR039163
14	*T. zaimeng* sp. nov.	MZMU-3036	Phungyar, Kamjong Dist., Manipur, India (4)	OQ996135	OR039164
15	*T. zaimeng* sp. nov.	MZMU-3037	Chingjaroi Ngachaphung, Ukhrul Dist., Manipur, India (6)	OQ996136	OR039165
16	*T. zaimeng* sp. nov.	MZMU-3038	Chingjaroi Ngachaphung, Ukhrul Dist., Manipur, India (6)	OQ996137	OR039166
17	*T. zaimeng* sp. nov.	MZMU-3041	Chingjaroi Ngachaphung, Ukhrul Dist., Manipur, India (6)	OQ996138	OR039167
18	*T. kachinorum*	ZMMU A5953	In Gyi Taung Mt., Indawgyi, Kachin, Myanmar (8)	MK095618	MK097273
19	*T. kweichowensis*	CIB Wg20080818014	Bijie City, Guizhou, China	KY800551	KY800823
20	*T. liuyangensis*	CSUFT 20100108	Liuyang City, Hunan, China	KY800606	KJ205598
21	*T. lizhenchangi*	KUHE 42316	Yizhang Co., Hunan, China	KY800621	KY800881
22	*T. maolanensis*	CIB Wg20090730001	Libo Co., Guizhou, China	KY800575	KY800842
23	*T. ngarsuensis*	LSUHC 13763	Ngar Su, Taunggyi Dist., Shan, Myanmar (14)	–	MH836585
24	*T. notialis*	FMNH 271120	Boualapha Dist., Khammouan, Laos	–	HM462061
25	*T. panhai*	PH019	Phu Hin Rong Kla NP, Phitsanulok, Thailand	–	AB830735
26	*T. panwaensis*	CAS 245418	Panwa, Myitkyina Dist., Kachin, Myanmar (10)	–	KT304279
27	*T. obsti*	IEBR 4471	Xuan Nha NR, Van Ho, Son La, Vietnam	–	MT210168
28	*T. pasmansi*	IEBR 4466	Phu Canh NR, Da Bac, Hoa Binh, Vietnam	–	MT201166
29	*T. phukhaensis*	CUMZ A-7717	Doi Phu Kha NP, Nan, Thailand	–	MN912573
30	*T. podichthys*	IEBR A.2014.1	Xam Neua, Huaphanh, Laos	–	LC017835
31	*T. pseudoverrucosus*	CIB WCG2012012	Ningnan Co., Liangshanyizu, Sichuan, China	KY800599	KY800860
32	*T. pulcherrimus*	CIB TY040	Lüchun Co., Yunnan, China	KY800626	KY800890
33	*T. shanjing*	KIZ 201306081	Yongde Co., Yunnan, China	KY800593	KY800856
34	*T. shanorum* (lineage 1)	CAS 230940	Taunggyi Township, Shan, Myanmar (16)	–	AB922823
35	*T. shanorum* (lineage 2)	KUHE 42348	Pinluang Township, Shan, Myanmar (17)	–	AB769544
36	*T. sini*	CIB XZ20091201	Xinyi City, Guangdong, China	KY800616	KY800876
37	*T. sparreboomi*	IEBR 4476	Sin Ho, Lai Chau, Vietnam	–	MT210162
38	*T. taliangensis*	CIB GG200110183	Shimian Co., Yan'an City, Sichuan, China	KY800559	KC147819
39	*T. thaiorum*	ZMMU A-7577	Pu Hoat NR, Nghe An, Vietnam	MW883482	MW883478
40	*T. tongziensis*	CIB WH10001	Wufeng Co., Hubei, China	KY800600	KY800863
41	*T. uyenoi*	KUHE 19037	Doi Inthanon, Chiang Mai, Thailand	–	AB830730
42	*T. verrucosus*	CIB TSHS1	Longchuan Co., Dehong, Yunnan, China (12)	KY800581	KY800847
43	*T. vietnamensis*	IEBR A.3674	Tay Yen Tu, Bac Giang, Vietnam	KY800614	KY800874
44	*T. wenxianensis* (lineage 1)	CIB 20090527	Wenxian Co., Gansu, China	KY800579	KC147813
45	*T. wenxianensis* (lineage 2)	CIB 20070638	Qingchuan Co., Sichuan, China	KY800543	KY800816
46	*T. wenxianensis* (lineage 3)	CIB 20090601	Wangcang Co., Sichuan, China	KY800571	KY800838
47	*T. wenxianensis* (lineage 4)	CIB 20080002	Yunyang Co., Chongqing, China	KY800540	KY800813
48	*T. wenxianensis* (lineage 5)	CIB 20080003	Yunyang Co., Chongqing, China	KY800541	KY800814
49	*T. yangi*	KUHE 42282	Pingbian Co., Yunnan, China	KY800624	KY800887
50	*T. ziegleri*	KUHE 55078	Quan Ba, Ha Giang, Vietnam	–	AB769541
51	*T. umphangensis*	CUMZ-A-8243	Umphang WS., Tak, Thailand	–	OK092618
	Outgroups:				
52	*Echinotriton chinhaiensis*	CIB ZHJY1	Zhenhai Co., Zhejiang, China	KY800627	KY800891
53	*Echinotriton raffaellii*	MVZ 232187	Tokunoshima, Kagoshima, Japan	EU880314	EU880314

and the J2+G model was considered the best fit for the third codon partition; TVM+G model was the best fit for the 16S rRNA partition. Mean uncorrected genetic distances (*p*-distances) between sequences were calculated in MEGA 7.0 with missing data and gaps excluded from the analysis (Kumar et al. 2016). The matrilineal genealogy was inferred using Bayesian Inference (BI) and Maximum Likelihood (ML) algorithms. BI analysis was conducted in MrBayes v.3.1.2 (Huelsenbeck and Ronquist 2001; Ronquist and Huelsenbeck 2003). Metropolis coupled Markov Chain Monte Carlo (MCMCMC) analyses were run with one cold chain and three heated chains for 20,000,000 generations and sampled every 2,000 generations. Five independent MCMCMC runs were performed, the MCMC simulations ran for 100,000,000 generations, were sampled every 10,000 generations and the first 10% of the trees from each run were discarded as burn-in. We checked the convergence of the runs and that the effective sample sizes (ESS) were all above 200 by exploring the likelihood plots using TRACER v.1.6 (Rambaut et al. 2014). Confidence in tree topology was tested by posterior probability (BI/PP) for the BI trees (Huelsenbeck and Ronquist 2001). Nodes with PP values over 0.95 were *a priori* regarded as sufficiently resolved, those between 0.90 and 0.95 were regarded as tendencies and values below 0.90 were considered as not supported. We conducted ML analysis in the IQ-TREE webserver. One-thousand bootstrap pseudoreplicates via the ultrafast bootstrap (UFBS; Hoang et al. (2018)) approximation algorithm were employed and nodes having ML UFBS values of 95 and above were *a priori* considered highly supported, while the nodes with values of 90–94 were considered well-supported and the nodes with values of 70–89 were considered as tendencies (Bui et al. 2013).

Results

Sequence variation

The final alignment of the ND2 gene contained 1157 aligned nucleotides, of which 716 sites were conserved and 441 sites were variable, of which 292 were parsimony-informative. The transition–transversion bias (R) was estimated at 4.45. Nucleotide frequencies were 37.49% (A), 23.82% (T), 28.10% (C) and 10.59% (G). The final alignment of the 16S rRNA gene contained 508 aligned characters, of which, 422 sites were conserved and 84 sites were variable, of which 51 were suggested as parsimony-informative. The transition–transversion bias (R) was estimated at 6.16. Nucleotide frequencies were 36.87% (A), 24.85% (T), 20.27% (C) and 18.01% (G) (all data given for ingroup only).

Molecular phylogenetic analysis

BI and ML phylogenetic analyses resulted in almost identical topologies (Fig. 2). The topology of the matrilineal genealogy was largely consistent with the phylogeny of *Tylototriton* presented by Wang et al. (2018), Than Zaw et al. (2019), Poyarkov et al. (2021b), Luy et al. (2021) and Dufresnes and Hernandez (2022) and recovered five main clades grouped into two major reciprocally monophyletic groups, corresponding to the subgenera *Tylototriton* (clades 1–2; Fig. 2) and *Yaotriton* (clades 3–4; Fig. 2).

Monophyly of the subgenus *Tylototriton* received strong support both in ML and BI analyses (100/1.0; Fig. 2); this group included two strongly supported clades:

1) Clade 1 joined the members of *T. verrucosus* species group from India, Nepal, Myanmar, China and northern Indochina (100/1.0) and included 16 nominal species of *Tylototriton*: *T. anguliceps* Le et al., 2015, *T. himalayanus*, *T. houi* Hernandez & Dufresnes, 2022, *T. kachinorum* Zaw et al., 2019, *T. kweichowensis* Fang & Chang, 1932,, *T. ngarsuensis* Grismer et al., 2018, *T. panwaensis* Grismer et al., 2019, *T. phukhaensis* Pomchote et al., 2020, *T. podichthys* Phimmachak et al., 2015, *T. pulcherrimus* Hou et al., 2012, *T. shanjing* Nussbaum et al., 1995, *T. shanorum* Nishikawa et al., 2014, *T. umphangensis* Pomchote et al., 2021, *T. uyenoi* Nishikawa et al., 2013, *T. verrucosus* Anderson, 1871 and *T. yangi* Hou et al., 2012 and a clade, including seven specimens of *Tylototriton* sp. from Manipur, India.
2) Clade 2, which herein is referred to as *T. taliangensis* species group (99/1.0), included two species from western China, namely *T. pseudoverrucosus* Hou et al., 2012 and *T. taliangensis* Liu, 1950.

Monophyly of the subgenus *Yaotriton* was poorly supported in ML analysis, but had significant support in BI analysis (86/1.0, hereafter node values given for ML UFBS/BI PP, respectively; Fig. 2). Subgenus *Yaotriton* included three highly-supported main clades with essentially unresolved phylogenetic relationships amongst them:

3) Clade 3 joined the members of *T. wenxianensis* is species group from China (98/1.0) and included ten species of *Tylototriton*: *T. anhuiensis* Qian et al., 2017, *T. broadoridgus* Shen, Jiang & Mo, *T. dabienicus* Chen et al., 2010, *T. daloushanensis* Zhou et al., 2022, *T. gaowangjienensis* Wang et al., 2022, *T. liuyangensis* Yang et al., 2014, *T. lizhenchangi* Hou et al., 2012, *T. maolanensis* Li et al., 2020, *T. tongziensis* Li et al., 2022 and *T. wenxianensis* Fei et al., 1984.
4) Clade 4 joined the members of *T. asperrimus* species group from China, northern Vietnam and Laos (100/1.0) and included nine nominal *Tylototriton* species: *T. asperrimus* Unterstein, 1930, *T. hainanensis* Fei et al., 1984, *T. notialis* Stuart et al., 2010, *T. pasmansi* Bernardes et al., 2020, *T. obsti* Bernardes et al., 2020, *T. sini* Lyu et al., 2021, *T. sparreboomi* Bernardes et al., 2020, *T. thaiorum* Poyarkov et al., 2021 and *T. ziegleri* Nishikawa et al., 2013.
5) Clade 5, which herein is referred to as *T. vietnamensis* species group (92/1.0), included two peculiar species of *Tylototriton* from northern Vietnam and north-eastern Thailand and adjacent Laos: *T. panhai* Nishikawa et al., 2013 and *T. vietnamensis* Böhme et al., 2005 .

The population of *Tylototriton* sp. from Manipur, India, belongs to clade 1 (Fig. 2), where it is reconstructed as a member of a clade also including *T. panwaensis* from Myanmar and *T. houi* from Yunnan, China. Although monophyly of this clade had strong node support (98/1.0; Fig. 2), genealogical relationships amongst the three included lineages appear to be insufficiently resolved, with *T. houi* and *T. panwaensis* forming a clade which had strong support in BI-analysis, but was poorly supported in ML-analysis (76/1.0; Fig. 2).

Genetic distances

The interspecific uncorrected genetic *p*-distances between the sequences of ND2 mtDNA gene of *Tylototriton* sp. from Manipur, India and other members of *T. verrucosus* species group varied from 3.0% (between *Tylototriton* sp. and its sister species *T. panwaensis*) to 8.6% (between *Tylototriton* sp. and *T. uyenoi*) (see Table 2). Genetic *p*-distances in the 16S rRNA gene were much lower and varied from 0.7% (between *Tylototriton* sp. and its sister species *T. houi*) to 3.3% (between *Tylototriton* sp. and *T. himalayanus*) (see Table 2). There was no intraspecific variation in ND2 and 16S rRNA gene sequences recorded for *Tylototriton* sp. from Manipur (Table 2).

Morphological analysis

Our phylogenetic analyses have unambiguously placed the Manipur population of *Tylototriton* sp. in the *T. verrucosus* species group as a sister lineage to a clade including *T. panwaensis* from Kachin State in northern Myanmar and *T. houi* from northern Yunnan, China. The detailed morphometric data are not available for *T. houi* which was described, based on a single partially decomposed subadult specimen (Dufresnes and Hernandez 2022); however, this species can be unambiguously diagnosed from the Manipur population of *Tylototriton* sp. by a different colouration pattern (black background pattern with bright orange-red markings on parotoids, vertebral ridge, rib nodules, vent, chest, light mid-ventral line, whole limbs and tail; see “Comparisons” section below) and is geographically isolated from north-eastern India.

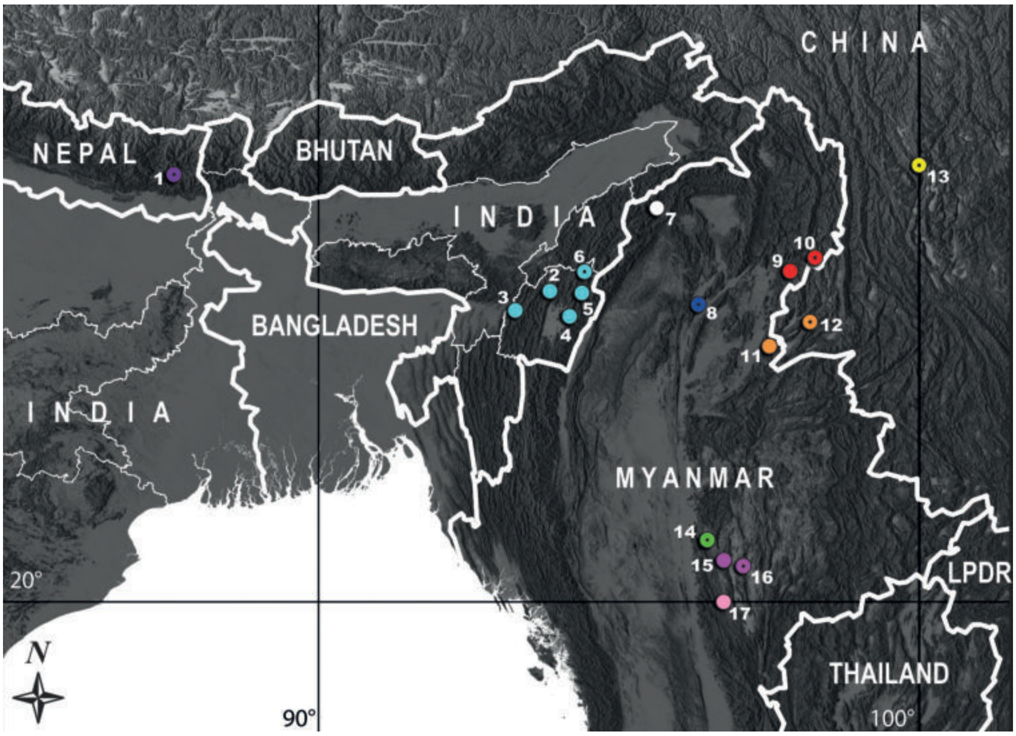

Figure 1. Distribution of *Tylototriton* ssp. in India and adjacent areas and sampling localities examined in this study. Colours of icons correspond to species marked in Fig. 2; a dot in a centre of an icon indicates the type locality. For locality info, see Table 1. *Tylototriton himalayanus*: 1 – Mai Pokhari, Illam, Mechi, Nepal; *T. zaimeng* sp. nov.: 2 – Zaimeng Lake, Koubru Forest Division, Kangpokpi, Manipur, India; 3 – Zeliad, Manipur, India; 4 – Phungyar, Kamjong, Manipur, India; 5 – Shiroy Lily Hills, Manipur, India; 6 – Chingjaroi Ngachaphung, Ukhrul, Manipur, India; *Tylototriton* sp.: 7 – Lahe, Khandi, Sagaing, Myanmar; *T. kachinorum*: 8 – In Gyi Taung Mt., Indawgyi, Kachin, Myanmar; *T. panwaensis*: 9 – Sadung, Myitkyina, Kachin, Myanmar; 10 – Panwa, Myitkyina, Kachin, Myanmar; *T. verrucosus*: 11 – Sinlum, Momauk, Banmaw, Kachin, Myanmar; 12 – Nantin, Momien and Hotha valleys, Yunnan, China; *T. houi*: 13 – Yulong, Lijiang, Yunnan, China; *T. ngarsuensis*: 14 – Ngar Su, Taunggyi, Shan, Myanmar; *T. shanorum*: 15 – Pindaya, Heho and Aungpan, Kalaw, Shan, Myanmar; 16 – Taunggyi, Shan, Myanmar; 17 – Pinlaung, Shan, Myanmar.

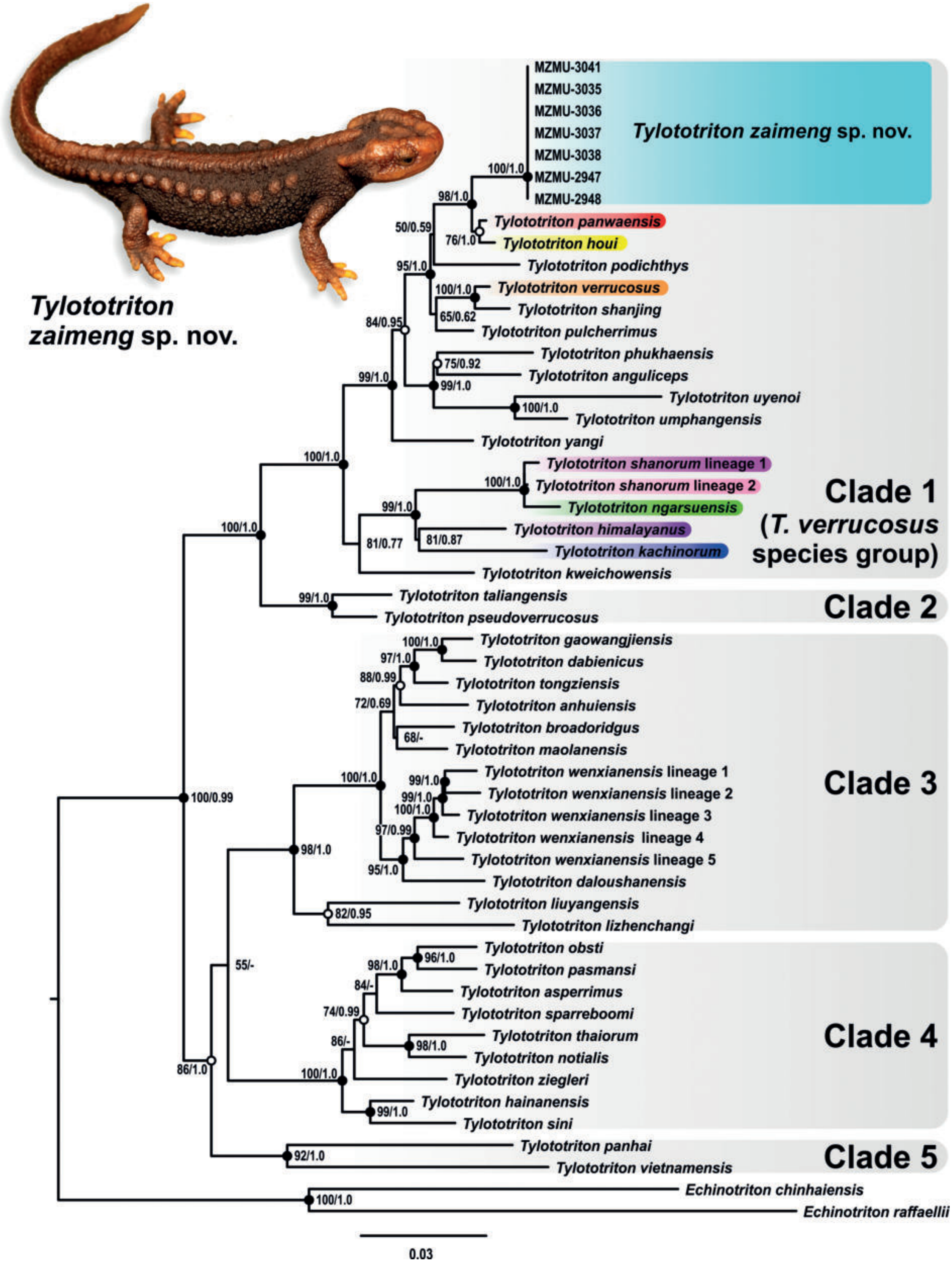

Figure 2. Maximum Likelihood inference consensus tree of genus *Tylototriton* derived from analysis of 1,157 bp ND2 and 508 bp 16S rRNA gene fragments. For voucher specimen information, geographic localities and GenBank accession numbers, see Table 1. Distribution of the species of *Tylototriton* highlighted in colour is shown in Fig. 1. Numbers at tree nodes correspond to ML UFBS and BI PP support values, respectively. Photograph by Andrey M. Bragin.

Table 2. Uncorrected *p*-distance (percentage) between the sequences of 1157 bp fragment of ND2 gene (below the diagonal) and 508 bp fragment of 16S rRNA gene (above the diagonal) of *Tylototriton verrucosus* species group members included in the phylogenetic analyses. Intraspecific genetic distances are shown on the diagonal.

	Taxon	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
1	*T. zaimeng* sp. nov.	**0.0\0.0**	0.9	0.7	–	1.3	1.3	1.3	–	1.8	2.9	–	1.3	2.2	–	3.3	3.1	2.4
2	*T. panwaensis*	3.0	–	0.7	–	0.9	1.3	1.3	–	1.8	2.9	–	1.8	2.7	–	3.6	3.3	2.5
3	*T. houi*	–	–	–	–	1.1	1.1	1.1	–	1.5	2.6	–	1.5	2.4	–	3.3	3.1	2.6
4	*T. podichthys*	4.4	3.3	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
5	*T. verrucosus*	3.3	2.5	–	3.6	–	0.9	1.8	–	2.2	2.9	–	2.2	3.5	–	4.0	3.7	2.9
6	*T. shanjing*	3.8	3.3	–	4.1	1.3	–	0.9	–	1.8	2.9	–	1.8	3.5	–	4.0	3.7	3.3
7	*T. pulcherrimus*	3.9	2.7	–	3.2	2.2	2.9	–	–	1.8	2.9	–	1.8	3.5	–	4.0	3.7	3.3
8	*T. phukhaensis*	6.5	5.6	–	5.8	4.9	5.2	4.3	–	–	–	–	–	–	–	–	–	–
9	*T. anguliceps*	5.9	4.9	–	5.7	4.8	5.1	4.4	4.6	–	2.4	–	1.3	3.1	–	3.5	3.3	2.9
10	*T. uyenoi*	8.6	7.6	–	8.1	7.2	7.3	6.9	7.0	7.7	–	–	2.4	4.6	–	4.6	4.8	4.4
11	*T. umphangensis*	6.5	5.5	–	7.0	5.0	5.1	5.6	6.0	5.4	5.1	–	–	–	–	–	–	–
12	*T. yangi*	5.3	4.0	–	5.0	3.9	4.6	3.7	5.5	4.5	7.8	5.6	–	2.2	–	2.4	3.1	2.4
13	*T. shanorum*	7.3	6.3	–	7.2	6.2	6.5	6.3	7.7	7.2	9.0	8.1	6.6	**0.6\0.1**	–	1.5	1.8	3.1
14	*T. ngarsuensis*	6.8	6.3	–	7.2	6.5	6.8	6.5	8.3	7.2	8.5	8.4	6.4	1.1	–	–	–	–
15	*T. himalayanus*	7.6	6.6	–	6.8	6.4	6.1	6.3	7.1	7.3	8.7	8.0	6.6	5.1	5.9	–	2.0	3.7
16	*T. kachinorum*	7.3	6.4	–	8.0	7.3	8.0	7.7	8.6	8.6	11.3	8.9	8.2	7.4	7.7	5.3	–	2.6
17	*T. kweichowensis*	6.5	5.7	–	5.8	5.6	5.9	5.3	6.2	6.4	7.9	7.8	6.2	5.9	6.4	5.4	6.6	–

Tylototriton panwaensis, a species recently described from Kachin State of northern Myanmar (Grismer et al. 2019), superficially more closely resembles the Manipur *Tylototriton* sp. population (Fig. 3A). We have compared external morphology of *Tylototriton* sp. from Manipur with a series of five adult *T. panwaensis* males from Sadung, Kachin, Myanmar (ZMMU NAP-09477, NAP-09479, NAP-09482, NAP-09484–09485; measurements taken by one of us (AMB), species identification confirmed by Than Zaw et al. (2020)). With respect to the SVL, our analysis revealed that males from Manipur have significantly smaller body size (65.5 ± 2.4 mm, 61.4–67.5 mm) than *T. panwaensis* (72.9 ± 4.3 mm, 68.9–79.3 mm). For SVL-standardised characters (%SVL), the males from Manipur population had significantly larger RHW, RMXHW RVL, RFLL, RHLL, RLJL, RSL, RIOD, RBTAW, RMXTAH and RON, but, in contrast, a significantly smaller RUEW, RUEL, ROL and DLWN than *T. panwaensis* (Table 3). The overall morphological differences were examined using PCA for the Manipur population of *Tylototriton* sp. and *T. panwaensis*. The first two principal components (PCs) explained 66.38% of the total variation in the dataset. Despite combining the sexes for the Manipur population, the two-dimensional PC1 vs. PC2 plot showed that the Manipur *Tylototriton* sp. population was completely separated from *T. panwaensis* and the two species occupy completely separate positions in morphospace (Fig. 3B). Morphological differences between the two populations were also present in the shape of the head, dorsolateral bony ridges, sagittal ridge, vertebral ridge, number of rib nodules, texture of skin and colouration (see "Comparisons" section below, Fig. 3A).

Finally, all specimens of *Tylototriton* sp. from Manipur examined had a peculiar shape of the vomerine tooth series, which was until now, to the best of our knowledge, not yet reported for any other *Tylototriton* species. In Manipur population of *Tylototriton* sp., vomerine teeth were arranged in two strongly-curved series, touching choanae, which start to widen in the anterior one third of series length, extends as two almost parallel lines in the middle of their length and, finally, widen again in the posterior one third of their length; thus, the two series form a curved shape somewhat resembling a bell or a bottle (Fig. 4A). In contrast to this, in all other *Tylototriton* species for which the shape of vomerine tooth series was reported, it is described as an inverted V-shape and it seems that the branches of the series are much less curved. This is also true in the case of *T. panwaensis*, where the vomerine teeth in five male specimens examined had a simple inverted V-shape with the anterior portion of the branches forming almost straight lines (Fig. 4B).

Systematic account

Despite the significant differences amongst *Tylototriton* sp. from Manipur, *T. panwaensis* and *T. houi* in external morphology, vomerine teeth arrangement and colouration, the genetic differentiation between these populations is not deep. However, the observed degree of pairwise divergence in ND2 mtDNA gene between these populations (p = 3.0%) is comparable or greater than the level of genetic divergence observed between many recognised species of *Tylototriton*, including members of the subgenus *Tylototriton*: *T. verrucosus* and *T. shanjing* (1.3%, though the latter was recently suggested as a subspecies or an ecological morph of *T. verrucosus*, see Dufresnes and Hernandez (2022)); *T. verrucosus* and *T. pulcherrimus* (2.2%); *T. verrucosus* and *T. panwaensis* (2.5%); *T. pulcherrimus* and *T. panwaensis* (2.7%); *T. pulcherrimus* and *T. shanjing* (2.9%); *T. taliangensis* and *T. pseudoverrucosus* (2.4%); and *T. ngarsuensis* and *T. shanorum* (1.1%) (see Table 2). This and the phylogeny raise the question as to whether or not the Manipur population of *Tylototriton* sp. may warrant taxonomic recognition as an independent taxon, as an integrative taxonomic approach might reveal.

In this study, based on newly-collected material of *Tylototriton* sp. from Manipur, north-eastern India, we provide a detailed examination of the external morphology and colouration of this population and report on a number of diagnostically important characters readily distinguishing this population of *Tylototriton* from all other congeners (summarised in “Comparisons” section below). Moreover, the presently known range of *Tylototriton* sp. is restricted to montane forests of Khongtheng Mountain Range in Manipur and is apparently isolated from the distribution of *Tylototriton* in Himalaya and north-eastern Myanmar (see Fig. 1). These arguments together support our hypothesis that the Manipur population of *Tylototriton* sp. represents a previously unknown species, which we formally describe below.

Family Salamandridae Goldfuss, 1820
Subfamily Pleurodelinae Tschudi, 1838
Genus *Tylototriton* Anderson, 1871
Subgenus *Tylototriton* Anderson, 1871

Tylototriton zaimeng sp. nov.

https://zoobank.org/2A07583B-5CEF-42DD-99E4-38F366575304
Tables 3, 4; Figs 2–9
Proposed English name: Zaimeng Lake Crocodile Newt

Type material. ***Holotype***. MZMU3041, an adult female from a swamp on forest clearing surrounded by montane evergreen tropical forest in Chingjaroi Ngachaphung, Ukhrul District, Manipur State, north-eastern India (coordinates 25.385°N, 94.458°E; elevation 1,630 m a.s.l.; datum = WGS84), collected on 20 November 2022 at 18:00 h by Ht. Decemson.

Paratypes. MZMU3035–3036, two adult males from a forest lake in Phungyar (environs of Tangkhul Hungdung), Kamjong District, Manipur State, north-eastern India (coordinates 24.811°N, 94.245°E; elevation 1,184 m a.s.l.), collected in July 2022 by Ht. Decemson and Shamungou; MZMU-2942–2947, six adult females from the Zaimeng Lake, Koubru Forest Division, environs of Chawangkining Village, Kangpokpi District, Manipur State, North-eastern India (coordinates 25.238°N, 93.944°E; elevation 2,212 m a.s.l.), collected on 18 July 2022 at 18:00 h by Ht. Decemson; MZMU-2948–2950, three adult males from the Zaimeng Lake, Koubru Forest Division, environs of Chawangkining Village, Kangpokpi District, Manipur State, north-eastern India (coordinates 25.238°N, 93.944°E; elevation 2,212 m a.s.l.; datum = WGS84), collected on 18 July 2022 at 18:00 h by Ht.Decemson.

Referred specimens. MZMU-3037–3040, four larvae premetamorphic stage 45 (Bernardes et al. 2017) from the same locality and with the same collection information as the holotype.

Diagnosis. The new species is assigned to the genus *Tylototriton* by having the following combination of morphological attributes: (1) the presence of dorsal granules, (2) dorsolateral bony ridges on the head, (3) the presence of dorsolateral series of rib nodules (knob-like warts); and (4) the absence of a quadrate spine and molecular data (see Fig. 2). *Tylototriton zaimeng* sp. nov. is distinguished from all other congeners by a combination of the following morphological attributes: (1) medium body size, adult SVL 61.4–67.5 mm in males, 61.6–68.7 mm in females; (2) tail comparatively short, subequal or slightly longer than body in both sexes, lacking lateral grooves; (3) skin roughly granulated; (4) head massive and wide, relative maximal head width comprising 24.3–27.9% of SVL; (5) snout rounded in dorsal view; (6) supratemporal bony ridges on head very wide, protruding, with rough surface, beginning at the snout; (7) sagittal ridge on head well-distinct, very wide and protruding; (8) limbs comparatively short, tips of fore-limb and hind-limb not overlapping when adpressed along body; (9) vertebral ridge distinct, wide and not segmented; (10) rib nodules distinct, 13–14 along each side of body; (11) background colouration brown; (12) head, vertebral ridge, rib nodules, palms, soles, vent and ventral tail ridge with dull orange to yellowish-brown markings; (13) vomerine teeth organised in two distinctly curved bell-shaped widening anteriorly series, with 81–113 teeth.

Comparisons. The new species can be easily distinguished from members of the subgenus *Yaotriton* (clades 3–5 in Fig. 2) by having light colour markings on head, vertebral ridge, rib nodules, palms, soles, vent and ventral tail ridge (vs. dark body colouration, except for palms and soles, vent region and ventral ridge of tail in most members of the subgenus *Yaotriton* with the exception of *T. panhai*). The new species can be further distinguished from *T. panhai* by having light colour markings on entire limbs (vs. distinct light markings only on palms, soles and fingers in *T. panhai*). *Tylototriton zaimeng* sp. nov. can be distinguished from *T. taliangensis* (member of clade 2, Fig. 2), by having light markings on distinct rib nodules, lips and parotoids (vs. lacking distinct rib nodules, generally dark charcoal-black body colouration with light orange to red markings only on the posterior part of parotoids, digits, palms, soles, vent and ventral tail ridge in *T. taliangensis*). *Tylototriton zaimeng* sp. nov. can be distinguished from *T. pseudoverrucosus* (clade 2, Fig. 2) and *T. kweichowensis* (clade 1, Fig. 2) by having isolated light markings on rib nodules (vs. connected markings forming light dorsolateral lines in *T. pseudoverrucosus* and *T. kweichowensis*).

Based the results of phylogenetic analyses (Fig. 2), *Tylototriton zaimeng* sp. nov. falls into clade 1 of the subgenus *Tylototriton* s. str. and, hence, morphological comparisons with members of the *T. verrucosus* species group members appear to be the most pertinent. Morphological comparisons on several diagnostic characters between *Tylototriton zaimeng* sp. nov. and the closely-related members of *T. verrucosus* species group are summarised

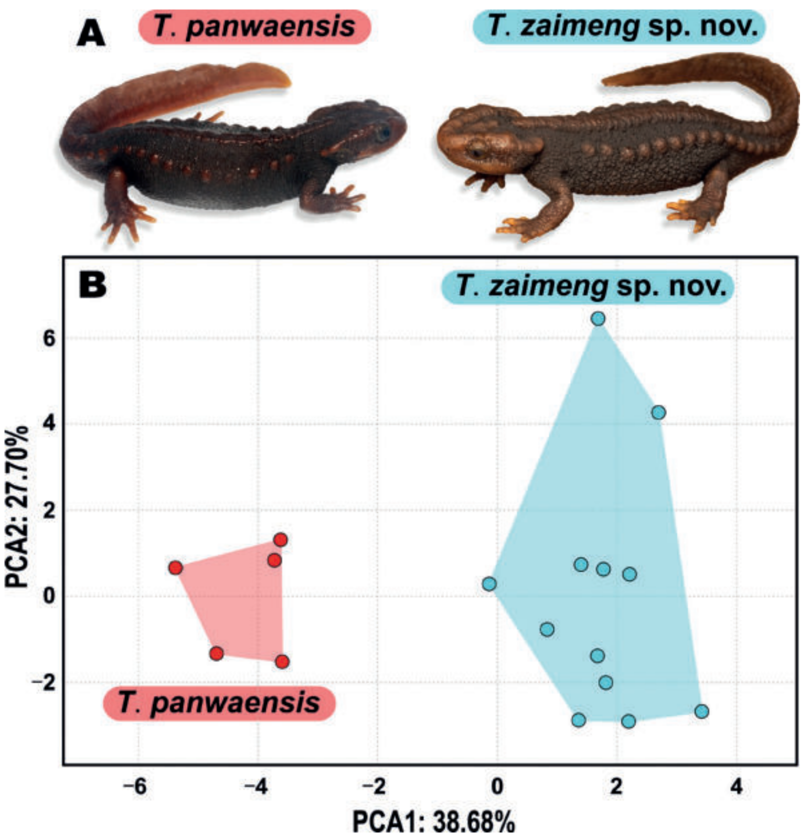

Figure 3. Morphological comparison between *Tylototriton zaimeng* sp. nov. and *T. panwaensis*. **A.** *Tylototriton panwaensis* (adult male ZMMU NAP-09477, from Sadung, Myitkyina, Kachin, Myanmar; photograph by Nikolay A. Poyarkov) and *Tylototriton zaimeng* sp. nov. (adult female MZMU3041, from Chingjaroi Ngachaphung, Ukhrul, Manipur, India; photograph by Andrey M. Bragin) in life; **B.** PCA plots of PCA1 versus PCA2 for morphological parameters of the two *Tylototriton* species.

Table 3. Measurements (mm) of adult *Tylototriton zaimeng* sp. nov. and *T. panwaensis*. Abbreviations defined in the text. Asterisk (*) indicates significant differences between relative character values in males ($p < 0.05$).

Character	*Tylototriton zaimeng* sp. nov.					*Tylototriton panwaensis*	
	MZMU-3041, female	5 males		6 females		5 males	
	holotype	paratypes				–	
		Mean	Min-Max	Mean	Min-Max	Mean	Min-Max
SVL*	76.09	65.5±2.4	(61.4–67.5)	64.7±2.6	(61.6–68.7)	72.9±4.3	(68.9–79.3)
HL	17.68	15.6±1.4	(13.8–17.1)	14.5±0.3	(14.3–15.1)	15.52±1.0	(13.7–16.3)
HW*	16.4	14.5±1.0	(13.2–15.8)	14.0±0.6	(12.9–14.7)	13.46±0.6	(13.0–14.2)
MXHW*	19.4	17.2±1.6	(14.9–18.8)	16.4±0.5	(15.7–17.3)	16.4±0.4	(16.1–17.0)
IND	5.08	3.8±0.5	(3.1–4.3)	3.7±0.4	(3.3–4.1)	4.64±0.3	(4.3–5.0)
AGD	42.97	34.6±2.7	(30.4–37.5)	32.2±2.4	(28.2–34.8)	38.8±1.5	(36.9–41.0)
TRL	63.12	55.2±1.5	(53.6–56.9)	54.9±1.7	(53.4–57.9)	59.44±2.0	(57.6–62.2)
TAL	73.8	72.5±3.3	(68.1–76.3)	69.0±4.4	(63.7–77.1)	71.46±12.5	(52.3–83.1)
VL*	4.08	6.4±0.4	(6.1–7.0)	5.0±0.7	(4.2–5.9)	4.1±0.9	(3.5–5.6)
FLL*	26.74	22.7±1.0	(21.3–24.1)	23.4±0.8	(22.2–24.5)	23.26±1.1	(21.9–24.9)
HLL*	28.6	23.8±0.8	(22.8–24.8)	24.7±1.9	(22.1–27.6)	25.4±0.9	(24.2–26.6)
LJL*	16.27	14.3±1.1	(13.5–15.9)	13.1±0.8	(11.7–13.9)	14.24±0.3	(14.0–14.6)
SL*	7.02	6.7±0.5	(6.1–7.5)	6.3±0.3	(5.8–6.7)	6.72±0.1	(6.5–6.9)
IOD*	9.8	8.9±0.6	(8.5–9.6)	8.6±0.3	(8.1–8.9)	7.36±0.4	(6.8–7.9)
UEW*	2.57	1.5±0.3	(1.0–1.8)	1.8±0.2	(1.6–2.1)	2.46±0.1	(2.4–2.5)
UEL*	4.1	3.6±0.2	(3.3–3.9)	3.9±0.3	(3.6–4.3)	4.76±0.2	(4.5–5.0)
OL*	2.6	2.6±0.5	(2.3–3.5)	2.6±0.3	(2.2–3.0)	4.54±0.2	(4.3–4.7)
BTAW*	8.72	8.8±0.9	(8.0–10.2)	7.3±0.7	(6.9–8.7)	6.36±0.2	(6.0–6.6)
MTAW	5.47	3.0±0.5	(2.6–3.7)	2.6±0.2	(2.4–2.9)	2.92±0.4	(2.3–3.3)
MXTAH*	10.17	9.4±0.7	(8.6–10.4)	8.2±0.7	(7.4–9.3)	7.0±0.6	(6.3–7.7)
MTAH	9.1	7.4±1.5	(5.7–9.5)	5.4±0.9	(4.6–7.0)	6.34±0.6	(5.9–7.4)
ON*	4.39	4.4±0.4	(3.9–5.0)	4.3±0.3	(4.0–4.8)	3.82±0.6	(2.8–4.4)
DLWN*	28	27.6±0.9	(27.0–29.0)	27.7±0.8	(27.0–29.0)	29.8±0.4	(29.0–30.0)

in Table 4. An important morphological difference which distinguishes *Tylototriton zaimeng* sp. nov. from all other members of *T. verrucosus* species group members is the shape of vomerine tooth series (VTS), which are distinctly curved and bell-shaped in the new species (Fig. 4A): VTS are distinctly widening in the anterior one-third of their length, further gradually widening posteriorly, reaching maximal width in the posterior one third of VTS length; this shape of VTS appears to be quite stable and was observed in all examined adult specimens of the new species. In contrary, in all members of *T. verrucosus* species group for which the shape of VTS was reported (in *T. houi*, *T. panwaensis*, *T. kachinorum*, *T. verrucosus*, *T. shanjing*, *T. uyenoi*, *T. anguliceps*, *T. podichthys* and *T. yangi*), it was described as inverted V-shape with VTS branches being comparatively straight in the anterior half of their length (see Fig. 4B).

Tylototriton zaimeng sp. nov. can be further distinguished from *T. uyenoi*, *T. pulcherrimus*, *T. shanjing*, *T. houi* and *T. yangi* by having dull orange-brown to yellowish-brown light markings (vs. much brighter orange to bright-yellow light markings in *T. uyenoi*, *T. pulcherrimus*, *T. shanjing* and *T. yangi* and vs. bright orange-red markings in *T. houi*). In particular, *T. houi* has bright orange-red markings on ventral surfaces including vent, chest, light mid-ventral line (vs. absent in the new species); *T. pulcherrimus* has a series of bright-orange glandular spots located ventro-laterally and on flanks (vs. flanks lacking light spots in the new species); while *T. yangi* has contrasting charcoal-black colouration of head and lips with only posteriormost part of parotoid colored bright orange and no light ventral markings on body and tail (vs. all head dull orange-brown, light markings present on ventral tail ridge and vent in the new species). *Tylototriton zaimeng* sp. nov. has relatively narrower head in both sexes (RHW 22.2 vs. 25.0 in males; 21.6 vs. 23.1–24.0 in females); shorter internarial distance (RIND 5.7 for males, 5.8 for females vs. 7.0–7.1 for both sexes); and very wide, protruding, and glandular dorsolateral and sagittal head ridges (vs. narrow steep ridges) than in *T. uyenoi* (see Table 4). Males of the new species can be further diagnosed from males of *T. umphangensis* by having shorter limbs which do not overlap when adpressed along the body (vs. overlap), by comparatively longer trunk (RTRL84.4 vs. 76.8), longer tail (RTAL 110.6 vs. 104.7), by having snout rounded in dorsal aspect (vs. truncate) and by having a non-segmented vertebral ridge (vs. distinctly segmented) (see Table 4). Males of the new species can be further diagnosed from males of *T. shanjing* by having comparatively shorter head (RHL 23.8 vs. 26.6), longer tail (RTAL 110.6 vs. 104.4), by shorter internarial distance (RIND 5.7 vs. 7.1) and by having a non-segmented vertebral ridge (vs. well-segmented) and brown to dark-brown background body colour (vs. blackish) (see Table 4). *Tylototriton zaimeng* sp. nov. can be distinguished from *T. verrucosus* by having light ventral markings on body and tail (vs. no light markings on body and tail). The new species can be further differentiated from *T. verrucosus* by having comparatively shorter internarial distance in both sexes (RIND 4.8–6.7 vs. 6.2–7.0) and longer tail in both sexes (RTAL 110.6 vs. 104.9 for males; 105.3 vs. 102.5 for females); the new species also has a non-segmented vertebral ridge (vs. well-segmented) and brown background colouration of body (vs. blackish) (see Table 4).

Tylototriton zaimeng sp. nov. can be distinguished from *T. podichthys* by having comparatively shorter head in both sexes (RHL 21.8–25.6 vs. 28.1–34.3), slightly longer tail in both sexes (RTAL in males 108.1–114.1 vs. 80.2–104.8; in females 96.6–112.2 vs. 79.2–81.4), in having 13–14 rib nodules (vs. 15–16 rib nodules), by comparatively shorter limbs which do not overlap when adpressed to body (vs. digit tips touching when limbs are adpressed to body) and by having duller colouration with orange to yellowish-brown light markings and brown background (vs. orange to dark-red light markings and blackish background) (see Table 4). The new species can be distinguished from *T. phukhaensis* by having very wide, protruding and glandular head ridges (vs. narrow steep head ridges), by having snout rounded in dorsal aspect (vs. truncate), by comparatively shorter limbs which do not overlap when adpressed to body (vs. digit tips touching when limbs are adpressed to body), by having comparatively wider head in both sexes (RHW 19.6–23.4 vs. 17.7–19.2), by having comparatively longer trunk in both species (RTRL 79.5–88.7 vs. 76.4–76.8) and by a having wide non-segmented vertebral ridge (vs. narrow, segmented) (see Table 4).

Tylototriton zaimeng sp. nov. can be distinguished from *T. anguliceps* by having a comparatively shorter head in males (RHL 22.5–25.6 vs. 26.2–29.5), by shorter internarial distance in both sexes (RIND 4.8–6.7 vs. 6.6–7.4), by having a wide non-segmented vertebral ridge (vs. weakly segmented), by having snout rounded in dorsal aspect (vs. truncate), by having very wide, protruding and glandular head ridges (vs. narrow steep head ridges, including narrow and long sagittal ridge), by having 13–14 rib nodules (vs. not less than 15 rib nodules), by comparatively shorter limbs which do not overlap when adpressed to body (vs. digit tips touching when limbs are adpressed to body) and by having duller colouration with orange to yellowish-brown light markings and brown background (vs. bright-orange markings and blackish background) (see Table 4).

The new species can be readily distinguished from *T. shanorum* by having smaller body size in both sexes (SVL 61.4–76.1 mm vs. 76.0–87.9 mm), by having comparatively narrower head in both sexes (RHW 19.6–23.4 vs. 24.8–26.3), by having comparatively longer trunk in both sexes (RTRL 79.5–88.7 vs. 74.3–77.6), by having snout rounded in dorsal aspect (vs. blunt to truncate), by comparatively shorter limbs which do not overlap when adpressed to body (vs. limbs overlapping when limbs are adpressed to body) and by having a wide non-segmented vertebral ridge (vs. weakly segmented) (see Table 4). *Tylototriton zaimeng* sp. nov. can be easily distinguished from

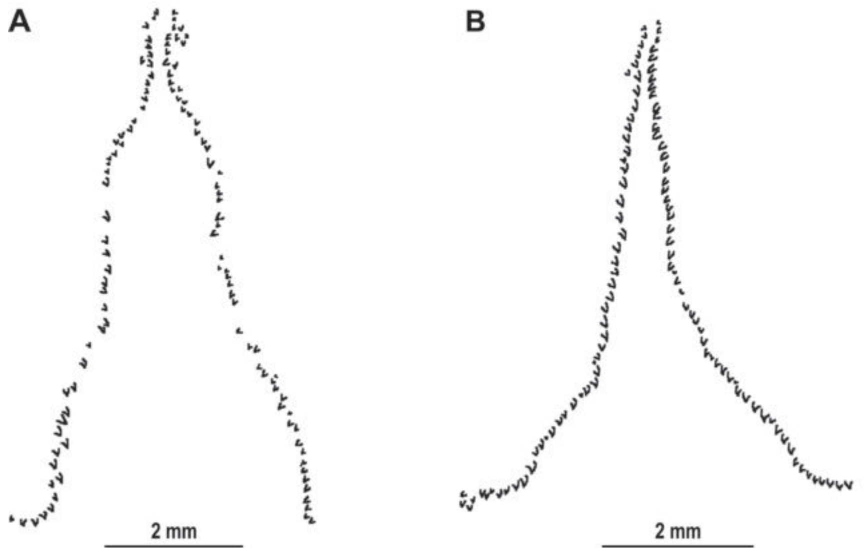

Figure 4. Vomerine tooth series of **A.** *Tylototriton zaimeng* sp. nov. (MZMU3041) and **B.** *T. panwaensis* (ZMMU NAP-09477). Drawing by Andrey M. Bragin.

Table 4. Morphological comparison between *Tylototriton verrucosus* species group members found in India and adjacent territories (Continued on next page).

Species	*T. ngarsuensis*		*T. panwaensis*		*T. phukhaensis*		*T. podichthys*	
	M	F	M		M	F	M	F
Character	2	1	5		1	2	2	2
SVL (in mm)	84.5	74.9–102.3	72.9	68.9–79.3	64.3	68.9–70.2	56.5–60.2	73.4–78.3
RHL	24.0	22.0–26.0	21.4	17.3–23.4	25.0	23.1–24.8	32.6–34.3	28.1–29.3
RHW	26.3	24.0–28.0	18.5	16.4–20.6	19.2	17.7–18.7	26.4–28.0	24.8–25.9
RIND	8.2	7.7–8.8	6.4	6.0–6.7	5.6	6.0–5.9	8.3–8.7	7.5–7.9
RAGD	49.7	48.0–51.0	53.4	46.5–55.9	56.6	54.7–56.6	48.0–50.7	52.1–53.5
RTRL	76.0	74.0–78.0	81.8	74.1–87.0	76.4	76.5–76.8	65.7–67.4	70.7–71.9
RTAL	102.3	98.0–105.0	98.7	66.0–116.5	108.7	88.1–87.0	80.2–104.8	79.2–81.4
RVL	10.3	8.0–12.0	5.6	4.8–7.9	10.5	4.0–3.4	14.2–14.8	6.3–7.7
RFLL	38.3	35.0–40.0	32.0	28.6–34.9	38.4	31.8–31.2	39.4–40.5	34.0–36.2
RHLL	42.7	39.0–47.0	34.9	33.5–36.2	36.7	30.1–34.5	38.2–40.2	35.8–36.1
RMXHW	–	–	22.6	20.4–24.4	22.1	22.0–22.4	–	–
Snout	truncate		truncate		truncate		rounded	
Dorsolateral head ridges	very wide, protruding		narrow, steep		narrow, very steep		very wide, protruding	
Sagittal ridge	very weak, indistinct		narrow, low		narrow, distinct		weak, glandular	
Surface of head ridges	rough		rough		rough		very rough	
Adpressed limbs	overlap		overlap		overlap		touch	
Vertebral ridge	not segmented		weakly segmented		narrow, weakly segmented		not segmented	
Rib nodules	distinct, 15		distinct, 15		prominent, 14–15		large, prominent, 15–16	
Ground colour	very dark brown		dark reddish-brown		dark brown		blackish	
Colour of light markings	light brown		reddish-brown		orange to light-brown		orange to dark–red	
Location of light markings	parotoids, palms, soles, vent, ventral tail ridge		parotoids, vertebral ridge, rib nodules, vent, whole limbs and tail		head, parotoids, vertebral ridge, rib nodules, limbs, vent region, and whole tail		head, vertebral ridge, rib nodules, vent, dorsal surface limbs, ventral tail ridge	
Lateral grooves on tail	absent		weak		absent		absent	
Shape of VTS	?		inverted V-shape		inverted V-shape		inverted V-shape	

T. ngarsuensis by having generally smaller body size in males (SVL 61.4–67.5 mm vs. 74.9–76.4 mm) and in females (SVL 61.6–76.1 mm vs. 102.3 mm), comparatively much narrower head in both sexes (RHW19.6–23.4 vs. 24.0–28.0), by notably more narrow internarial distance in both sexes (RIND 4.8–6.7 vs. 7.7–8.8), by having comparatively longer trunk in both sexes (RTRL 79.5–88.7 vs. 74.0–78.0), by having snout rounded in dorsal aspect (vs. truncate). The new species can be further distinguished from *T. ngarsuensis* by having dorsolateral head ridges starting at the snout (vs. posterior to orbit), by having a non-segmented vertebral ridge (vs. weakly segmented) and by having 13–14 rib nodules (vs. 15 rib nodules). *Tylototriton zaimeng* sp. nov. has much lighter and duller colouration than *T. ngarsuensis*: background colour brown (vs. nearly black) with light orange-brown markings on rib nodules and parotoids and limbs (vs. no light markings on rib nodules and parotoids) (see Table 4).

Tylototriton zaimeng sp. nov. can be easily distinguished from *T. himalayanus* from Nepal by the following morphological attributes: by notably narrower internarial distance in both sexes (RIND 4.8–6.7 vs. 8.2–8.4), by having generally longer trunk in both sexes (RTRL 79.5–88.7 vs. 77.1–79.9), by having longer tail in males (RTAL 108.1–114.1 vs. 98.0), by having snout rounded in dorsal aspect (vs. blunt), by comparatively shorter limbs which do not overlap when adpressed to body (vs. limbs overlapping when limbs are adpressed to body), by having 13–14 rib nodules (vs. 16 rib nodules) and by lacking lateral transverse grooves on tail (vs. clearly distinct) (see Table 4). The new species can be further distinguished from *T. kachinorum* from Kachin State of Myanmar by shorter head in both sexes (RHL 22.5–25.6 vs. 27.6 in males, 21.8–23.6 vs. 23.9–24.9 in females), by shorter tail in both males (RTAL 108.1–114.1 vs. 120.5), by notably narrower internarial distance in both sexes (RIND 4.8–6.7 vs. 7.7–8.0), by having snout rounded in dorsal aspect (vs. truncate) and by comparatively shorter limbs which do not overlap when adpressed to body (vs. limbs overlapping when limbs are adpressed to body) (see Table 4).

Phylogenetically and morphologically, *Tylototriton zaimeng* sp. nov. is most closely related to two species of *Tylototriton* inhabiting northern Myanmar and Yunnan Province of China – *T. panwaensis* and *T. houi* (see Fig. 2, Table 4). From *T. houi*, the new species can be easily distinguished by its much duller colouration (see above and Table 4) and can be further distinguished by having snout rounded in dorsal aspect (vs. truncate), by having very wide, protruding and glandular head ridges with rough surface (vs. narrow steep head ridges with smooth surface, including short sagittal ridge) and by having 13–14 rib nodules (vs. 16 rib nodules).

From its sister species *T. panwaensis*, *Tylototriton zaimeng* sp. nov. can be distinguished by having snout rounded in dorsal aspect (vs. truncate), by having very wide, protruding and glandular head ridges including prominent sagittal ridge (vs. narrow steep head ridges with smooth surface, including very weak and low sagittal ridge), by having 13–14 rib nodules (vs. 15 rib nodules), by having a very wide non-segmented vertebral ridge (vs. narrow, weakly segmented), by lacking lateral transverse grooves on tail (vs. weak grooves present) and by comparatively shorter limbs which do not overlap when adpressed to body (vs. limbs overlapping when limbs are adpressed to body). *Tylototriton zaimeng* sp. nov. has generally lighter colouration than *T. panwaensis*: brownish-ground colour with orange-brown light markings (vs. more contrasting dark reddish-brown to black background colour with reddish-brown light markings in *T. panwaensis*) (see Table 4). Moreover, males of the new species are different from males of *T. panwaensis* in a number of morphometric characters. Males of *Tylototriton zaimeng* sp. nov. have greater maximal head width (RMXHW 24.3–27.9 vs. 20.4–24.4), generally longer snout (RSL 9.2–11.2 vs. 8.7–9.6), wider interorbital distance (RIOD 12.3–14.3 vs. 9.9–10.7), smaller eyes (ROL 3.3–5.2 vs. 5.9–6.4) and wider tail base (RBTAW 10.4–15.2 vs. 7.9–9.5) than in *T. panwaensis* (see Table 3).

Description of holotype. A medium-sized specimen in a good state of preservation (Figs 5, 6). ***Head.*** Head slightly longer than wide (HW/HL ratio 92.8%) (Fig. 5C–E), head slightly wider than body; pentagonal in shape in dorsal view, flattened in profile (Fig. 5E); snout long, about three times longer than eye (UEW/SL ratio 36.6%), gently rounded in dorsal view (Fig. 5C), rounded in lateral view (Fig. 5E), notably projecting beyond lower jaw; nostrils on anterior margin of snout located notably closer to snout tip than to eye (NSD/ON ratio 39.9%), nostrils with antero-lateral orientation, not visible from dorsal view; eyes small, not projecting in lateral view (Fig. 5E), slightly projecting in dorsal view (Fig. 5C); labial folds absent; tongue oval, attached to anterior floor of mouth cavity, laterally and posteriorly free; vomerine teeth arranged in a bell-shaped distinctly curved series (Fig. 4A), distinctly widening in the anterior one-third of vomerine tooth series length, further gradually widening posteriorly, reaching maximal width in the posterior one third of vomerine tooth series length; vomerine tooth series notably longer than wide (VTW/VTL ratio 40.4%), anteriorly reaching beyond the level of choanae, numbers of vomerine teeth 113 (62/51 in right and left branches, respectively), upper jaw teeth 72 and lower jaw teeth 90; parotoids distinct, large and protruding, bean-shaped, slightly projecting posteriorly (Fig. 5E); dorsolateral supratemporal bony ridges on head very wide, with rough surface, notably protruding, continuing from the snout tip to the anterior end of parotoid, becoming wider towards the posterior end (Fig. 5C); sagittal bony ridge on head very wide and protruding, becoming higher and wider posteriorly (Fig. 5C); gular fold indistinct (Fig. 5D). ***Body.*** Body habitus stout (Fig. 5A); costal folds absent; vertebral mid-dorsal ridge very wide, not segmented, beginning at the occiput region and continuing to the anterior one fourth of tail length, separated from sagittal head ridge with gap subequal to eye in length (Fig. 5C); rib nodules prominent, distinct, forming knob-like glandular warts, 14 on both sides of body, arranged in two longitudinal lines on dorsolateral surfaces of dorsum, running from area posterior to axilla to tail base (Fig. 5A); on body, rib nodules almost of the same size, rounded, decreasing in size posteriorly on sacrum and tail basis. ***Limbs.*** Limbs comparatively short and slender (Fig. 5A); fore-limbs slightly shorter than hind-limbs; relative length of fore-limb FLL/SVL ratio 35.1%, relative length of hind-limb ratio 37.6%; fore- and hind-limbs not overlapping when adpressed towards each other along sides of the body; fingers and toes well developed (Fig. 5F–I), free of webbing; fingers four, comparative finger lengths: 1FL < 4FL < 2FL < 3FL; toes five, comparative toe lengths: 1TL < 2TL < 5TL < 4TL < 3TL. ***Tail.*** Tail comparatively short and thick, slightly shorter than body length (TAL/SVL ratio 97.0%); tail laterally compressed on all of its length, gently tapering posteriorly, lateral grooves on tail absent; dorsal tail fin starting at the anterior one fourth

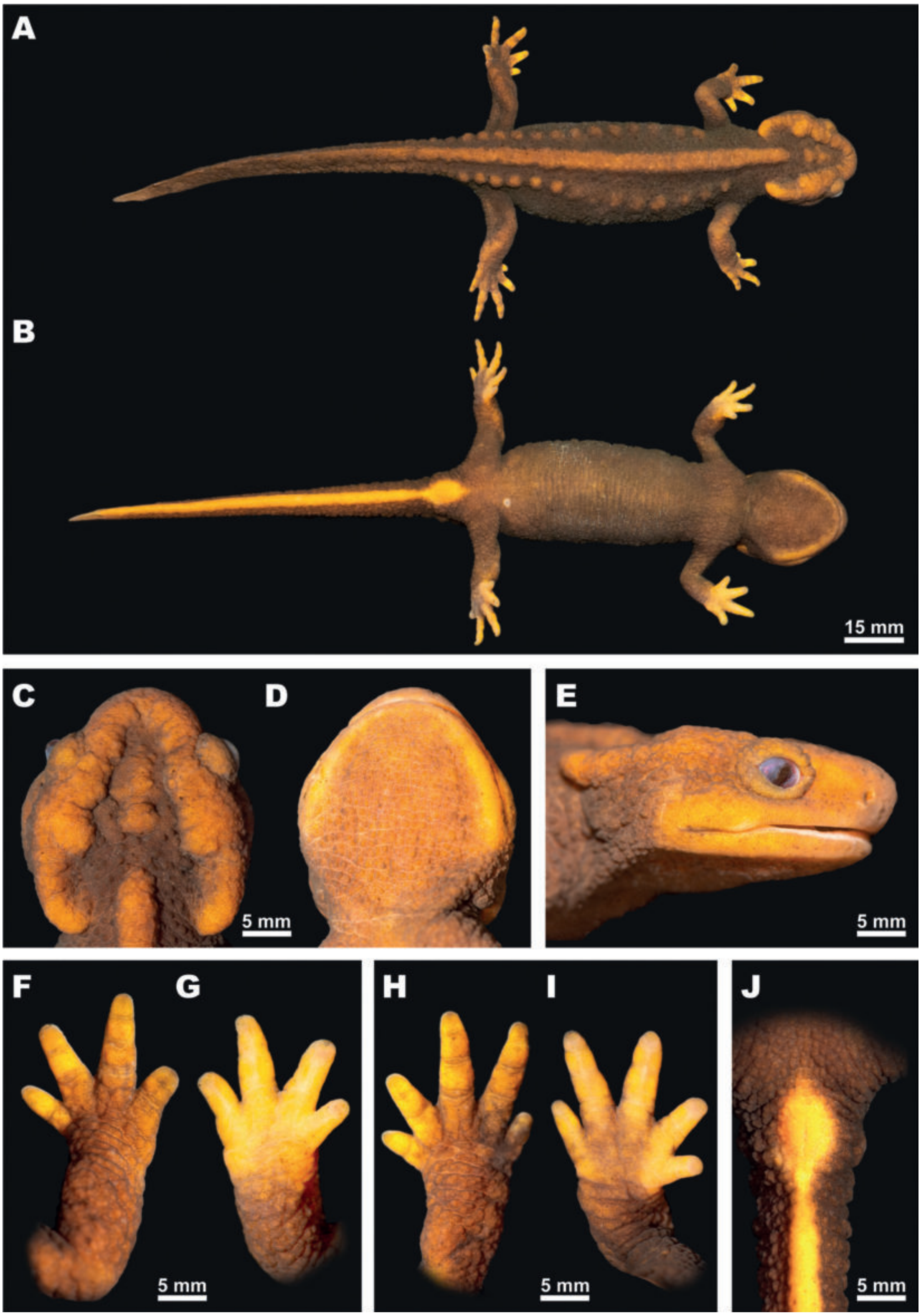

Figure 5. Holotype of *Tylototriton zaimeng* sp. nov. (MZMU3041, female) in life. **A.** Dorsal view; **B.** Ventral view; **C.** Head, dorsal view; **D.** Head, ventral view; **E.** Head, lateral view; **F.** Opisthenar view of right hand; **G.** Volar view of right hand; **H.** Opisthenar view of right foot; **I.** Plantar view of right foot; **J.** Ventral view of cloacal area. Photographs by Andrey M. Bragin.

of tail length, becoming more distinct posteriorly, with maximal tail height at posterior two thirds of tail length, dorsal and ventral tail fins smooth; tail tip pointed. ***Skin texture and skin glands.*** Skin dorsally very rough, with numerous small granules present on dorsal surfaces of head and dorsum (Fig. 5A, C), lateral sides of body and tail; ventral surfaces more smooth with smaller granules arranged in transverse striations (Fig. 5B); small granules regularly arranged on throat (Fig. 5D); head ridges and parotoids with rough surface; skin on dorsal surfaces of

Figure 6. Holotype of *Tylototriton zaimeng* sp. nov. (MZMU-3041, female) in situ. Photograph by Andrey M. Bragin and Ht. Decemson.

limbs granular, on volar and plantar surfaces of hands (Fig. 5G) and feet (Fig. 5I) with tiny grooves forming a reticulated pattern; metacarpal or metatarsal tubercles absent. Cloacal region slightly swollen, vent as a longitudinal slit (Fig. 5J), vent edges with small transverse folds.

Colour of holotype in life. Ground colour of dorsal surfaces of head and trunk dark brown (Figs 5A, 6); dorsal surfaces of limbs and lateral surfaces of tail yellowish-brown to light orange (Fig. 5A); iris dark-brown with copper speckles along its outer margins (Fig. 5E); throat, belly and ventral surfaces of limbs light brown (Fig. 5B); anterior parts of head, including snout, light orange to yellowish-brown; rib nodules and vertebral ridge light orange-brown, notably discernible from dark brown trunk colouration; upper and lower lips, head bony ridges and parotoids, palms and soles light-orange.

Colour of holotype in preservative. After preservation in ethanol for six months, the colouration pattern of the holotype resembles that observed in life; however, yellowish and orange tints faded turning light brownish-grey.

Measurements and counts of the holotype. Measurements of the holotype are presented in Table 3. Additional morphometric characters (all in mm) include: ICD 9.1; CW 13.0; NSD 1.8; 1FL 3.8; 2FL 5.7; 3FL 6.7; 4FL 4.1; 1TL 3.1; 2TL 4.7; 3TL 8.6; 4TL 8.4; 5TL 4.8. Meristic characters: UJTN 72; LJTN 90; VTN 62/51 (right/left); DLWN: 14/14 (right/left). Body weight (when alive): 13.5 g.

Variation. All individuals in the type series are generally similar in morphology and agree well with the description of holotype in body proportions and colouration; variation of morphometric characters within the type series is shown in Table 3. Variation of the dorsal colouration in eleven paratypes in preservative is presented in Fig. 7. The state of preservation of nine paratypes MZMU-2942–2950 is much worse than that of the holotype and the paratypes MZMU-3035–3036: they are desiccated what caused partial discolouration and skin damage (Fig. 7). Colouration of the paratypes MZMU-3035–3036 closely agrees with that described for the holotype. In general, males have more slender bodies than females**.** Males and females do not differ in body size (SVL 61.4–67.5 mm in males vs. SVL 61.6–68.7 mm in females) (Table 3).

Larval morphology. Description of larval morphology is based on four premetamorphic larval specimens (MZMU-3037–3040, Bernardes et al. (2017) stage 45) (see Referred specimens for details).

Larval measurements (n = 4; in mm). SVL 35.1 ± 2.5; HL 10.2 ± 0.7; HW 10.3 ± 0.7; OL 2.7 ± 0.2; AGD 21.1 ± 1.5; TAL 35.3 ± 2.5; FLL 11.9 ± 0.8; HLL 12.1 ± 0.8; MXTAH 5.2 ± 0.4.

Larval external morphology. Body elongated, as high as wide (Fig. 8). Head large, ovoid in dorsal view, wide and slightly depressed with a short and rounded snout, gently sloping in lateral view, slightly wider than body in dorsal view. Snout rounded in dorsal view (Fig. 8B), as well as in lateral view (Fig. 8A). Tail equal to body length comprising 100.4% of SVL; myotomes on body and tail not discernible in lateral view. Nostrils rounded, small, orientated anterolaterally, located much closer to snout tip

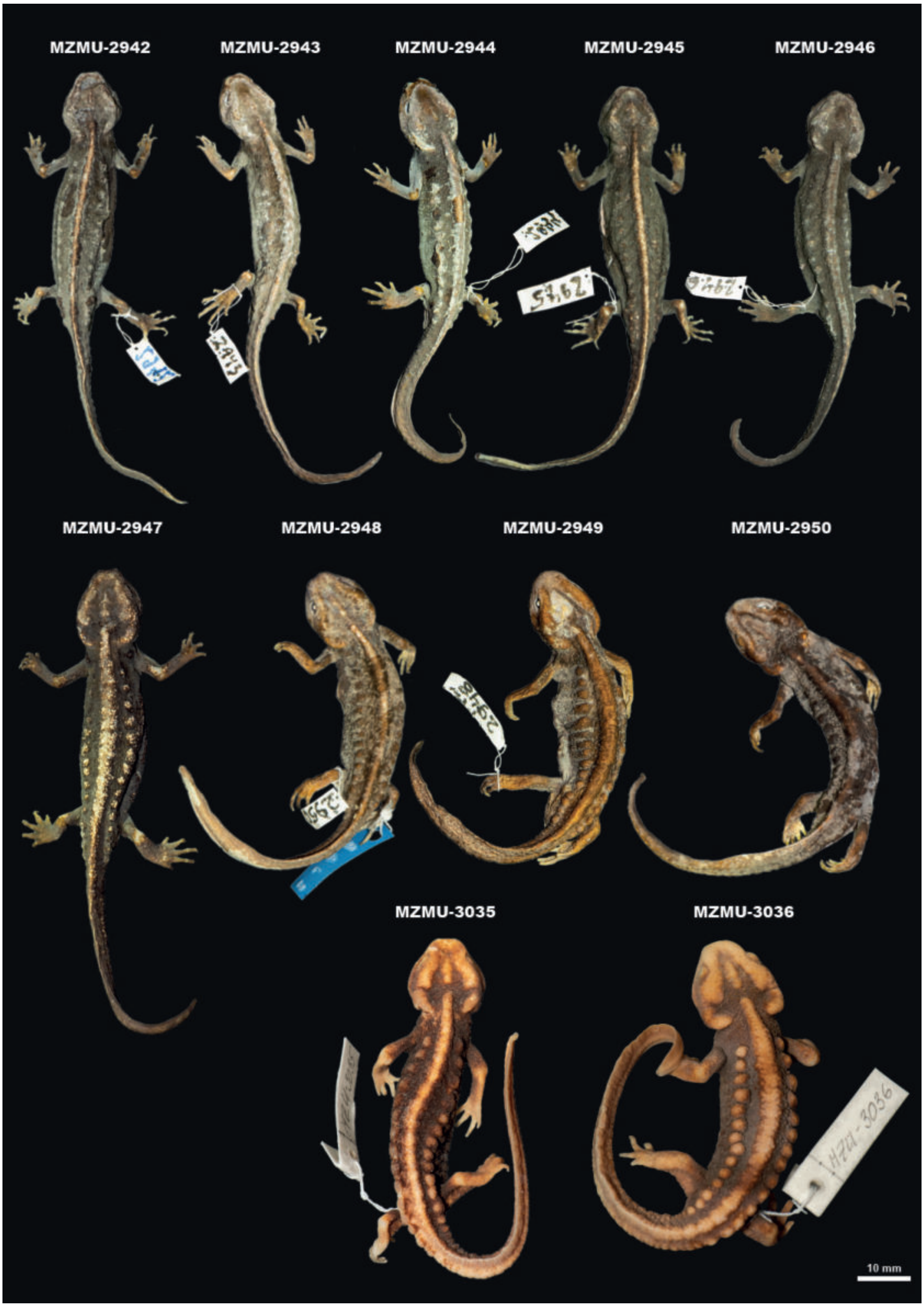

Figure 7. Variation of dorsal colouration in paratypes of *Tylototriton zaimeng* sp. nov. Scale bar: 10 mm. Photographs by Andrey M. Bragin.

than to eye (Fig. 8A). Eyes large, rounded, orientated dorsolaterally, well visible in dorsal view (Fig. 8B). Limbs short, fore-limbs subequal to hind-limbs, FLL/HLL ratio 98.5%. Fore-limbs with four well-developed elongated fingers; relative finger lengths: 4FL < 3FL <1FL < 2FL. Hind-limbs with five well-developed toes; relative toe lengths: 5TL < 1TL < 2TL < 4TL < 3TL. Orbit diameter (OL) 7.7% of SVL. Vent a short longitudinal slit. Tail fins reduced; maximum height of dorsal tail fin ca. 10% of maximum tail height. Ventral tail fin almost completely reduced. Dorsal tail fin starts roughly above the cloaca, though it remains still visible as a thin mid-dorsal line starting at the level of axilla (Fig. 8B). Tail tip pointed in lateral view (Fig. 8A). Skin completely smooth; lateral line organs visible on dorsal surface of head; three pairs of gills partially reduced, not reaching the level of axilla.

Larval colouration in life. In life, larval background colour ochre to light brown dorsally (Fig. 8A, B), ventral surfaces of body off-white to pinkish, translucent, ventral surface of tail and vent bright yellow (Fig. 8C). Dorsal surfaces of body, tail and head with indistinct dark-grey marbling. Eyes, except for pupil, fully pigmented, iris copper-coloured (Fig. 8A).

Position in mtDNA genealogy and sequence divergence. According to our mtDNA genealogy, *Tylototriton zaimeng* sp. nov. belongs to clade 1 of the subgenus *Tylototriton* s. str., corresponding to the *T. verrucosus* species group (see Fig. 2). The new species is grouped together with *Tylototriton* species from northern Myanmar (*T. panwaensis*), northern Indochina (*T. podichthys*) and Yunnan Province of China (*T. verrucosus*, *T. shanjing*, *T. houi* and *T. pulcherrimus*), forming clade with *T. panwaensis* and *T. houi*. Uncorrected genetic *p*-distances between *Tylototriton zaimeng* sp. nov. ND2 sequences and all homologous sequences of other members of *T. verrucosus* species group included in our analyses varied from 3.0% (with its sister species *T. panwaensis*) to 8.6% (with *T. uyenoi*) (see Table 2).

Distribution and Natural history. To date *Tylototriton zaimeng* sp. nov. is known from five localities in montane areas of Manipur State, north-eastern India (see Fig. 1: localities 2–6) on elevations from 1,180 to 2,210 m a.s.l. The actual extent of distribution of the new species remains unknown; it is likely that *Tylototriton zaimeng* sp. nov. occurs further northwards along the Khongtheng Mountain Range and other heavily forested highlands in the Nagaland State of India and even may penetrate to the easternmost parts of Arunachal Pradesh State of India and Sagaing Division of Myanmar. The taxonomic status of the existing record of *Tylototriton* sp. from Sagaing, Myanmar (Fig. 1: locality 7), tentatively identified as *T.* cf. *panwaensis* by Grismer et al. (2019), requires further studies as it may represent a lineage closely related to *Tylototriton zaimeng* sp. nov.

Our knowledge on biology of *Tylototriton zaimeng* sp. nov. is incomplete. Adult animals were encountered both at night and during the day-time on the shallow parts of the lake (Fig. 9). The Zaimeng Lake, where the new species was for the first time encountered, is situated on top of Khongtheng Mountain Range at Thonglang Village (Bena Tababang); it is located on an elevation of ca. 2,212 metres above sea level and is one of the highest lakes in Manipur (Fig. 9A). The lake total area measures about 90,580.46 m^2 and is surrounded by evergreen montane forest composed of *Michelia champacca*, *Phoebe hainensenia*, *Magnolia* sp. and *Quercus* sp. with dense *Arundinaria munro* bamboo undergrowth (Sebastian 2015; Singh et al. 2018). Adult male and female newts were observed slowly moving along the muddy bottom in clear water. *Tylototriton zaimeng* sp. nov. is locally abundant: in July, hundreds of adult newts could be seen on the bottom of Zaimeng Lake (Fig. 9B, C). The new species is known to local Liangmei people as "*Takope*", or "*Tadui taku*" in Liangmei dialect (Singh et al. 2018), "Lengva" in Tangkhul and "Hangoi mamei panba" in Manipuri meaning "Tailed amphibian" (Selim 2001). Other species of amphibians recorded syntopically with the new species at the type locality include *Polypedates* sp. and *Zhangixalus* sp.

Etymology. The specific name "*zaimeng*" is given as a noun in apposition and represents a Latinised version of the Liangmei dialect word "*zaimeng*" literally meaning "Puzzle Lake" or "Mystery Lake". The name is given in reference to the Zaimeng Lake – a high-elevation mountain lake in Koubru Forest Division, the famous location where the crocodile newts were for the first time recorded in Manipur. It is believed that the name of the Lake was given by the Zeliangrong ancestors of Thonglang Village who passed by the Lake, but could not find their way to their destination as they used to return to the same spot again and again and circled the Lake over and over again (Sebastian 2015).

Recommended vernacular names. We recommend the following trivial name in English: Zaimeng Lake Crocodile Newt. The vernacular name in Liangmei dialect: Tadui taku; Tangkhul: Lengva; Manipuri: Hangoi mamei panba.

Conservation status. *Tylototriton zaimeng* sp. nov. is to date known from not more than five localities in montane areas of Manipur State of north-eastern India; the actual extent of range of the new species is unknown (see Fig. 1). The new species is anticipated to inhabit elevations above 1,000 m a.s.l. on mountains of the Khongtheng Mountain Range and could be possibly found in the adjacent parts of Nagaland and perhaps even the Sagaing Division of Myanmar. Further studies are needed to understand the current distribution range, population trends and possible threats to *Tylototriton zaimeng* sp. nov. The montane forests where the new species occurs are affected by growing forest destruction and anthropogenic pressure. Given this information, we tentatively suggest *Tylototriton zaimeng* sp. nov. to be considered as a Vulnerable (VU) species, following IUCN's Red List categories (IUCN 2019).

Discussion

The discovery of *Tylototriton zaimeng* sp. nov. brings the total number of the nominal species of the genus *Tylototriton* to 40. Our study also suggests that *T. verrucosus* sensu stricto likely does not occur in India and its range is likely restricted to north-eastern Myanmar, south-western Yunnan Province of China and northernmost Thailand (Pomchote et al. 2020b). It has to be noted, however, that many taxonomic issues regarding Indian *Tylototriton* remain unresolved, even after our publication and are awaiting further research. In particular, the taxonomic

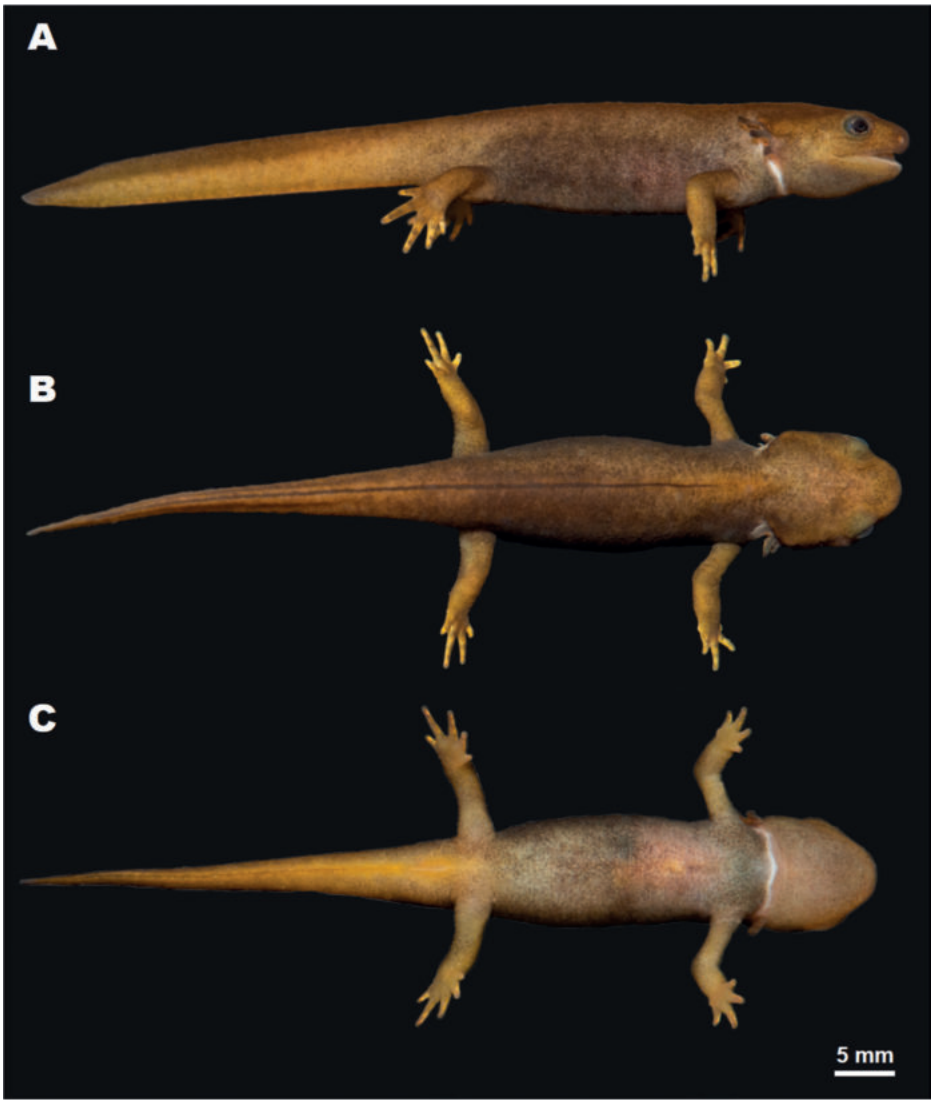

Figure 8. Lateral (**A**), dorsal (**B**) and ventral (**C**) views of larval specimen (MZMU3037; Bernardes et al. (2017) stage 45) of *Tylototriton zaimeng* sp. nov. in life. Scale bar: 5 mm. Photographs by Andrey M. Bragin.

status of the *Tylototriton* populations in Indian Eastern Himalaya (West Bengal, Sikkim, Arunachal Pradesh) remains to be assessed: these populations may be conspecific to *T. himalayanus* originally described from eastern Nepal or may represent new, previously unknown lineages that could be candidates for future taxonomic assessment (Hernandez et al. 2018). The extent of distribution of *Tylototriton* salamanders in north-eastern India also remains insufficiently understood: the records of *Tylototriton* from Meghalaya, previously tentatively assigned to *T. himalayanus* (Hernandez et al. 2018) may be found to be closely related to *Tylototriton zaimeng* sp. nov. and further integrative taxonomic studies are needed to clarify the taxonomic status of these populations. Finally, the taxonomic status of the existing record of *Tylototriton* from Sagaing Division of north-western Myanmar (Fig. 1, locality 7) has to be clarified using an integrative taxonomic approach. Previously, this population was listed as *T.* cf. *panwaensis* (Grismer et al. 2019); however, as it was recorded in the mountain system connected to Khongtheng Mountain Range where *Tylototriton zaimeng* sp. nov. occurs, these populations may be conspecific.

Our work further emphasises the importance of the integrative taxonomic approach, combining data from multiple lines of evidence, including molecular and morphological differentiation, for assessing the diversity and evolutionary relationships of *Tylototriton* salamanders. Numerous previous studies have demonstrated that evolutionary independent lineages of *Tylototriton* may demonstrate only subtle differences between each other in morphological characters (Khatiwada et al. 2015; Phimmachak et al. 2015; Grismer et al. 2018, 2019; Than Zaw et al. 2019; Bernardes et al. 2020; Li et al. 2020; Pomchote et al. 2020a, 2021; Poyarkov et al. 2021b). It has to be noted, however, that species delimitation, based on just two mtDNA markers used in the present study (16S rRNA and ND2 genes), has certain limitations. For example, high mtDNA polymorphism was reported within single geographic populations of several *Tylototriton* species, such as *T. ziegleri* and *T.*

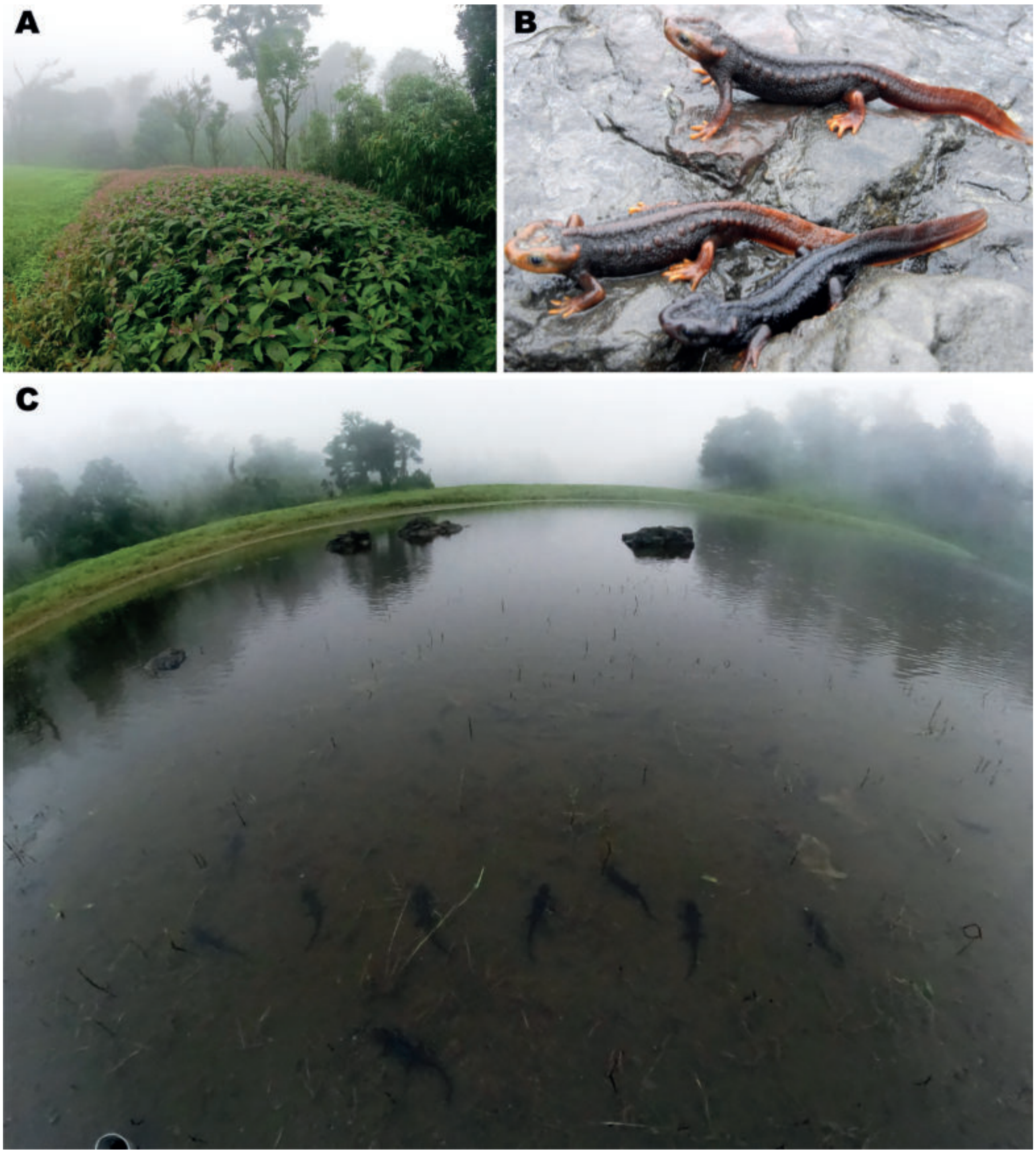

Figure 9. Natural habitat of *Tylototriton zaimeng* sp. nov. at Zaimeng Lake, Koubru Forest Division, Kangpokpi, Manipur, India. **A.** Vegetation surrounding the Zaimeng Lake; **B.** Males of *Tylototriton zaimeng* sp. nov. *in situ*; **C.** Breeding habitat of *Tylototriton zaimeng* sp. nov., numerous individuals can be seen on the muddy bottom of the lake. Photographs by Parag Shinde and Ht. Decemson.

maolanensis (Dufresnes and Hernandez 2022). Furthermore, matching the review by Dufresnes and Hernandez (2022), in our study the genetic distances in 16S rRNA gene, which was earlier proposed as a 'barcoding' gene for amphibians (Vences et al. 2005a, b; Vieites et al. 2009), did not closely correlate with distances of the more fast-evolving locus (ND2; see Table 2). Therefore, it seems that, at least in *Tylototriton*, estimating taxonomic ranks, based on 16S rRNA thresholds is not reliable and a truly multilocus approach combining data from mtDNA and nuDNA genetic markers using larger sample sizes is required for more accurate species delimitation in this group (Dufresnes and Hernandez 2022).

Despite comparatively shallow divergences amongst *T. panwaensis*, *T. houi* and *Tylototriton zaimeng* sp. nov. in mtDNA sequences, these taxa show unique phenotypic features allowing distinguishing them as independent species. The peculiar curved bell-shape of the vomerine tooth series observed in Manipur populations of crocodile newts was never previously reported in any other member of *T. verrucosus* species group; therefore, this feature appears to be an important diagnostic character supporting the full species status of *Tylototriton zaimeng* sp. nov. Moreover, our study revealed significant morphological differentiation between *Tylototriton zaimeng* sp. nov. and its sister species *T. panwaensis*, which are clearly separated in the morphospace of PCA analysis (Fig. 3). Despite the small sample size for *T. panwaensis* (five male specimens) which may potentially affect the results of PCA analysis, the complete absence of an overlap between the two species in morphospace, along with diagnostic characters, such as the shape of vomerine tooth series and number of rib nodules, overall support the status of Manipur populations of crocodile newts as a separate species.

Though significant progress was recently achieved in summarising the available information of evolutionary relationships and phylogeography in *Tylototriton* salamanders (e.g. Dufresnes and Hernandez (2022), many questions still await to be answered. Future survey efforts might focus on insufficiently explored regions where new lineages and species are likely to be found, such as the remaining states of north-eastern India (Nagaland, Arunachal Pradesh, Meghalaya), northern and eastern Myanmar, Bhutan and southern China. Taxonomy of several species complexes within the *T. verrucosus* species group still remains insufficiently understood. For example, as in case of *T. verrucosus* – *T. shanjing* and *T. shanorum* – *T. ngarsuensis* species complexes, significant differentiation in morphology and colouration are combined with very shallow divergence in mitochondrial loci examined. Further comprehensive taxonomic studies, including genomic-level analyses and examination of a broader geographic sampling, are required for a better understanding of the diversity and distribution of these iconic amphibians.

The recognition of Manipur populations of *Tylototriton* as a distinct species, *Tylototriton zaimeng* sp. nov., would benefit its conservation, as well as further underline the importance of the montane subtropical forests of north-eastern India as one of the key centres of herpetofaunal diversity in Asia (Selim 2001; Sebastian 2015; Mirza et al. 2022). Further intensified survey efforts are required to document the amphibian fauna of the region and to elaborate the corresponding conservation measures. Mountain forests in north-eastern India are threatened to a greater degree than in other parts of India and, hence, immediate efforts to document the biodiversity of the region are imperative to ensure its conservation.

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Appendix 1

Specimens examined:

Tylototriton zaimeng sp. nov.: Zaimeng Lake, Koubru Forest Division, environs of Chawangkining Village, Kangpokpi District, Manipur State, Northeastern India (MZMU-2942–2950)

Tylototriton zaimeng sp. nov.: Chingjaroi Ngachaphung, Ukhrul District, Manipur State, Northeastern India (MZMU-3037–3041)

Tylototriton zaimeng sp. nov.: Phungyar (environs of Tangkhul Hungdung), Kamjong District, Manipur State, Northeastern India (MZMU-3035–3036)

Tylototriton panwaensis: Myitkyina area, Sadung area, environs of Sadung village, Kachin, Myanmar (ZMMU NAP-09477–09485)

Tylototriton verrucosus: Bhamo area, Momauk Dist., Lawmun village environs, Sen Lum Mt., Kachin, Myanmar (ZMMU NAP-09486–09493)

Tylototriton verrucosus: Doi Chang Mt., Chiang Rai, Thailand (ZMMU NAP-11668, ZMMU NAP-12905)

Tylototriton kachinorum: Ingyin Taung Mountain, Indawgyi Lake area, Mohnyin Township, Kachin State, Myanmar (ZMMU A5953–A5957, ZISP 13721, ZDUM-0101–0105)

Tylototriton uyenoi: Chiang Mai, Thailand (ZMMU NAP-08220)

Tylototriton uyenoi: Doi Suthep, Chiang Mai Province, Thailand (NSMTH 1073-1077)

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Expanded description of *Hemiphyllodactylus hongkongensis* (Squamata, Gekkonidae)

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https://zoobank.org/C4FC529A-B70C-4E9E-9239-586ADA4D140A

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Abstract

The expanded description of *Hemiphyllodactylus hongkongensis* Sung, Lee, NG, Zhang & Yang, 2018 is provided based on four newly collected specimens from eastern Guangdong Province, China, which is beyond its previously known range. The new collection also fills the distribution gap of the genus *Hemiphyllodactylus* in the region. Due to the absence of sufficient data, we recommend *Hemiphyllodactylus hongkongensis* be listed as Data Deficient (DD) in the IUCN conservation status categorization.

Key Words

conservation, molecular phylogeny, morphology, slender gecko

Introduction

The genus *Hemiphyllodactylus* Bleeker, 1860, commonly known as the half leaf-fingered geckos, dwarf geckos or slender geckos, are a group of extremely diverse yet morphologically conserved lizards that are widespread in the Indo-Pacific realm with 54 recognized species (Zug 2010; Grismer et al. 2013; Agarwal et al. 2019; Agung et al. 2021, 2022; Uetz et al. 2023). The rapid advancement and application of molecular techniques in taxonomic studies have led to a great increase in the discovery of cryptic species in recent years (Zug et al. 2010; Grismer et al. 2013; Grismer et al. 2015; Cobos et al. 2016; Grismer et al. 2018; Agarwal et al. 2019; Grismer et al. 2020).

The Hong Kong Slender Gecko, *Hemiphyllodactylus hongkongensis* Sung, Lee, NG, Zhang & Yang, 2018 was originally described based on two male and six female specimens from Aberdeen Country Park (22°15.51'N, 114°9.69'E; 120 m a.s.l.) and Po Toi Island (22°9.83'N, 114°15.33'E; 50 m a.s.l.), Hong Kong SAR, China, with molecular evidence in the mitochondrial ND2 gene and the following diagnostic characters: (1) 5–6 chin scales in a unique combination, (2) manual lamellar formula 3-3(4)-4-4, (3) pedal lamellar formula 3(4)-4(5)-4(5)-4, (4) 24–25 continuous femoral and precloacal pores, (5) 12–15 dorsal scales contained in diameter of eye, (6) 9–10 ventral scales contained in diameter of eye (Sung et al. 2018). It was previously thought to be endemic to Hong Kong. However, during recent herpetological surveys in eastern Guangdong Province, China where there is no record of the genus *Hemiphyllodactylus*, we collected four specimens which generally match the morphological diagnosis of *H. hongkongensis* but also show variations in the manual lamellar formula, pedal lamellar formula, the number of continuous femoral and precloacal pores, dorsal and ventral scales contained in diameter of eye. Subsequent phylogenetic analysis demonstrates the newly collected specimens to be conspecific with *H. hongkongensis* with subtle genetic divergences. Herein, a detailed morphological redescription includes the revised diagnostic characters, sexual dimorphism and ecology information, as well as the expanded geography range are provided.

Materials and methods

Morphometrics

Morphological examinations were performed on the four newly collected specimens from eastern Guangdong Province, China (Fig. 1). The collection information is given in the taxonomy account below. All specimens were fixed in 10% buffered formalin and later transferred to 70% ethanol for preservation, and deposited at the Guangdong Polytechnic of Environmental Protection Engineering (GEP), Foshan City, Guangdong, China; tissue samples were preserved in 95% ethanol for molecular studies.

Measurements followed Zug. (2010) and were taken with a digital caliper to the nearest 0.1 mm. These measurements are as follows: snout-vent length (SVL, distance from the tip of snout to the vent), tail length (TailL, from the vent to the tip of tail), trunk length (TrunkL, taken 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), eye diameter (EyeD, the horizontal diameter of the eyeball); head length (HeadL, distance between retroarticular process of jaw to snout-tip), head width (HeadW, maximum width of head), nares-eye length (NarEye, measured from the anterior margin of the eye ball to the posterior margin of the external nares), snout-eye length (SnEye, distance between anterior most point of eye and the tip of snout), internarial width (SnW, distance between external nares). Evaluation of meristic characters and color pattern followed Zug (2010) and Grismer et al. (2013).

Phylogenetic analyses

In total, 156 samples including four outgroup samples were used in this study, encompassing four newly sequenced individuals and others downloaded from GenBank. Detailed information for all samples was given in Suppl. material 1.

The NADH dehydrogenase subunit 2 gene (*ND2*) fragment was amplified for new samples with the primers ND2f101A and HemiR followed Sung et al. (2018). DNA extraction, PCR, and sequencing followed Agung et al. (2022). Sequences were aligned with Clustal X 2.0 (Thompson et al. 1997) with default parameters. PartitionFinder2 was used to test the best partitioning scheme and jModelTest v2.1.2 was used to test the best fitting nucleotide substitution models, resulting the *ND2* gene partitioned by codon position, and the best fit models

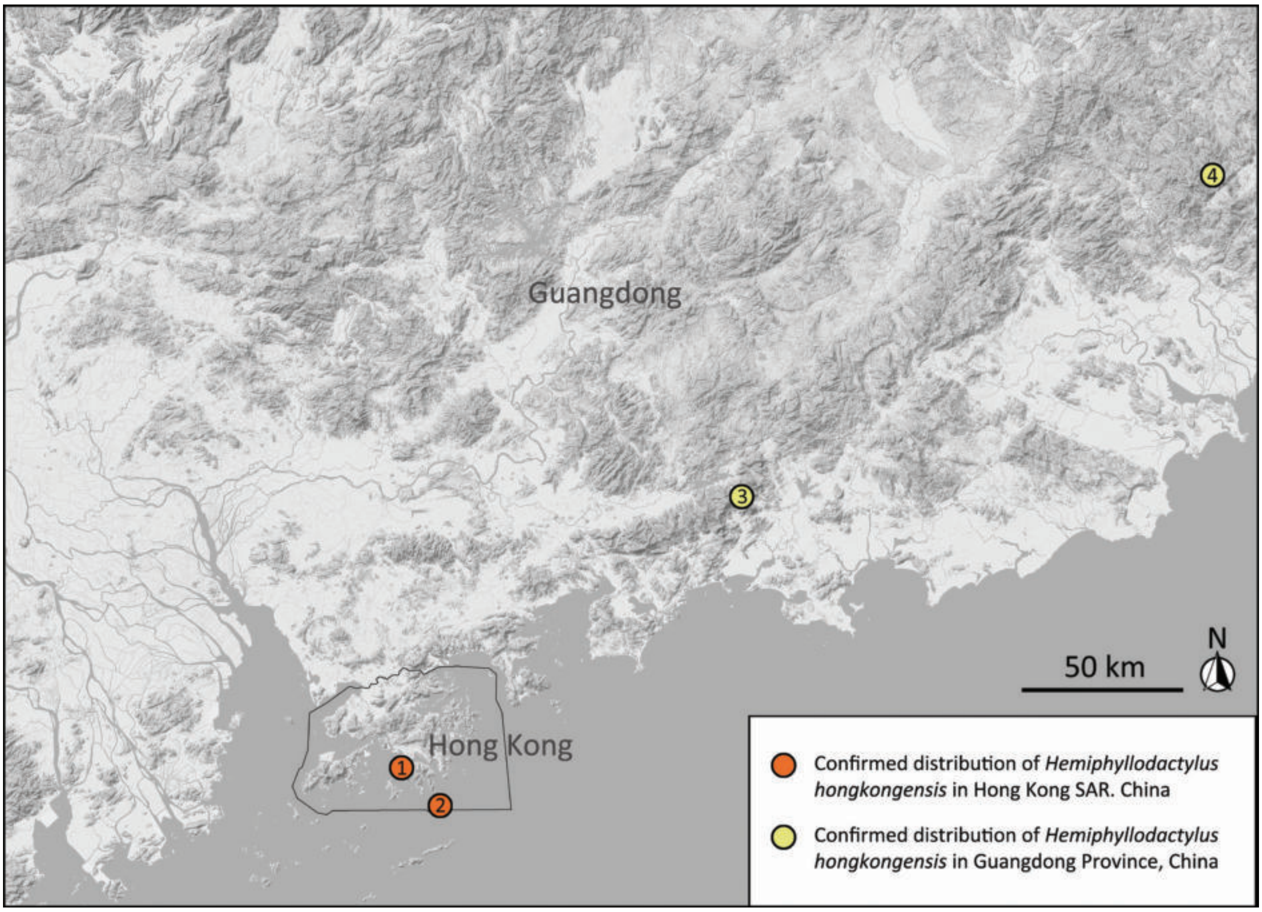

Figure 1. Map showing the confirmed distribution of *Hemiphyllodactylus hongkongensis*: Aberdeen Country Park **(1)** and Po Toi Island **(2)**, Hong Kong SAR, China, and Mt. Lianhua **(3)** and Mt. Fenghuang **(4)**, Guangdong Province, China.

for all partitions was GTR+I+G (General Time Reversible model, with variable sites modelled according to the Gamma distribution, and a portion of the sites invariant). Phylogenetic analysis was conducted using Bayesian inference (BI) in MrBayes 3.2.4 (Ronquist et al. 2012) and maximum likelihood (ML) in maximum likelihood (ML) in Randomized Axelerated Maximum Likelihood (RAxML, Stamatakis 2006) with RAxML GUI 1.3 (Silvestro and Michalak 2012). For the ML analysis, an optimal tree was obtained and branch supports were evaluated with 1000 rapid bootstrapping replicates. For the BI analysis, two independent runs with four Markov Chain Monte Carlo simulations were performed for ten million iterations and sampled every 1000 iterations. The first 25% of the samples were discarded as burn-in, leaving 7500 samples in the final summary. Convergence of the Markov Chain Monte Carlo simulations was assessed by PSRF < 0.01 and ESS (effective sample size) value > 200 using Tracer 1.4 (http://tree.bio.ed.ac.uk/software/tracer/). Nodes having ML bootstrap values (BS) ≥ 70 and BI posterior probabilities (BPP) ≥ 0.90 were considered well supported. Genetic distances among *Hemiphyllodactylus hongkongensis* with its phylogenetically close congeners were calculated in MEGA 6 using the uncorrected *p*-distance model.

Results

The ML and Bayesian results show identical topologies, and all *Hemiphyllodactylus* samples form a monophyletic clade with strongly-support in both phylogenetic trees (Fig. 2). *Hemiphyllodactylus hongkongensis*, *H. yanshanensis*, *H. huishuiensis*, *H. nahanensis*, *H. dushanensis*, *H. dupanglingensis*, *H. zugi* and *H. ngocsonensis* form a robust clade (BS = 100; BPP = 1.00), and their uncorrected *p*-distance are calculated and listed in Suppl. material 2. In addition, the *Hemiphyllodactylus* samples from Mt. Lianhua and from Mt. Fenghuang cluster with topotypic samples of *H. hongkongensis* with shallow divergence (BS = 100; BPP = 1.00; *p*-distance 0.002–0.017). This result indicates that the newly collected specimens from eastern Guangdong should be identified as *H. hongkongensis*.

Taxonomic account

Hemiphyllodactylus hongkongensis Sung, Lee, NG, Zhang & Yang, 2018

Fig. 3

Hong Kong Slender Gecko / xiāng gǎng bàn yè zhǐ hǔ (香港半叶趾虎)

Chresonymy. *Hemiphyllodactylus* sp. – Chan et al. 2018.

Examined materials. GEP r024, adult male, and GEP r025–026, adult females, collected by Jian Wang and Zhao-Chi Zeng from Mt. Fenghuang (26°54'33.61"N, 116°36'26.51"E; ca. 1180 m a.s.l.), Chaozhou City, Guangdong Province, China. GEP r032, adult male, collected by Jian Wang and Zhao-Chi Zeng from Mt. Lianhua (23°4'3.51"N, 115°14'14.39"E; ca. 870 m a.s.l.), Huizhou City, Guangdong Province, China.

Description of new specimens. Morphometric data are listed in Tables 1, 2. Head triangular in dorsal profile, depressed, distinct from neck; lores and interorbital regions flat; rostrum relatively long (NarEye/HeadL 0.27–0.33); prefrontal region flat; canthus rostralis smoothly rounded, snout moderate, rounded in dorsal profile; eye large (EyeD/HeadL 0.22–0.24, EyeD/NarEye 0.80–0.84); ear opening oval, small; eye to ear distance greater than diameter of eye; rostral wider than high, bordered posteriorly by large supranasals; 1–3 internasals in contact with rostral anteriorly; circumnasals 3–4; supralabials 10–13; infralabials 9–12; dorsal superciliaries flat, rectangular, imbricate; mental triangular, bordered laterally by first infralabials and posteriorly by two (GEP r024–026) or three (GEP r032) large postmentals; gular scales triangular small, granular, grading posteriorly into slightly larger, subimbricate, throat and pectoral scales which grade into slightly larger, subimbricate ventrals.

Body slender and small, dorsoventrally compressed, ventrolateral folds absent; dorsal scales small, granular, 18–20 scales contained within one eye diameter; ventral scales, flat, subimbricate, larger than dorsal scales, 10–13 scales contained within one eye diameter; in males, 23–24 pore-bearing scales extending from midway between the knee and hind limb insertion of one leg to the other.

Forelimbs short, robust in stature, covered with granular scales dorsally and with slightly larger, flat, subimbricate scales ventrally; palmar scales flat, imbricate; all digits except digit I well developed; digit I vestigial, clawless; distal, subdigital lamellae of digits II–V undivided, angular and fan-shaped; lamellae proximal to these transversely expanded; lamellar formula of digits II–V 3–4–4(5)–4 on both hands; 4–5 transversely expanded lamellae on digit I; claws on digits II–V well-developed, unsheathed; distal portions of digits strongly curved, terminal joint free, arising from central portion of lamellar pad. Hind limbs short, more robust than forelimbs, covered with slightly pointed, juxtaposed scales dorsally and by larger, flat subimbricate scales ventrally; all digits except digit I well-developed; digit I vestigial, clawless; distal, subdigital lamellae of digits II–V undivided, angular and fan-shaped; lamellae proximal to these transversely expanded; lamellar formula of digits II–V 4–4(5)–4(5)–4(5); 4–5 transversely expanded lamellae on digit I; claws on digits II–V well-developed, unsheathed; distal portions of digits strongly curved, terminal joint free, arising from central portion of lamellar pad; posterior section of tail broken, round in cross-section; all caudal scales flat, subimbricate, not forming distinct caudal segments.

Coloration of new specimens in life. Dorsal surface of head, body, and limbs pale-brown, densely mottled with irregular darker markings; a dark brown preorbital tripe extending from external nares to anterior corner of eye; a dark brown postorbital tripe through extending from posterior corner of eye to just anterior of forelimb insertion

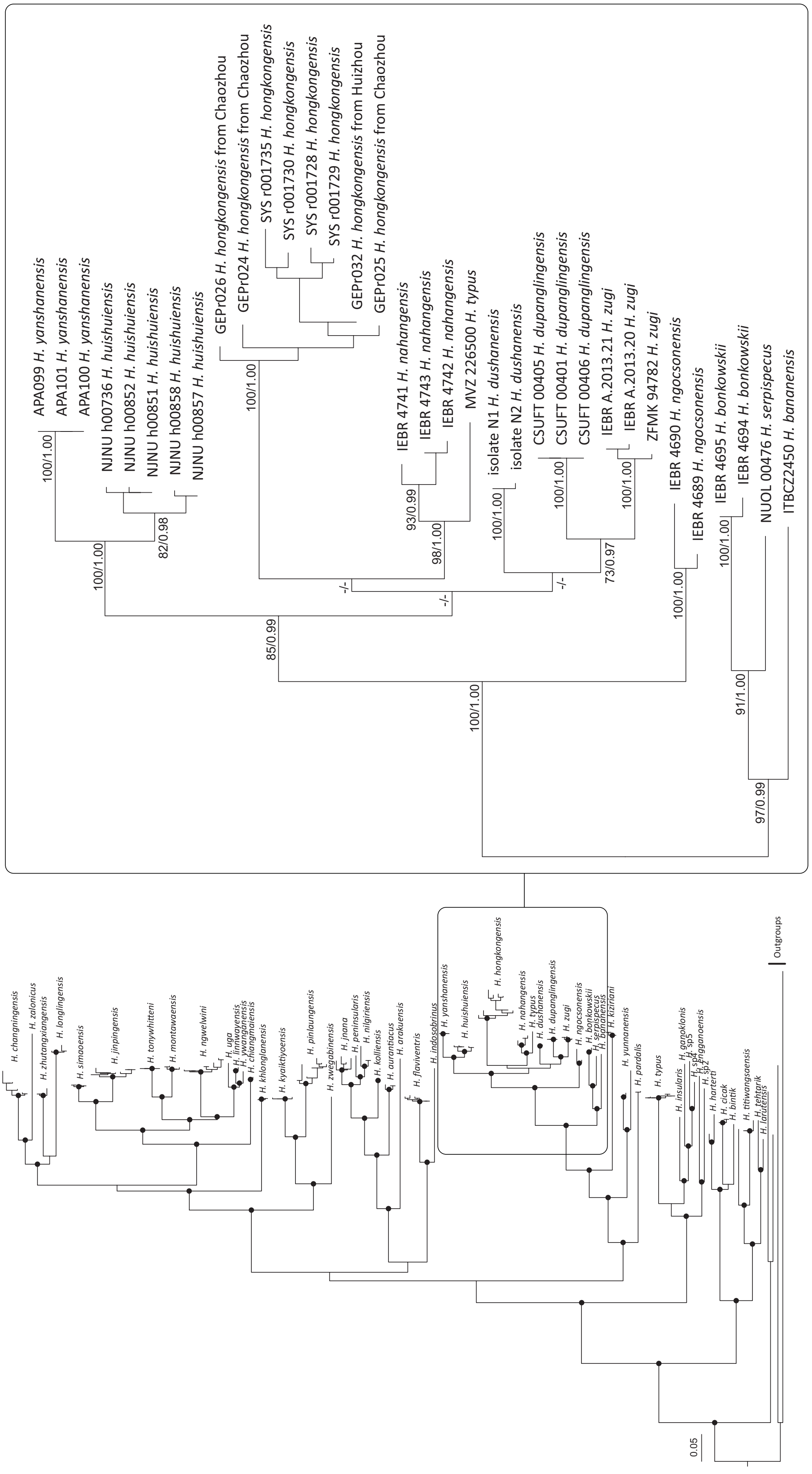

Figure 2. Bayesian Inference and Maximum Likelihood phylogenies. Black dots at nodes in the left part of the trees represents the Bayesian posterior probabilities (BPP) > 0.90 and the bootstrap supports (BS) > 70. Numbers at nodes in the right part of the tree represents the BS/BPP and '-' represents Bayesian posterior probabilities (BPP) < 0.90 or the bootstrap supports (BS) < 70.

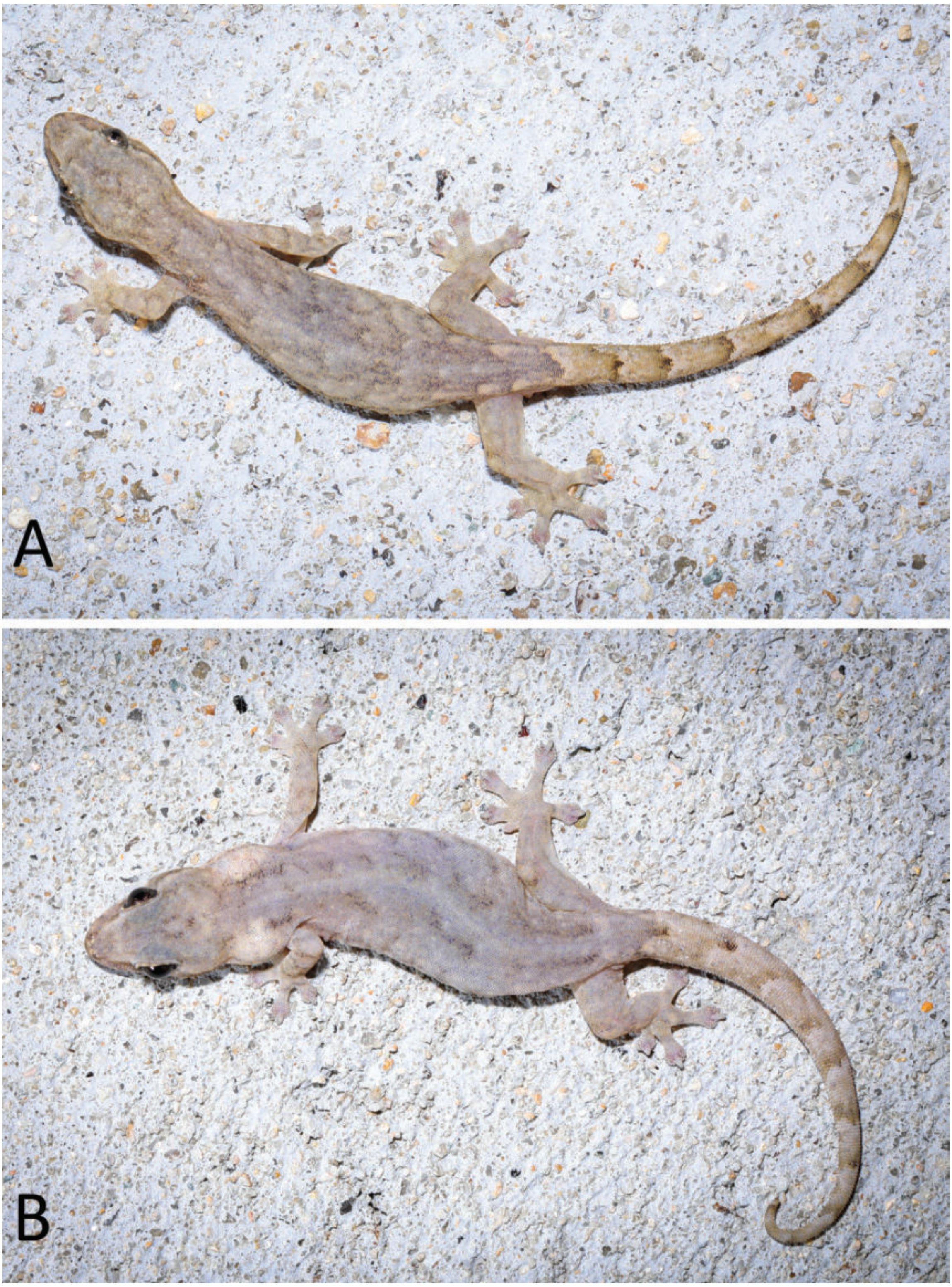

Figure 3. Life aspect of *Hemiphyllodactylus hongkongensis* **A.** Male (GEP r032) and **B.** Female (uncaptured) from Mt. Lianhua, Huizhou City, Guangdong Province, China. Photos by Jian Wang.

on body; limbs and digits with irregularly shaped dark markings; ventral head, body and limbs unicolor beige; dorsal tail olive-brown, with several irregularly shaped dark markings and gray-white mottling, ventral tail unicolor gray-brown.

Coloration of new specimens in preservative. Dorsal surface of head, body, limbs and tail dorsal dark brown, irregular darker markings more distinct; ventral head, body and limbs gray-brown; pre- and postorbital tripes, irregularly shaped dark markings on limbs and digits, dark

Table 1. Scalation of *Hemiphyllodactylus hongkongensis*.

Vocher	**SYS r001735 (Holotype)**	**SYS r001734 (Paratype)**	**GEP r024**	**GEP r032**	**SYS r001728 (Paratype)**	**SYS r001729 (Paratype)**
Sex	Male	Male	Male	Male	Female	Female
Dorsal scales	15	14	19	20	13	14
Ventral scales	10	9	13	12	10	9
Cloacal spurs on each side	1	1	1	1	1	1
Circumnasal scales	3	3	4	3/4	3	3
Scales between supranasals	3	3	2	3	3	3
Supralabial scales	10/10	11/11	11/13	12/10	11/11	12/12
Infralabial scales	9/10	10/11	10/11	10/11	10/10	10/10
Chin scales	5	6	6	6	6	5
Manual lamellar formula	3444	3444	3454	3444	3444	3444
Pedal lamellar formula	4554	4554	4554	4445/4554	4444	4554
Subdigital lamellae on 1st finger	5	5	4	5	4	4
Subdigital lamellae on 1st toe	5	5	4	5	5	5
Precloacal and femoral pores	24	25	24	23	0	0
Vocher	**SYS r001730 (Paratype)**	**SYS r001731 (Paratype)**	**SYS r001732 (Paratype)**	**SYS r001733 (Paratype)**	**GEP r025**	**GEP r026**
Sex	Female	Female	Female	Female	Female	Female
Dorsal scales	13	12	14	13	18	20
Ventral scales	9	9	9	10	10	12
Cloacal spurs on each side	1	1	0	1	1	1
Circumnasal scales	3	3	4	3	3	3
Scales between supranasals	3	4	3	3	2	1
Supralabial scales	12/12	10/10	12/11	11/11	13/12	11/11
Infralabial scales	10/10	10/10	11/10	11/10	11/9	10/12
Chin scales	6	6	5	6	6	6
Manual lamellar formula	3444	3344	3444	3444	3444	3444
Pedal lamellar formula	4554	3444	4554	4554	4554	4554
Subdigital lamellae on 1st finger	4	3	4	4	4	4
Subdigital lamellae on 1st toe	5	5	5	5	5	4
Precloacal and femoral pores	0	0	0	0	0	0

markings on dorsal tail more distinct; gray-white mottling on dorsal tail absent; ventral tail unicolor dark brown.

Sexual dimorphism. Males possess a pair of hemipenis and 23–25 precloacal and femoral pores; gravid females harbor two calcareous eggs.

Revision of diagnostic characters. (1) 5–6 chin scales in the unique combination, (2) manual lamellar formula 3-3(4)-4(5)-4, (3) pedal lamellar formula 3(4)-4(5)-4(5)-4(5), (4) 23–25 continuous femoral and precloacal pores, (5) 12–20 dorsal scales contained in diameter of eye, (6) 9–13 ventral scales contained in diameter of eye.

Distribution and ecology. *Hemiphyllodactylus hongkongensis* is currently known at low altitude from Aberdeen Country Park (ca. 120 m a.s.l.) and Po Toi Island (ca. 50 m a.s.l.), Hong Kong SAR, China, and Mt. Lianhua (ca. 870 m a.s.l.) and Mt. Fenghuang (ca. 1180 m a.s.l.) in eastern Guangdong Province, China. However, the taxonomic status of *Hemiphyllodactylus* populations from Shek Kwu Chau and Pokfulam Country Park in Hong Kong SAR, China still remained further confirmation (Karsen et al. 1998; Sung et al. 2018; Zug, 2010).

Hemiphyllodactylus hongkongensis is a forest dwelling species which can be found among the bark of large trees, abandoned buildings and rock crevices (Chan et al. 2008). Both newly discovered populations are found in the forest area at high altitude above ca. 800 m a.s.l.. Specimens from Mt. Lianhua are collected on the wall of an abandoned house, while those from Mt. Fenghuang are collected on the bare rocks. This species is oviparous so that each adult female including uncaptured individuals harbors two mature calcareous eggs during surveys on February (Mt. Lianhua) and July (Mt. Fenghuang).

Discussion

The genus *Hemiphyllodactylus* is an extremely diverse but taxonomically complicated group with dozens of morphological conservative congeners distributed in various habitats (Zug et al. 2010). The rapid advancement and application of molecular biology techniques have made great advancements in the taxonomic studies of this genus (Agarwal et al. 2019; Cobos et al. 2016; Grismer et al. 2013; Grismer et al. 2015; Grismer et al. 2018; Grismer et al. 2020; Zug et al. 2010). However, using single gene data, the phylogenetic relationships among *H. hongkongensis* and its genetically close congeners still remain unclear (Do et al. 2020; Sung et al. 2018; Zhang et al. 2020), More comprehensive genetic data from multiple populations are needed to solve this issue.

Even though the distribution range has been vastly expanded in this study, *Hemiphyllodactylus hongkongensis* likely has an even wider range in southeastern China due to its ability to adapt to diverse habitats in different elevations (see Distribution and ecology section above). More data based on exhaustive investiga-

Table 2. Measurements and body proportions of *Hemiphyllodactylus hongkongensis*.

Vocher	SYS r001735 (Holotype)	SYS r001734 (Paratype)	GEP r024	GEP r032	SYS r001728 (Paratype)	SYS r001729 (Paratype)	SYS r001730 (Paratype)	SYS r001731 (Paratype)	SYS r001732 (Paratype)	SYS r001733 (Paratype)	GEP r025	GEP r026
Sex	Male	Male	Male	Male	Female	Female	Female	Female	Female	Female	Female	Female
SVL	33.6	32.3	37.2	43.1	37.5	37.4	40.8	42.1	43.0	38.9	40.4	41.5
TailL	27.3	23.9	31.3	38.8	3.8	29.4	36.3	36.1	34.2	12.7	35.8	14.0 (broken tail)
TrunkL	15.8	15.6	17.3	17.9	19.4	19.3	21.0	21.3	22.0	20.4	20.4	18.8
HeadL	9.3	8.7	9.7	10.4	9.5	9.9	10.4	10.3	11.2	10.4	10.3	9.9
HeadW	6.9	7.0	7.1	8.1	5.2	6.4	8.0	7.5	8.2	8.2	7.2	8.0
SnEye	3.1	3.2	3.8	3.9	3.2	3.7	3.8	3.6	4.0	3.6	4.1	3.9
NarEye	2.4	2.6	2.8	2.9	2.6	2.5	2.9	3.1	3.0	3.0	2.8	2.7
EyeD	2.0	2.3	2.3	2.4	2.2	2.3	2.4	2.3	2.5	2.3	2.2	2.2
SnW	1.1	1.1	1.1	1.2	1.2	1.1	1.2	1.3	1.4	1.3	1.1	1.2
TrunkL/SVL	0.47	0.48	0.47	0.42	0.52	0.52	0.51	0.50	0.51	0.53	0.51	0.45
HeadL/SVL	0.28	0.27	0.26	0.24	0.25	0.27	0.26	0.24	0.26	0.27	0.26	0.24
HeadW/SVL	0.21	0.22	0.19	0.19	0.14	0.17	0.20	0.18	0.19	0.21	0.26	0.24
HeadW/HeadL	0.74	0.80	0.73	0.78	0.55	0.64	0.76	0.73	0.74	0.79	0.70	0.81
SnEye/HeadL	0.33	0.37	0.39	0.38	0.33	0.37	0.36	0.35	0.36	0.35	0.40	0.39
NarEye/HeadL	0.26	0.30	0.28	0.33	0.27	0.26	0.28	0.30	0.27	0.29	0.27	0.27
EyeD/HeadL	0.22	0.27	0.24	0.22	0.24	0.23	0.23	0.23	0.22	0.22	0.22	0.22
SnW/HeadL	0.12	0.13	0.12	0.14	0.12	0.11	0.11	0.12	0.12	0.12	0.16	0.17
EyeD/NarEye	0.85	0.89	0.84	0.83	0.87	0.89	0.81	0.76	0.82	0.78	0.80	0.81
SnW/HeadW	0.24	0.23	0.17	0.19	0.33	0.28	0.26	0.24	0.23	0.22	0.22	0.21

tions are still lacking in the mountain belt that stretches from Hongkong SAR and eastern Guangdong Province, to Fujian Province, China. Thus, we recommend *H. hongkongensis* be listed as Data Deficient (DD) in the IUCN categorization, pending further investigation. Moreover, the taxonomic status of *Hemiphyllodactylus* populations from Shek Kwu Chau and Pokfulam Country Park in Hong Kong SAR, China still require further confirmation (Karsen et al. 1998; Zug, 2010; Sung et al. 2018).

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Supplementary material 1

Localities, voucher information, and Genbank accession numbers for specimens used in this study

Authors: Xiang-Yi Li, Shi-Shi Lin, Zhao-Chi Zeng, Yan-Jun Sun, Jian Wang

Data type: xlsx

Link: https://doi.org/10.3897/herpetozoa.36.e106566.suppl1

Supplementary material 2

Pairwise distances based on ND2 gene among *Hemiphyllodactylus hongkongensis* and its phylogenetically close congeners

Authors: Xiang-Yi Li, Shi-Shi Lin, Zhao-Chi Zeng, Yan-Jun Sun, Jian Wang

Data type: xls

Link: https://doi.org/10.3897/herpetozoa.36.e106566.suppl2

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Occurrence and tentative population status of the Balkan Terrapin (*Mauremys rivulata*, Valenciennes, 1833) on Greek islands

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Abstract

The distribution of *Mauremys rivulata* on Aegean islands was summarized by Broggi in 2012. Here, the study area encompasses all Greek islands, including the Ionian Islands, and the two Turkish islands of Gökceada and Bozcaada. For the first time, I attempt to estimate the status and size of the populations on the islands. This is a subjective assessment based on my personal visits to most islands and not on IUCN criteria. *Mauremys rivulata* was found on 29 Greek islands, plus two Turkish islands in the Aegean Sea. Five previously mentioned sites are doubtful, and on the three islands of Sifnos, Syros and Ithaca the species appears to be extinct. On 12 islands I assess its status as "threatened with extinction". On seven, mostly larger, islands its populations are probably less vulnerable. Efforts must be made for the long-term protection of *M. rivulata* on the Greek islands.

Key Words

conservation, Reptilia, status, Testudines, threats

Species description

The first description of the Balkan Terrapin *Mauremys rivulata* was provided by Valenciennes, on Bory de Saint-Vincent's scientific expedition (1833) to Morea (Peloponnese), under the name *Emys rivulata* (Mantziou and Rifai 2014). In 1913, Siebenrock (1913) split the species into three subspecies. A clear geographical distinction between the former *M. caspica caspica* and *M. caspica rivulata*, and the elevation to species rank, was made by Fritz and Wischuf (1997). This species status was later confirmed by DNA analyses (e.g. Mantziou et al. 2004).

The Balkan Terrapin is an impressive animal, reaching a carapace length of 25 cm, while the 4–10 hatchlings that leave the nest measure 3–4 cm. The species is distributed from the Croatian coast southwards in south-eastern Europe, including Greece, via Turkey to Israel and Syria in the Middle East. It is adapted to a warm Mediterranean climate. In the northern parts of its range it hibernates; while in southern climates it may aestivate over the summer. It occurs in running waters, seasonal ponds and pools, lakes, brackish lagoons, but also drainage systems, e.g. ditches, as well as reservoirs. Only fast-flowing streams are not colonised. The Balkan Terrapin is more tolerant of poor water quality than the European Pond Terrapin (*Emys orbicularis*) which co-occurs in part of the range of *M. rivulata*, and is thus also found in over-fertilised waters or, as mentioned, brackish water.

Due to its tolerance to salinity, the Balkan Terrapin may cross extensive distances at sea, as demonstrated by Vamberger et al. (2014). Since the Asia Minor islands are separated by sea from mainland Anatolia, one would have expected the populations there to have developed differently compared to the Anatolian populations. "The amazing thing is that even turtles that are spatially distant from each other show an almost identical genetic pattern," says

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Prof. Uwe Fritz, who was involved in the study (Vamberger et al. 2014). In other species, this could happen when animals are transported by humans intentionally or accidentally. But in the case of the Balkan Terrapin, this explanation is unlikely. *Mauremys rivulata*, unlike the European Pond Terrapin, often empties the contents of its cloaca when captured. Additionally, the unpleasant, musky-smelling secretion it produces is an effective protection. So there was no reason to transport the turtles on a large scale. On the other hand, turtles may be carried into the sea during storms and river floods and some could survive in the sea until they were washed up on a coast again.

Mauremys rivulata does not normally feed at water temperatures below 13 °C. It is a generalist and opportunist, feeding on plants as well as on small animals. It can occur in densities of 19–217 individuals, in eutrophic waters even up to 2000 individuals per hectare (Wischuf and Busack 2001). There are no such high densities on the Greek islands because such extensive habitats do not occur there, but populations of several hundred animals are possible. Populations on small Mediterranean islands, where wetlands are usually small and under heavy human pressure, are usually very vulnerable. This is true for the majority of their occurrences on Greek islands today. As Beutler and Froer (1980) said: “Many island populations are now threatened with extinction.”

On the herpetological exploration of the islands

From the early 19th century on, the Greek herpetofauna has fascinated the international research community. The first herpetological “discoverer” was the Russian Jacques von Bedriaga, publishing his work “Die Amphibien und Reptilien Griechenlands” in German (Bedriaga 1883). After the turn of the century, with a peak in the 1930s, the Viennese Professor Franz Werner published 12 articles on the herpetofauna of the Greek islands. He was accompanied and followed by the Viennese Otto Wettstein with “Herpetologia aegaea” in 1953 and a supplement in 1957. This has been described as the “German wave” in herpetological research as far as the language of the written contributions was concerned. Since the early 1970s, the number of field herpetologists working on Greek islands has increased. They are referred to by Pafilis (2010) as the “International Brigade” and included, among others, the Britons Richard Clark and David Buttle. Most of the contributions were made by Augusto Cattaneo from Rome, and the author of this paper from Liechtenstein. Tortoises were not always the primary interest for herpetologists, who often concentrated on lizards and/or snakes. Thus, data on the island herpetofauna for *Mauremys* and *Emys* are rather meagre. For Greece, herpetological checklists were compiled by John Ondrias (Ondrias 1968) and Basil Chondropoulos (Chondropoulos 1986, 1989), but these did not include turtles. In the last 20 years, molecular biology has contributed significantly to further research and allowed the recognition of cryptic species in particular. With the standard work “The Amphibians and Reptiles of Greece” by Valakos et al. (2008), researchers from Greece in particular are taking over the further study of the country’s herpetofauna. Our recent knowledge is complemented by numerous European nature photographers who have reported on their travels on various websites and include excellent animal photographs.

Methods: specific procedures for finding *Mauremys rivulata*

The Mediterranean region has been visited annually since 1972 by a group of nature lovers from the Botanical-Zoological Society Liechtenstein-Sargans-Werdenberg from the Alpine Rhine Valley (Table 1). Due to advancing age, the number of excursion participants has decreased from a maximum of 10 to 3. The majority of the 49 excursions to date have taken place on Greek islands, starting in 1975 with Samos. Since 2005, only Greek islands have been visited, a different one every year, and usually for a fortnight. Only the islands of Ikaria, Kythera and Kefalonia have been visited twice so far.

My tasks as excursion leader were island selection, finding literature and, for some time, logistical organisation, which I have since handed over to a colleague. Strikes, strong winds, and Covid-19 have presented logistical challenges. Procuring literature used to be much more difficult before the internet, as the writers came from many states and often used their respective native languages. There was an exchange of writings and experiences, and the number of field herpetologists working on Greek islands was manageable. During the past 20 years, the number of researchers has increased dramatically. Mostly young Greek university graduates have joined, and the language of publication is now predominantly English.

Maps of the islands to be visited were not commercially available in the early days. They were partly hand-drawn and very rudimentary. Today we have suitable GIS-based maps of most (e.g., Anavasi, Skai, Orana, Terrain Map). On them, it is easier to locate hydrological objects with appropriate signatures, such as the indication of springs, permanently flowing watercourses, swamps, lagoons, old water mills, cisterns and wells. Google Maps also provides an overview of hydrological catchment areas with its aerial photographs. The WWF-Greece’s Wetland Inventory of the Greek Archipelago is an important reference source (www.oikoskopio.gr/ygrotopio). In addition, the location data from the literature for the water-loving species of the herpetofauna were used. These are all transferred to the maps.

Once we arrived on an island, we first took a tour to familiarise ourselves with the topographical features. Then the objects marked on the map were systematically visited. The hydrologically productive units were verified in the field and searched if the terrain allowed. In the process, there were always opportunities to talk to local people, who usually wanted to know where we came from

Table 1. Dates of excursions to Greek and Turkish Islands. This list includes all the islands visited in the Aegean and Ionian Sea. No Balkan Terrapins were observed on the islands not mentioned in the article.

Samos 12–20.4.1975
Lesbos 9–23.4.1978
Naxos 28.4–4.5.1984
Lefkas 19.5–24.5.1985
Ikaria 17–30.4.1986
Samothrace 25.4–6.5.1987
Rhodes 23.4.1988
Karpathos 24.4–6.5.1988
Kythira 30.4–4.5.1989
Chios 23.4–3.5.1991
Kefalonia 15–23.4.1993
Andros 8.4–19.4.1995
Thassos 25–29.5.1996
Kalymnos 19–24.4.1997
Leros 25–28.4.1997
Kos 19–20.4.1997
Gökçeada 27.4–7.5.1998
Milos 18–24.4.1999, 18.4.2013
Sifnos 25–30.4.1999
Ikaria 24–4.5.2000
Astypalea 23–29.4.2001
Symi 23.4–1.5.2002
Sesklia 28.4.2022
Skyros 19–30.4.2003
Tilos 8–11.10.2004, 15–23.4.2005
Nisyros 24–27.4.2005
Amorgos 19–28.4.2006
Lipsi 10–20.4. 2007
Patmos 17.4.2007
Kos 9.4.2007
Ithaca 15.4–24.4.2008
Alonissos 13–25.4.2009
Serifos 11–19.4.2010
Paros 20.4.2010
Kea 9–19.4.2011
Crete 10–12.4.2012
Gavdos 12–19.4.2012
Kimolos 5–17.4.2013
Polyegos 15.4.2013
Elafonissos 6–14.4.2014
Kythira 6–18.4.2015
Limnos 17–28.2016
Kefalonia 13–26.4.2017
Skopelos 9–15.6.2019
Skiathos 16.6.2019
Kythnos 28.5–8.6. 2021
Ios 8–17.4.2022
Santorini 18–19.4. 2022
Fourni 14–24.4.2023

and what we were looking for. This was explained in basic terms. In addition, word typically got around on smaller islands about what aspect of the island's natural history these "strangers" were particularly interested in. An island diary was kept, and the geographical information system was used to record the locations and the observations. Towards the end of the island stay, work was already being done on the manuscript for possible publication, so as to record impressions while they were still fresh.

Occurrence of *M. rivulata* on all Greek islands as well as on two Turkish islands in the Aegean Sea (in alphabetical order)

Mauremys rivulata is still found on 29 Greek islands plus on two Turkish islands. Five sites are doubtful and on three islands the species appears to be extinct (Fig. 1). On 12 islands *Mauremys* is threatened with extinction. Only on seven islands its populations are less vulnerable.

Present on island

Andros (380 km^2)

The first record is by Werner (1937). Buttle (1995) includes *Mauremys* for Andros in a species list, without comment. Buttle (1997) states: "Often seen in quite large numbers, especially in still water pools, along the riverine Ateni valley, in hillside streams around Katakilos, and in pools on coastal marshes at the bays of Atemi and Vori."

According to our own experience (Broggi 1996), the Balkan Terrapin occurs in the larger beach lagoons, but in mostly small populations. It migrates sometime up the streams and lives up to its German name "Eastern Mediterranean Stream Turtle". The abundance of water on the island in general, and the scours of the creeks in particular, make it possible for it to survive in isolated populations. Seven location records for *Mauremys* included no more than 20 specimens seen per location (see map in Broggi 1996). The separate populations appear to be stable on the island, especially as the species also inhabits streams (Broggi 1996).

Bozcaada (Tenedos) (37 km^2)

The first record on this Turkish Aegean island comes from Tosunoglu et al. (2009) in a description of the island's herpetofauna. However, only one location is given. Similarly, Gül et al. (2014) write: "Only one freshwater habitat in Lake Azmak; with a fish trap a total of 29 *M. rivulata* were sampled." I have not visited the island personally. Bozcaada is the second smallest island with a *M. rivulata* population. With only one location, the species is threatened.

Chios (843 km^2)

Werner (1935) was the first to describe *M. rivulata* on Chios, followed by Wettstein (1953), and since then its occurrence has been confirmed several times, e.g. in Tsunis and Dimitropoulos (1994), as well as travel descriptions by nature photographers (for example: iNaturalist 2023). In the work on the herpetofauna of Samos and Chios by Cattaneo (2003), however, the species is not mentioned. In my own diary notes for Chios there is only one observation at Kateros, a wetland, with 10 specimens, on 23.4.1991. Thus, it can be stated that the Balkan Terrapin occurs on Chios, but seems not to form large populations. Its status remains to be verified.

Corfu (585 km^2)

First recorded by De Betta (1868), while Werner (1894) also has an early record of *M. rivulata* on the island. Mertens (1961), in particular, dealt intensively with the herpetofauna of Corfu. Buttle (1995) confirms the occurrence: "*Mauremys rivulata* can be found in every gully and lake." In Toth et al. (2002) there is an overview of the occurrence of *M. rivulata* on the island, but without population data. Hill (2003) says: "The two terrapin species could only be found as single individuals." The rarity of both species was striking and contradicts the information of Wütschert (1984), who mentions a wide distribution, with *E. orbicularis* predominating. According to him, the two terrapins can be found in practically

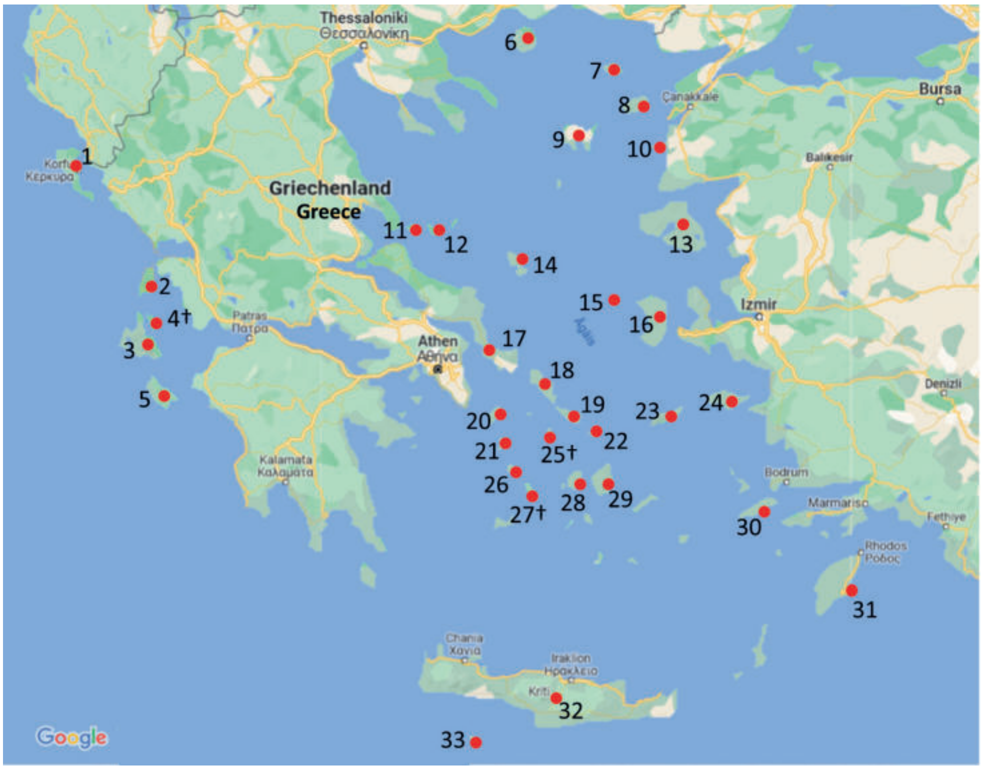

Figure 1. Distribution map of the Balkan Terrapin (*Mauremys rivulata*, Valenciennes, 1833) on Greek islands. Red dot – occurrence, cross – extinct. 1. Corfu; 2. Lefkada; 3. Kefalonia; 4. Ithaca †; 5. Zakynthos; 6. Thassos; 7. Samothrace; 8. Gökçeada (Imbros); 9. Limnos; 10. Bozcaada (Tenedos); 11. Skiathos; 12. Skopelos; 13. Lesbos; 14. Skyros; 15. Psara; 16. Chios; 17. Euböa (Evia); 18. Andros; 19. Tinos; 20. Kea; 21. Kythnos; 22. Mykonos; 23. Ikaria; 24. Samos; 25. Syros †; 26. Serifos; 27. Sifnos †; 28. Paros; 29. Naxos; 30. Kos; 31. Rhodes; 32. Crete; 33. Gavdos.

all watercourses and ponds on the island. Stille et al. (2021) point to an increase in invasive Red-eared Slider Turtles (*Trachemys scripta*).

Crete (8 450 km^2)

The first record after Bedriaga (1882) was by Duméril and Bibron (1839). Wettstein (1931) gives an overview of the herpetofauna of Crete. He names five locations, although these held only individual animals. Nevertheless, he thought “that along the whole of Crete in estuaries and marshes the species was widespread”. Werner (1938) lists 13 different locations of the species in Crete. In the article by Mantziou and Rifai (2014), the recent known locations are shown on a distribution map. According to this, the species is distributed all over the island. On Crete, there is a chance of encountering the Balkan Terrapin in fresh or brackish water, even in larger aggregations. Nature photographers such as Speybroeck (2009) and others have documented such occurrences in their travel reports. There is also a bibliography of Crete’s reptiles by Midtgaard (2019). I was active in Crete for only three days and was unable to gain an overview of the large island.

Euboea (Evia) (3 684 km^2)

First mentioned by Cyren (1933). Werner (1938) also reports a record. Pictures of *Mauremys* on Euboea can be found on the internet, for example iNaturalist (2023), taken on April 16, 2022 from the north-east coast of Evia. I do not know the exact status of the species on the island, as so far I have only crossed it to catch the ferry to Kea Island.

Gavdos (33 km^2)

Valakos (1987) presented the first record of *M. rivulata* in a short note. He observed four adults and 5–6 juveniles. This is probably the most peripheral locality for this species on the islands, as Gavdos covers only 33 km^2 and lies 48 km south of Crete, making it the southernmost point in Europe. The stream serving as habitat for *M. rivulata* flows into the sea at Lavrakas and an only partially watered section is found about 1.5 km upstream at the chapel of Agios Georgios. Mantziou and Rifai (2014) estimated the population at 50 animals. It is genetically and morphologically distinct from the Crete populations (Mantziou et al. 2004). We visited the island in April 2012 (Broggi 2014). Only two adults and one sub-adult and later one juvenile specimen were noted in two visits. The geology university professor Apostolos Alexipoulos from Athens, who is familiar with the locality, estimated the population at 10–12 individuals (pers. comm.). This means that there is a great risk of extinction, especially since the stream was already showing signs of drying out in April 2012. The species likely aestivates here.

Gökçeada (Imbros) (279 km²)

First record by Baran (1981). Balik et al. (1993) found five sites for *M. rivulata*. Our visit in 1998 allowed the following observation: The Balkan terrapin occurs at most of the stream estuaries, and migrates up the stream systems (Broggi 1999). One location of animals between two waterfalls was particularly interesting, especially considering the difficulty in accessing it. The paper by Bayrakci et al. (2016) states: "The species is distributed almost all around the island, particularly in the western part, with a low density. The population size in the largest river on the island, Büyükdere, was estimated at 136 individuals." There are, therefore, large occurrences on this island.

Ikaria (255 km²)

First recorded by Broggi (1994). During our excursion in May 1986, 200–300 *M. rivulata* were found at the Misonas estuary near Caliskari, and over 100 at the Charakas River near Armenistis. These are backwaters at the mouths of streams, which are created by the formation of beach walls and form ideal habitats for terrapins (Fig. 2). The turtles also ascend into the streams here, as the example at Charakas showed. For example, *Mauremys* have been observed in a drinking water catchment at Oros Fytron at over 500 metres above sea level (Broggi 1994). Dug, open-water basins are colonised on the mountain plateau. Clark (1996) confirms this for Ikaria as follows: "*Mauremys* was found nearly everywhere – in large congregations in bodies of water behind shorelines and in significantly smaller populations in streams."

Our second visit to the island in 2000 confirmed the wide distribution in the north-west at all altitudes. However, the large populations seem to have halved (Broggi 2001). With this wide distribution in the north-west clear, the species should not yet be endangered as such. Oefinger (2019) reports hand-tame terrapins feeding at the Myrsonas stream on Ikaria in June 2019 (Fig. 3). For his part, Grano (2020) notes at the same site the invasive Red-Eared Slider Turtle (*Trachemys scripta elegans*), which is occurring on more and more Greek islands and could endanger the native species because of its more pronounced aggressiveness.

Figure 2. Ikaria-Armenistis: Due to beach wall formation, the running waters are dammed back and form suitable habitats for the Balkan Terrapin (April 1986).

Figure 3. Peter Oefinger, German herpetologist with a tame *Mauremys rivulata* on Ikaria-Armenistis (13.6.2019).

Kea (132 km²)

First record by Grillitsch and Tiedemann (1984), of a dense turtle population, including juveniles, in tributary waters at the mouth of the Pisses River. Four offshore sites in the middle and upper reaches of the Mylopotamus are also mentioned: "It is to be expected that such water-rich valleys will in future serve as refuges for threatened coastal populations."

When we visited the island in 2011, the estuary at Pisses was completely occupied by tourist infrastructure and there was no longer any space for *M. rivulata*. However, the manager of our accommodation, the "Red Tractor" in Korissia, told us that there are still Balkan Terrapins in the Mylopotamos valley, which is difficult to access, and that they are periodically washed out to sea during heavy rainfall. During our stay on the island, we were able to find three more occurrences in water-rich valleys away from the estuary (Broggi 2012). Thus, the prediction of Grillitsch and Tiedemann (1984) could come true.

Kefalonia (787 km²)

First recorded by Werner (1894). Wilson (2006) writes: "Unfortunately, however, on Kefallinia its presence may soon become a thing of the past. On this island it was found to be relatively common at only one locality... At this locality about a dozen adults and juveniles were observed. However, like other such habitats throughout Greece they have a tendency of being turned into local rubbish dumps." We visited the island twice, in 1993 and 2017. In 2017, I found *M. rivulata* at five sites on the island, with small populations. The largest population was found in the extensive Livadi Marshes on the Lassi Peninsula (Fig. 4), where dozens of animals were seen

Figure 4. The Livadi Marshes on Kefalonia are the last suitable habitats for the Balkan Terrapin (16.4.2017).

(Broggi 2017b). Based on these observations, the Balkan Terrapin must be considered endangered on the island.

Kos (287 km^2)

First recorded by Werner (1902), with later observations by other herpetologists. I was able to do only a one-day tour of the island, in 1988, with a visit to the lagoon of Tigaki, which did not allow for an overview. Buttle (1995) gives Kos as a location in his island species lists without further comment. Cattaneo (2003) in his herpetological island work mentions six sites with rather small populations and a denser occurrence in the marsh at Pythos. Wilson (2015) gives some observations of *M. rivulata* in his herpetological travelogues. Cattaneo (2020) says the populations are declining due to wetland encroachment by land use, tourism and intensive agriculture. Brueckers et al. (2006) suggest that the Red-eared Slider Turtle is already reproducing naturally on Kos and could become a competitor to the native species.

Kythnos (99 km^2)

First recorded by Bedriaga (1882). Werner (1935) saw *M. rivulata* at the lagoon near Lutra, as did Wettstein (1953). Grillitsch and Tiedemann (1984) mentioned two sites on the island. Cattaneo (1990) studied the snakes of Kythnos and confirmed an occurrence in Episcopi Bay (pers. comm, 21.2.2020). The three occurrences on the island known from the literature could not be confirmed by us in 2021. On the other hand, we were able to find a small population close to the coast on the basis of information from the WWF wetland inventory (WWF-Greece 2014), as well as a population in a stream near the Chora, acting on information from the landlord of our apartment (cf. distribution map in Broggi 2021). This population, found after a long search, thrives with more than 50 specimens in the discharged wastewater of the Chora and shows once again that the species can also thrive under eutrophic conditions (Fig. 5). This stream, which is fed by sewage, is endangered by the installation of a sewage treatment plant, as paradoxical as this may sound. Occurring in only two localities, however, the species is endangered on the island.

Figure 5. *Mauremys rivulata* in sewage water of the Chora on Kythnos (8.6.2021).

Lefkada (325 km^2)

First record by De Betta according to Bedriaga (1882), and cited in Werner (1894). Sindaco (2020) gives a literature checklist of all *M. rivulata* observed on Lefkada (also known as Lefkas). Apollonoi, Nidri and Vadiliki are named as localities. We visited the island in 1985, and individual herpetological observations were noted in Broggi (1994). *Mauremys rivulata* was found at several aquatic sites, especially in water-bearing ditches. The Balkan Terrapin is frequently captured by nature photographers and pictured on websites, such as Schmid (2018) and iNaturalist (2023), with the last picture from Nidri (12.6.22). The Balkan Terrapin does not appear to be threatened in water-rich Lefkada, although there is no record of large populations.

Lemnos (476 km^2)

First found by Werner (1930). Schneider's Herpetofauna of the island states: "In almost all water accumulations, even in shallow rivulets, lived large animals whose dorsal carapaces were higher than the water depth" (Schneider 1996). Cattaneo (2001) also says: "Present in most wet places". Strachinis and Roussos (2016) offer the next overview: "Species found at almost all existing wetlands, the largest population being in a pond in the middle of the island, with 350 specimens." Broggi (2017a) confirms 13 of 20 occurrences named in the wetland inventory (WWF-Greece 2014), with coordinates, and indicates two newly found wetland sites. The new discovery at the Moschilos volcanic crater turned out to be the largest previously unknown occurrence in the island's interior with hundreds of specimens: "Lemnos is the turtle island of the Aegean. The populations on Lemnos are among the most numerous in the Aegean."

Lesbos (1 633 km^2)

First recorded by Werner (1935). Wettstein (1953) points out the significant size reached by the turtles of Lesbos. Particularly large specimens live in the clear, brackish water of the Gulf of Hiera, in the south-east of the island. Broggi (1978) states: "At the northern end of Geras Bay, in the brackish backwaters, hundreds of terrapins lay crowded together on the shore and on some small islands to sun themselves. Similarly, observations were made in streams." I described the carapace length as up to 30 cm (Broggi 1978). This was an estimate without measuring, as in the literature the lengths are described as being a maximum of 25 cm (Wischuf and Busack 2000). I think, however, that this size could be exceeded here. Wettstein (1953) also speaks of particularly large specimens living in the brackish water of the Gulf of Hiera and describes one as a "giant specimen", but without specifying the size. Sonnenschein (1980) writes: "On sandy shores, where vegetation cannot gain a foothold, numerous terrapins sun themselves. The space is limited, and some of the animals lie on top of each other. If you get closer to them, the dark mass starts to move and they plop into the water. Bog turtles are frequently seen, they inhabit even the smallest water hole; even in brackish waters on the coast they can be met by the hundreds, the European Pond and the Balkan Terrapin." Buttle (1995) also writes: "*Mauremys rivulata* especially numerous on Lesbos in wetlands and streams", while Kasapidis et al. (1996) give only a location with 4 individuals observed, but refer to Tsunis and Dimitropoulos (1994) saying the species is common on the Aegean islands.

On 5.7.2005, a peculiar behaviour of the Balkan Terrapin was observed on the beach of Skala Eressos in the southwest of the island (Gemel et al. 2008): "More than 50 conspicuously large specimens were present in the adjacent lagoon; juveniles were missing. Most of them swam towards the tourists standing at the edge of the lagoon to beg for food. Some even ran to the shore to take food from the tourists' hands." Busack (2009) also mentions the same behaviour. On 24.6.2008, he saw several dozen Balkan Terrapins near a bridge west of Skala Eresou. A bus driver dropped bread into the water, and many turtles came and fought for their share. Uwe Fritz had seen something similar in western Crete, as well as on the southern coast of Turkey. The threat to native populations by a new invasion of the Common Slider (*Trachemys scripta*) is also mentioned for Lesbos (Christopoulos and Levgolis 2022).

Milos (151 km^2)

First recorded by Bedriaga (1882). Schweizer (1935), the self-taught "Snake Hansi" from Allschwil (Canton Basel-land) and acquaintance of my father, states: "[*M. rivulata*] is found in large numbers in the pond area of freshwater accumulations on Milos, furthermore I also saw them on the beach in rush-covered, salty marsh ponds". Werner (1938) and Wettstein (1953) mention the island in their works. Pérez Mellado et al. (1999) found only one specimen at each of two sites on the island, Adamas and Alytes. Broggi (2000) also found two sites on the island and saw only one and three specimens, similar to Speybroeck (2006, 2013). Schweiger (2020) states: "Quite a common sight in Lake Provatas in the 1970s. Completely disappeared due to draining." Obviously, the habitats are dwindling rapidly. *Mauremys rivulata* is endangered on Milos.

Mykonos (105 km^2)

First recorded by Bedriaga (1882). The species is later mentioned several times as occurring on Mykonos, but seems to be cited only from older sources. Not so by Wettstein (1953), who visited the island and found *M. rivulata* there. Beutler and Froer (1980) give two sites on the island, but note that one is already largely destroyed. The number of animals at Ftelia was estimated at 100 specimens, which was recorded on 30.5.1977. They are considered to be critically endangered by Beutler and

Froer (1980). That the species still exists on Mykonos is confirmed by a recent photographic travel report by Wilson (2022) from the Cyclades in 2022: "Terrapin in particular seem to be an endangered species in the Cyclades in general, seen at Mykonos." So *M. rivulata* still exists on Mykonos but seems to be endangered by extinction. Personally, I have never been to Mykonos.

Naxos (389 km²)

First record by Werner (1899), mentioning the species for Naxos without further comment as follows: *Clemmys caspica G.* var. *rivulata* Val. In his monograph Werner (1938) does not mention his previous work under the species description. Wettstein (1957) writes: "Not rare on Naxos in small streams in small populations." Buttle (1993), for his part, observes two adult specimens in a drying watercourse. Oefner (2016) observes: "South coast some very shy *Mauremys* and 1 juvenile *M.*, Potamia Valley." A student at the university of Copenhagen wrote to me on 14.4.2022, saying: "Large population in Galanthis Bay, south end of Naxos." We were on Naxos for a week in April 1984 and encountered *Mauremys* eight times in the estuaries of watercourses and in streams (stream SW bay of Appollones, end of stream Myloperama, beach of Galini Ormos Amyti, end of stream Pyrgaki, lagoon Aghiason, stream Potame, lagoons behind Ormos Kalandou, stream after Kouronochori, direction Galini, nearby lagoon Alikes). *Mauremys rivulata* seems to be common on the island in the lower reaches of streams.

Paros (193 km²)

Werner (1938) and Wettstein (1953) do not mention *M. rivulata* for Paros. Gruber and Fuchs (1977) give the following mention as first recorders: "It seems to be rare on Paros in general. We found our specimen in a slow-flowing, muddy stream near Naoussa." Buttle (1995) does not mention it on his species list for Paros, or describes it as "absent". We were on the island for one day that allowed us to get an overview of the landscape. We were shocked by the degree of overdevelopment on the island and have so far refrained from a natural history excursion there. Troidl and Troidl (2021) describe the Balkan Terrapin as the most endangered reptile on the island: "According to our research, and also according to the statements of Johannes Foufopoulos, these animals only occur in a very small area of a temporary stream in the north of Paros." There is talk of a population of 8–12 animals at two water points 150 metres apart. This means that the Balkan terrapin must be described as threatened with extinction on Paros.

Psara (44 km²)

Pafilis et al. (2018) report a record on the island, finding 3 specimens of *M. rivulata* in smaller areas of water near Xirolambos. Psara is isolated, 22 km west of Chios. How is settlement at all possible under the given suboptimal conditions? Transoceanic dispersal must be expected here. "We assume that from time to time turtles are swept out by storms and drifted away by sea currents" (Vamberger et al. 2014). Will the species be able to survive on the island under these conditions, considering there are probably no other suitable habitats available? This is the most unusual record in recent decades.

Rhodes (1 401 km²)

First recorded by Calabresi (1923). Not listed by Werner (1938), but Calabresi's record was included by Wettstein (1953). Wettstein (1965) later reports three specimens taken from the "Seven Springs", noting that he had already seen the species in 1935 in a pond near Apolon on Rhodes. Furthermore, he wrote in 1953: "Not less rare on Rhodes than on Crete." Bader et al. (2009) give an overview and write: "We think that the Balkan Terrapin populates most rivers and streams on the island." They give 12 locations in a distribution map (Fig. 2 there). INaturalist (2023) mention 14 objects on the island with *M.rivulata* observations. Among them are also some occurrences in streams. However, there is no mention of larger populations. Nevertheless, the species seems to be widespread on the island. In 1988 we took only a one-day trip to get an idea of the landscapes.

Samos (477 km²)

First recorded by Werner (1935). The first natural history excursion to a Greek island took us to Samos in April 1975. I likely saw in the distance both species of terrapin at the archaeological site of the Temple of Hera, but I could not confirm this, as, at a time of political tension between Turkey and Greece, we were expelled by the local military and had some problems with our cameras and binoculars and we were forced to hand in slide films.

The island of Samos has been herpetologically studied several times, by Ioannidis et al. (1994), Cattaneo (2003, 2019) and Speybroeck et al. (2019). Speybroeck et al. (2019) give 68 records for *M. rivulata*, found within 3.5% of all grid cells (1 × 1 km) on Samos: "The species seems largely restricted to the south-eastern wetland areas of the island. Single record from the north-western coast near Karlovasi. A relatively high tolerance for elevated levels of salinity and eutrophication." The Balkan Terrapin penetrates streams up to 400 metres above sea level.

The island has been visited by several nature photographers, such as Bok (2009). In his Field Report from October 2009 he writes: "In the dried riverbed of the river Imvrassos, Balkan Terrapins were quite common though the river was reduced to small, shallow pools." Samos, therefore, seems still to have secure populations of *M. rivulata.*

Samothrace (178 km²)

First found by Werner (1935). Wettstein (1953) also mentions the species. We visited the island for the first time in 1986 in a two-week stay (Broggi 1988), and from then on we took two weeks for each of these island visits. The Balkan Terrapin was observed in small populations at two lower reaches of creeks dammed by beach riprap and three pools along the north coast road. Buttle (1989) also mentions *M. rivulata* from Samothrace. Cattaneo (2001) reports them in good numbers along the creeks, including some backwaters along the beach. Ochsenhofer (2012) names *M. rivulata* as the most common reptile observed, with 10 individuals sighted behind beach walls in impounded estuaries and smaller wetlands. Zagoris (2014) found a new site with *M. rivulata* at Polypoudi in Alonia village. There are, then, multiple populations without larger aggregations on Samothrace.

Serifos (75 km^2)

First recorded by Bedriaga (1882). Werner (1933) confirms the record and states: "I found a juvenile in the shore marshes on Seriphos and received two more from there. The species is very common here, for towards the end of my stay I was offered another number." Cattaneo (1980) confirms the occurrence without naming a place. He later names occurrences at two sites, one on a stream through Livadi and the other on the Potamia stream below the village of Potamia in the north of the island, as a result of a personal communication with me on 8.6. 2010. Our excursion in 2010 yielded four more site records. The unused Steno reservoir, still full of water, forms one of the refuges for *M. rivulata* (Broggi 2011). Thus, the species is currently still secure on Serifos.

Skiathos (49 km^2)

Bergmann (1995) reports news on the herpetofauna of Skiathos, but nothing on the Balkan Terrapin. First recorded by Cattaneo (1997), who refers to an observed specimen with a length of 16.6 cm, from a small ditch together with green frogs. He writes in Italian, the English translation of which reads: "Because of the progressive extinction of wetlands, all associated species, especially the Balkan Terrapins, are threatened with extinction." I was on Skiathos for only one day in 2019 on my way back from Skopelos. I expected to see *M. rivulata* in the lagoon behind Koukounari beach but did not. In the northern part of the island I heard green frogs calling but saw no terrapins there either. Whether there are any left on Skiathos is questionable. Tourist development is well-advanced on the island, which is only 49 km^2 in size. One must reckon with the extinction of the species on Skiathos as well as on Skopelos.

Skopelos (96 km^2)

First recorded by Cattaneo (1998) – a few specimens in a backwater, including juveniles. As on Skiathos, he considers *M. rivulata* to be threatened with extinction. I visited the island for a week in June 2019. The WWF Greece inventory of island wetlands (WWF Greece 2014) indicates three sites, two of which were still present. An artificially created pond near Lautsa is said to have been home to the Balkan Terrapin a short time ago, according to an internet blog (skopelosculture.org 2011). Despite two visits, I did not see any animals there. Behind Panormos beach, a backwater has been preserved in a depression in the sandy area, and an adult specimen was observed there. Thus, any further occurrence on Skopelos should be assessed very critically.

Skyros (209 km^2)

First recorded by Werner (1930). Cattaneo (1998) observed a few specimens in the final section of a stream before it flows into the sea. Our excursion to Skyros took place in 2003 (Broggi 2006a). I noted three occurrences on the Kephisos stream, with about 25 individuals. In another stream, which flows into the bay of Aberounes and creates a backwater due to the formation of beach walls, a few *M. rivulata* were found. Overall, the population on the island is endangered. The alluvial plain of Kalamatsa, covering about 70 ha, is the largest wetland on the island worthy of protection. Agricultural intensification, with drainage, is threatening the habitat there.

Thassos (380 km^2)

First reported by Cyren (1933). Buttle (1995) includes it in his species list for Thassos, without comment. Clark (1993, 1999) mentions *M. rivulata* but also does not comment. Cattaneo (2001) saw them in the locality of Prinos, where he noted various specimens. Searching for "Thassos *Mauremys rivulata*"on Google gives an overview of a Natural History of Thassos, including *Mauremys* (Fowles 2001). I was on the island for a week in 1996, but the herpetological results were not sufficient for publication. I did encounter *M. rivulata* on the island at that time in Skala Prinos and Skala Panagia. The population status is not known sufficiently and thus can only be described as occurring.

Tinos (194 km^2)

First recorded by Bedriaga (1882). Werner (1938) puts the species on his island list, as does Wettstein (1953). Beutler and Froer (1980) write: "It was probably the best preserved wetland in the Northern Cyclades, the lagoon of Kolibithra. There is another lagoon near Ormos Panormou. Smaller ponds were found around Tinos town, Tripotamos, Kardiani and Isternia." Bohlmann et al (1981) mention only one specimen, near Panormos, in their herpetological notes. Broggi (2019) benefits from using the wetland inventory of WWF-Greece (2014) in his research. The recorded significant habitat of Agios Ioanni no longer exists, as is the case with other, smaller wetlands near the island's capital. All these sites have been searched. The large *Mauremys* populations of Panormou and Kolibithra can be confirmed. In Broggi (2019), Fig. 3 gives an overview of all recorded occurrences on the island. The Livadi Valley sightings were the largest stream occurrences on a Greek island to date. The Tinos populations are, therefore, still viable.

Zakynthos (406 km^2)

First recorded by Werner (1894). Keymar (1986, 1988) dealt with amphibians and reptiles of the Ionian Islands but recorded *M. rivulata* only in the species lists, including for Zakynthos, without comment. This also applies to Buttle (1995), who recorded the species in his list for Zakynthos. Wilson (2006) writes: "It was found to be abundant in the rivers of Limni Keri, and a specimen was also seen in the Laganas river." Wilson (2009) also records two population sizes for Zakynthos in his observations: "5.5.2005, 10 specimens, and 6.9.2009, 20 specimens. He sees an "overpopulation" of *Emys orbicularis* in Lake Keri, including *M. rivulata*. We have not yet visited the island.

Extinct

Ithaca † (118 km^2)

Keymar (1986) refers to Cyren (1935) in his herpetological survey of the Ionian Islands. He sighted water turtles in a well in Ithaca but was unable to identify the species. Keymar said this was all the more remarkable because Ithaca had no permanent open water. Cisterns were probably the last habitat refuges of amphibians and hydrophilic reptiles that colonised the archipelago during a "wetter" geological period. Broggi (2009) confirms that there are no suitable habitats for aquatic turtles today. If Cyren's (1935) observation is correct, *M. rivulata*, which has fewer habitat requirements than *Emys orbicularis*, would be extinct.

Sifnos † (74 km^2)

Erhard (1858), the first who described the species from Sifnos, wrote that *Mauremys* populations were rare in the wetlands. The species was later reported there by Bedriaga (1882), Werner (1933) and Schweizer (1938). Wettstein (1953) was the last to record two specimens. Grillitsch and Tiedemann (1984) and Broggi (2000) saw no specimens, even in the inland waterholes that had *Pelophylax kurtmuelleri*. Toth (2001) also did not report the species. The once-secure site in the coastal marshes of Kamares has been destroyed by tourist use. Wilson (2022) writes in his blog: "The Balkan Terrapin is probably extinct now that the coastal wetland at Kamares no longer exists."

Syros † (102 km^2)

First recorded by Bedriaga in 1882. Werner (1938) refers to Bedriaga in his island list, and no record has been made later. Beutler and Froer (1980) believe the Balkan Terrapin was probably extinct here. Dimitropoulos (2016) has nothing new to report on this either. It must be assumed that the species is extinct, as it is on Sifnos.

Absent / status unknown

Amorgos? (126 km^2)

Erhard (1858) mentions the Balkan Terrapin, and Bird (1935) repeats this information. Werner (1938) no longer mentions it, however. Old data may be based on confusion of islands, or the species may have disappeared early. Lotze (1979) dealt with the herpetofauna of Amorgos but could not confirm this species there and thought that suitable habitats were missing. This was confirmed by me after a visit to the island (Broggi 2007).

Chalki? (27 km^2)

Grano and Cattaneo (2017) report an observation of a well-preserved dead specimen of *M. rivulata*. They found it on the Dodecanese island of Chalki, near Rhodes, in a dried-up well. This observation requires further clarification. Is it a specimen that was washed here from another island? According to studies by Vamberger et al. (2014), this must be assumed as probable. Suitable habitats are likely to be lacking on the island.

Kythera? (278 km^2)

So far, *M. rivulata* has not been recorded here, although Pieper (1970) does not rule out its occurrence. I have been on the island twice, in 1989 and 2015, and think Kythera offers potential habitats for the species, especially in the north of the island and in the beach lagoon of Kakia Lagada. Stavros Emmanuel, a local biologist, said a friend of his had seen terrapins in the Karavas area in the north of the island. My search was in vain, but this is something to keep in mind.

Symi? (58 km^2)

Dimaki (2002) mentions *M. rivulata* for Symi, without giving any further location. I could not confirm this on my visit to the island (Broggi 2002), nor could Cattaneo (2007), Wilson and Grillitsch (2009) and Wilson (2012). It must be a case of mistaken identity, since with the exception of a pond, suitable habitats are scarcely present here.

Tilos? (61 km^2)

There are no historical records for Tilos. When I visited the island, an adult *M. rivulata* was seen in a water reservoir covered with rubber sheeting south of Megali Chori on 18.4.2005 and subsequent days. There used to be two wetlands on the island, which no longer exist. As a result, the green frog has become extinct. Is this observation a release or a relict? The present situation is not suitable for the species to persist (Broggi 2006b).

Discussion

Faunistic aspects and occurrence

My favourite observations on the Greek islands for the last three decades have been of terrapins in their habitats. These possible habitats are consistently searched on every island visited. With experience, it is possible to spot the shy animals in their sunny positions from afar. The distribution of the Balkan Terrapin (*Mauremys rivulata*) in the Aegean Sea was described in the journal Herpetozoa (Broggi 2012), and here the statements are updated on the basis of new findings, with the Ionian Islands now taken into account. For the first time, the focus is on population sizes.

In the Aegean and Ionian Islands, *M. rivulata* has been recorded on 29 Greek and two Turkish islands. Five further occurrences are described as questionable, with two of them probably due to misinformation; one of them has potential but remains without confirmed evidence; one refers to a dead deposit; and one is probably due to a release. On Syros, Sifnos and probably Ithaca it can now be assumed that the Balkan Terrapin is extinct. Population sizes appear to be small on most Greek islands but can only be based on estimates. Only from Samos counts are available (Speybroeck et al. 2019). Of all the islands mentioned as having *M. rivulata* present, I have no personal experience of Bozcaada, Corfu, Mykonos, Psara, Syros and Zakynthos.

Wettstein (1953, p.660) writes that "*Clemmys caspica rivulata*" occurs on all larger water-bearing Aegean islands. This statement is still valid, but with limitations. The recent record of the species presented here now includes 29 Greek and two Turkish islands. These occurrences refer to islands covering more than 50 km^2 of land area, except for Gavdos (33 km^2), Bozcaada (37 km^2), Psara (44 km^2) and Skiathos (49 km^2). This is understandable, as it usually takes a larger land mass to form surface waters. In presenting the distribution and assessing the populations, it was possible to draw on our own observations in many cases, as so far around 60 Greek islands have been visited during the last decades (see Table 1).

The Mid-Aegean Trench (MAT) from Crete to Thassos created a sea barrier in the Aegean Sea. Except for sea turtles, *Mauremys rivulata* is the only local reptile species for which the MAT is practically non-existent. Just being a Terrapin is not sufficient to overlook the sea as a barrier. The pattern of occurrence of *Emys orbicularis* is contrary and more similar to the other reptiles and amphibians (Lymberakis and Poulakakis 2010). The optimal habitats of *M. rivulata* are in the estuaries of flowing waters and the beach lagoons that may have formed there. The turtle benefits from the formation of beach walls, which block the outflow of freshwater into the sea by means of currents and backwater, especially when the freshwater pressure towards the sea decreases. Especially in sandy bays, this backwater effect can lead to prolonged water accumulations up to lagoon size. Werner (1935) described this situation with the following statement: "On Sifnos and Samothraki they live in a pool formed at the end of a stream which fails to reach the sea but rather builds up and spreads out on the sandy beach." The largest and particularly impressive populations are thus found in intact lower reaches and in the estuaries of watercourses, as seen on Lesbos in the Gulf of Hiera and at Mirsonas and Charakas on Ikaria.

On vulnerabilities and protection

Substantial threats to *M. rivulata* also exist in other regions of Greece, see Walters (1998) and Erzenberger (2018). The dependence on hydrological phenomena shows the danger and vulnerability on the islands due to land-use conflicts. Tourist development, in particular, is taking place on the sandy beaches that are less common here. Such bays are still being redeveloped and the hydrological systems destroyed, as last seen in 2022 on Ios at Pappa Aulaki Bay, where a backwater was destroyed for a luxury hotel development. The flat areas of the estuaries are also of interest for agriculture. For this purpose, the terrain is levelled, the watercourse is narrowed and thus the possibility of egg-laying is also impaired. In addition, water is taken from the water-holding streams over long distances (Fig. 6). Increasingly, groundwater pumps are also used to lower the groundwater level, with a corresponding impact on the surrounding area.

In sections where there is still a long period of water retention in the watercourse, and the water in scours is retained for longer, refugial populations of *M. rivulata* can settle best. I have noticed such stream colonisations away from the estuary in Thassos, Samothrace, Andros, Lemnos, Kythnos, Kea and Serifos. But even in these more favourable conditions, water is being extracted for agriculture, accelerating their drying out. In addition, most of

Figure 6. Water abstraction for agriculture endangers the habitats of the Balkan Terrapin (Limnos, April 2016).

Figure 7. Open well on Kea (2011).

the natural springs are exploited. The Balkan Terrapin can also resort to using anthropogenic water accumulations such as open cisterns and sites for livestock watering (Fig. 7). However, such artificial biotopes are becoming rarer. They are replaced by closed cisterns and groundwater pumps, and the once-open waterholes for amphibians and hydrophilic reptiles are no longer maintained. The loss of open water areas is also exacerbated by ongoing climate change. In the last two decades, drying out has intensified (Fig. 6). For example, on some islands such as Lipsi, Fourni, Ithaca or Alonissos, no flowing water could be seen from April onwards. On the other hand, winter flash floods can carry turtles all the way into the sea and thus endanger their populations, as experienced in Kea (Broggi 2012). If turtles wash up in new areas, the probability of finding suitable habitats is extremely low.

For *M. rivulata*, there are no sufficient temporal data of its distribution on Greek islands with population estimates. My assessment of the endangerment status therefore does not follow the IUCN criteria for Red Lists of threatened and rare species. These are my personal subjective assessments based on visits to the islands. However, I have been systematically searching Greek islands for *Mauremys* occurrences for the last 30 years. Some of my observations are older, however, and a lot may have happened at the individual sites in the meantime. The threat to the species on Greek islands is generally considered to be high. The current occurrences are highly isolated and exchange with other populations is becoming increasingly difficult. Habitat destruction, but also climate fluctuations, can accelerate extinction. On 12 islands, namely Skyros, Skopelos, Skiathos, Psara, Paros, Mykonos, Milos, Kythnos, Kefalonia, Zakynthos, Gavdos and Bozcaada, the status “threatened with extinction” is appropriate. The status of some other islands is not yet known well-enough to make an assessment. On the other hand, larger confirmed occurrences are found on Gökçeada, Crete, Ikaria, Lemnos, Lesvos, Samos and Tinos. On some of these islands, populations are also found in streams, where they are more protected from threats.

The EU lists *M. rivulata* in Annexes II and IV of the Habitats Directive. It is a species in need of strict protection. Under Greek law (Presidential Decree 67/1981) the Balkan Terrapin is a protected species. It is also listed in the Red List of Threatened Animals of Greece (Legakis and Maragos 2009).

The causes of threats have been presented. It is no longer possible to determine how many wetlands were destroyed in the last century, but a large number of small biotopes were likely affected. Catsadorakis and Paragamian (2007) started in 2004 by compiling an inventory of wetlands for the Aegean islands, excluding Crete. They recorded 352 wetlands, including lagoons, on 51 islands, that covered about 40 km^2. The inventory was subsequently extended to the entire Greek archipelago (WWF Greece 2014; oikoskopio.gr/ygrotopio). Thus, 824 wetland structures were identified on 76 islands, of which 100 were wetlands on Ionian islands, with 526 sites on 64 Aegean islands and 192 on Crete. In a presidential act of June 2012, 350 natural wetlands on 58 islands were strictly protected. Protection is now said to have been extended to 565 wetlands, accounting for 70% of the recorded inventory (Paragamian et al. 2014). Monitoring of the conservation status is recommended.

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Occurrence and status of the European Pond Turtle, *Emys orbicularis hellenica* (Valenciennes, 1833), on Aegean and Ionian Islands (Greece, Turkey)

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Abstract

A study on the occurrence of *Emys orbicularis* in the Aegean, published in 2012, is here extended to the Ionian Islands. For the first time, a status analysis has also been carried out for the individual islands. *Emys orbicularis* is found on 11 Greek islands and one Turkish. Its presence on the big islands of Rhodes and Chios has not been ascertained so far, while for four previously mentioned islands there is no confirmation. On Corfu and Lesbos there are still viable larger *Emys* populations. Most other island occurrences are characterized by small populations. On Kefalonia, Zakynthos, Thassos and Samos, *E. orbicularis* is in danger of extinction because the wetland biotopes are threatened. In the meantime, many wetlands have been placed under protection. The enforcement of these nature conservation regulations needs to be monitored, and *E. orbicularis* can be a lead species for such monitoring.

Key Words

conservation, Emydidae, Insular Greece, Reptilia, Testudines, threats

Short portrait of the species

Emys orbicularis occurs in freshwater habitats, with a range extending from the Maghreb over the Iberian Peninsula, southern France and central Europe to the Baltic States, and in the south over Italy, the Balkan peninsula and Turkey to the Caspian Sea (Fritz 2001). In this large distribution area, a number of different subspecies are described. In Greece, the Eastern Mediterranean *Emys orbicularis hellenica* (Valenciennes, in Bibron and Bory de Saint-Vincent 1833) is described (Fritz 1998). This subspecies is small to medium-sized and its carapace is relatively high and narrow, with a usual maximum length of 25 cm. Its plastron is yellowish (Fritz 2001). The only similar species in Greece is the Balkan Terrapin *Mauremys rivulata* (Valenciennes, 1833), from which *E. orbicularis* differs in neck colouration pattern – striped in *M. rivulata*, spotted in *E. orbicularis*. *Emys orbicularis* lives in still or slow-flowing water in the shore area of lakes, ponds and ditches. Also, along the Mediterranean coasts, it is found in slightly brackish water in the backwaters of estuaries. Dense vegetation with a muddy substrate is preferred. It feeds mostly carnivorously, on snails, crustaceans – in fact, virtually anything it can catch – but will also eat aquatic plants (Fritz 2001).

Suitable habitats on the islands are similar to those on the mainland except that I never saw *E. orbicularis* in streams at higher altitudes. However after observations, it is noticeable that it only occurs on the peripheral larger islands along the continental shelf. There, *M. rivulata* and *E. orbicularis* usually live in the same habitat, thus sharing it sympatrically (Fritz 2001). *Mauremys* then shows itself to be in the majority, perhaps with the exception of Corfu. *Emys* also demands in my estimation less polluted waters than *Mauremys* and is less tolerant of salinity. It is striking that *Emys* on the Greek islands – contrary to

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the mainland – has not been observed by me in flowing streams and seems not to use them as a refugial habitat, unlike *Mauremys*. Their occurrences are concentrated in the lowlands, especially on the estuaries of water flowing into the sea with their network of hydrological structures.

Exploration on Greek islands

Herpetological research on Greek islands goes back almost 200 years. This begins with the Excursion de Morée in 1828–1833, which was a French military action in the Peloponnese as part of efforts for Greek independence. This military action was accompanied by a natural science commission under Bory de Saint-Vincent with 17 experts, including Gabriel Bibron, who was active in herpetology (Pafilis 2010). Werner (1938) and Wettstein (1953) made significant progress in herpetological research on Greek islands in the 20th century. The history of such research in Greece is described by Pafilis (2010). Valakos et al. (2008) present the amphibians and reptiles of Greece with distribution maps, which give us a good overview, as do Lymberakis et al. (2018) in a shorter contribution.

How does this present itself for *E. orbicularis* in particular? In the course of herpetological research in Greece, the Ionian Islands were initially the focus of attention. They were perhaps easier to reach for central European herpetologists, and they already belonged to the Greek state, in contrast to the islands of Asia Minor. The Cyclades then became the focus of herpetological research in the 20th century. However, no *Emys orbicularis* have been recorded there. In the Aegean, records are restricted to the larger peripheral islands on the Anatolian side, which were not the focus of the Austrian studies by Werner (1938) and Wettstein (1953). Another reason for late records is that *E. orbicularis* associates with *M. rivulata* and is less conspicuous, with its smaller populations, among Balkan Terrapin. This was at least the case for the observations by the author during the field research on Lesbos and Samothrace. It could also be true that for many field herpetologists the snake and lizard species are more in the foreground of consideration, and therefore the habitats of pond turtles were less well searched.

Occurrences on Greek islands and on Gökçeada (Turkey)

In the following, an overview of *Emys* occurrences on Aegean and Ionion islands (Fig. 1) is given in alphabetical order. A question mark appears against islands where a past occurrence is questionable. In each case the first description is mentioned, and further later observations from the literature are evaluated. Likewise, my own observations recorded in the field books of the excursions were included. As far as possible, the *Emys orbicularis* status on each individual islands is also described. This present contribution is the continuation of studies published in 2012, which referred only to the Aegean Sea (Broggi and Grillitsch 2012). Also in 2012, the species *M. rivulata*, which is sympatric with *E. orbicularis*, was dealt with for the Aegean (Broggi 2012). This study has now been updated (Broggi 2023).

Andros? (380 km^2)

Bedriaga (1882) referred to Erhard's "Fauna of the Cyclades" (Erhard 1858) in his overview of the amphibians and reptiles of Greece with the following statement: "Erhard has seen *Emys* on Naxos, Amorgos, Andros and Mykonos. I suspect that Erhard rather confused *Clemmys caspica* ssp. *orientalis* with *Emys orbicularis*.". Werner (1938), therefore, does not take Erhard's statement into account. *Mauremys* occurs today on the above islands, except Amorgos. After my visit to Amorgos in 2006, an earlier record of *M. rivulata* on that island is assumed to be rather unlikely (Broggi 2007). According to Fritz (1992), a colonization axis of Andros-Tinos-Mykonos would be possible in principle. Corresponding habitats would also be available. However, apart from the reference for Mykonos, which is to be confirmed, there are no indications of past or present *Emys* occurrences on these islands. Our intensive search on Andros in 1995 was also fruitless (Broggi 1996). I do not believe in the Andros-Tinos-Mykonos colonization axis for *Emys orbicularis*.

Chios? (843 km^2)

While there are *Emys* records for Lesbos and Samos, this is not yet the case for Chios. The herpetological contributions by Tsunis and Dimitropoulos (1995), Kasapidis et al. (1996) and Cattaneo (2003) do not provide any information in this regard. In Kasapidis et al. (1996), a distribution overview of *Emys* on the North-Eastern Aegean islands is published but without reference to Chios. I visited the island in 1991, without any *Emys* record, but with some *Mauremys* observations. Is it just that a record for Chios is still missing, or does *Emys* really not occur here? A possible occurrence is to be expected.

Corfu (Kerkyra) (585 km^2)

Werner (1894) mentions the occurrence of *Emys* on Corfu, which is probably the best herpetologically researched Greek island, including turtles (see Stille and Stille 2017). Wütschert (1984) reported that in the central part of the island *Emys orbicularis* is 9–10 times more common than *Mauremys*. He calls it widespread – in a pond near Tebloni in central Corfu he saw 150 specimens. Mertens (1960, 1961), on the other hand, described it as less common than *Mauremys*. In any case, the ratio between the two species is nowhere else on the Greek islands as balanced as on Corfu. Kattinger (1972) mentions *E. orbicularis* several times, for example on 3.8.1965 in a stream on the coastal plain, flowing from a walled well to Lake Korission; on 5.8.1965 several specimens there; and on

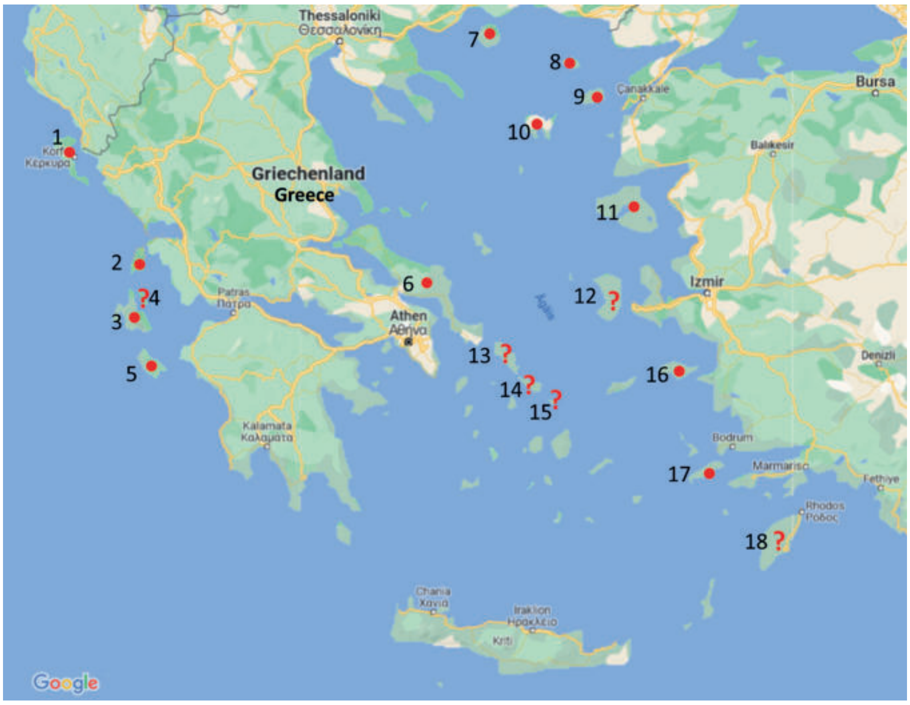

Figure 1. Occurrence of *Emys orbicularis* on Aegean and Ionian Islands in categories present and question mark absent/status unknown. 1. Corfu; 2. Lefkas; 3. Kefalonia; 4. Ithaca; 5. Zakynthos; 6. Euboea; 7. Thassos; 8. Samothrace; 9. Gökçceada; 10. Lemnos; 11. Lesbos; 12. Chios; 13. Andros; 14. Tinos; 15. Mykonos; 16. Samos; 17. Kos; 18. Rhodos.

21.8.1965 several *Emys orbicularis* were seen among *Mauremys rivulata* in a stream in the lower reaches of the Sutoria. Toth et al. (2002) give an overview of *Emys* occurrences on the island. They also refer to other sightings: at Linia, Lake Korission (Kattinger 1972), Temblisi (Gundke 1988), Paleokastritsa (Werner 1894; Wütschert 1984), the Sidari region (Mertens 1960; Gundke 1988; Fritz 2001). Larger occurrences are mentioned several times for the Antinioti Lagoon and Lake Korission. Wilson (2014) mentioned *Emys* in his Corfu report for 2014.

There are also numerous photographic descriptions of trips, for example by Balej and Jablonski (2006, 2021) with sites at Acharavi 10.5.2007 and 2.8.2009; Agios Georgios 6.8.2011 and 8.5.2014; Ormos Ermones (38 m above sea) 13.8.2011 and 8.5.2011; as well as Stroggili 10.8.2011 and 8.5.2011. Kalter (2013) mentions for his trip in July 2010 five different sites with *Emys orbicularis*, three of them in small rivers and two in marshes and standing water. Some travel reports, among others by Vandenbroeck (2014), can also be found on the webpage of fieldherping.eu, where *Emys* is mentioned in a river near Agios Georgios, as well as in a pond near Sidari, which has as many as three species of water turtle, namely *Emys*, *Mauremys* and the invasive Red-eared Slider Turtle *Trachemys scripta*. The most recent report is by Speybroek (2022) covering an excursion from 10–17.4.2022. *Emys* cannot normally be seen in flowing water on Greek islands, so the above are exceptions, but probably the water was very slow flowing. Lake Antinioti, with about 40 hectares in the north of the island, is called "heaven for turtles". It is located between the beaches of Almyros and Agios Spyridonas. *Emys* is, therefore, widespread on Corfu and has suitable habitats here to ensure its survival. It probably has a larger population here than on any other Greek island. Nevertheless, Stille and Stille (2017) consider *E. orbicularis* to be highly endangered on Corfu.

Euboea (Evia) (3,660 km²)

In Boettger's (1891) overview of the reptiles of Euboea, *E. orbicularis* is not yet listed. Cyren (1935) mentioned the species on the island for the first time. He saw some *M. rivulata* and an *E. orbicularis* in brackish water on the beach in the north of the island, the latter having a necrosis on its dorsal shield. He wondered if this was caused by the brackish water. Cyren (1941) mentioned again that he had captured, in brackish water, an *E. orbicularis* that had a dorsal carapace badly affected by algae. Werner (1938) refers to Cyren (1935) for his cited occurrence on Euboea. There are no later herpetological contributions with further *Emys* references, but there are individual references such as in Fritz (2001), and in Moysiadis and Efthimiou (2012), on a Natura 2000 object sheet GR 2420004, at Megalo Livari in the north of Euboea. *Emys* photos are also known from the island, for example by Davranoglou (2020) on a beach near Profitis Ilias. The status of this species on the island is thus not clarified; we can only state here that it does occur.

Gökçeada (Imbros) (279 km²)

The first record was from the estuary of a stream near Aydmerk on 29.4.1998 (Fig. 2), then on 5.5.1998 and in a residual pond two kilometres away on 6.5.1998 on the east

Figure 2. First record of *Emys orbicularis* on Gökçeada (Imbros) (May 1998) (Photo: M.F. Broggi).

coast see location map in Broggi (1999). Bayrakey et al. (2016) also confirm this evidence. As frequently observed in the Aegean, European Pond Turtles were only found sporadically among *Mauremys rivulata*. Suitable habitats are present on Gökçeada, although only small *Emys* populations can be expected, based on the observations.

Ithaca? (118 km^2)

Keymar (1986) refers to Cyren (1935) in his herpetological survey of the Ionian Islands. Cyren sighted water turtles in a well on Ithaca but was unable to identify the species. Keymar said this was all the more remarkable because Ithaca had no permanent open water. Cisterns are probably the last refugial habitats of amphibians and hydrophilic reptiles on the island. Broggi (2009) can confirm that there are no suitable habitats for aquatic turtles on Ithaca today. If Cyren's (1935) observation is correct, *M. rivulata*, which has fewer habitat requirements than *Emys*, would probably be more likely. Strachinis and Artavanis (2017) found a new record of *Bufo bufo*, but equally no terrapins.

Kefalonia (773 km^2)

Werner (1894) mentions *Emys* for Kefalonia, but little is known about its occurrence on the island since then. Wilson (2006a) does not mention *E. orbicularis* at all. One occurrence known to me is in the Livadi marshes, where I was able to observe about a dozen *Emys*, some of them mating, during a visit to the island on 16.4.1992. The observation was repeated in April 2017. An observation was also made in a stream near the artificial Karovomilos Lake at Sami. The Livadi marshes are described in Archipelagos (2016) in a natural history article titled "Wetlands in Kefalonia". On the webpage of the Kefalonia-Ithaca Geopark, *Emys* is described as "rarely seen in Kefalonia" (kefaloniapark.gr/en/node/293, later unavailable). In any case, it seems that the status of *Emys* on Kefalonia is endangered.

Kos (287 km^2)

The first recorder of the species for Kos is probably Fritz (1989) with a collected specimen between Kos Town and Psalidi. Kos is otherwise not mentioned for *Emys* in the herpetological literature (see e.g. Cattaneo 2005). In Cattaneo et al. (2020), a paper on the herpetofauna of the Dodecanese, a photo with *Emys* on Kos is included without further comment. Further data on finds, in taxonomic contributions by Fritz (1989) and Fritz et al. (1998), prove that the species occurs on Kos, while Bader et al. (2009) point out in their contribution on Rhodes, that on 13.4.1984 a specimen was collected between Kos Town and Psalidi (Naturhist. Museum Wien, NHMW 28291). There is also photographic evidence of *Emys* on Kos on the internet, for example in a field report from 8.-22.6.2006 by Wilson (2006b), who saw two *Emys*. Troidl (2022) presented a photo of *E. orbicularis* from Kos in iNaturalist. These are single data, indicating sparse populations.

Lefkas (325 km^2)

De Betta (1868) is named as the first describer by Werner (1894). Another early mention of the species is by Lehrs (1912), where he refers to several occurrences on the island. During my visit to Lefkas I saw three specimens, on 19.5.1985 in Nidri Bay, and on 20.5.1985 and 23.5.1985 in Vasili Bay. For further *Emys* data, see Sindaco and Rossi (2020). They found 15 specimens along Lake Maranthodi and two in a small channel near Vasiliki. The species is, therefore, present on Lefkas but probably only in small populations.

Lesbos (1,633 km²)

I succeeded in making the first record for the island in April 1978, when I found a dead *E. orbicularis* on the country road at the northern end of Geras Bay (Broggi 1978). In the following days, every fifth turtle observed turned out to be *Emys*. More juveniles were found in a swamp south of the main town of Mytilene. Buttle (1995) did not succeed in confirming *Emys*, nor did Kasapidis et al. (1996). Only Perez Mellado et al. (1999) confirm this species for Lesbos with an observation of a specimen at Kopster-Myrtiotissas-Filia. Hofstra (2003) found an *Emys* with a carapace length of 13 cm in a polluted river near Kalloni Lake. He describes it as extremely shy, much shyer than *Mauremys*. In the wetlands of Kalloni Bay, the two species share the habitat. Some nature photographers have posted examples on their websites, for instance of some *Emys* sites, on iNaturalist (2022). Christopoulos and Zevgotis (2022) report from a 12-year study. They surveyed 119 wetlands on the island. They found them within stable populations of *Emys* and *Mauremys*. They present a distribution map with six *Emys* sites, compared to 83 for *Mauremys*. All these *Emys* sites were found in Kalloni Bay. The populations do not seem large but are stable according to Christopoulos and Zevgotis (2022). These authors also found three sites where Red-eared Slider Turtles (*T. scripta*) co-occurred with the native terrapins.

Limnos (476 km²)

Werner (1938) does not mention *Emys* on Limnos. The first record was made by Schneider (1986), who found a site with two specimens behind the beach of Evgatis. Strachinis and Roussos (2016) studied the herpetofauna of the island for many years, and Strachinis found a dead specimen near Plaka on 30.7.2007, which had been killed by heavy machinery during reed harvesting. This was followed in April 2015 by his further observation of three specimens in a narrow ditch near the airfield. The authors describe *E. orbicularis* as threatened with extinction and refer in particular to attempts to drain back-dammed water towards the sea near Aliki. I was able to detect 11 *Emys* sites on the island, each with low numbers, during intensive surveys (Broggi 2017). *Emys* and *Mauremys* shared the habitat, with *Mauremys* being more common (Fig. 4). The assessment of the threat to the species remains valid despite these new findings.

Mykonos? (105 km²)

Bringsøe (1985) writes that a Swedish tourist brought him a live European Pond Turtle on Mykonos. This was the first, and so far the only, find in the Cyclades. We know about the endangered status of *Mauremys rivulata* on the island (Broggi 2012; Broggi in press), and *E. orbicularis* has not been seen again since this single record. It is difficult to assess this 1985 find. Is it a release, is it a relict? Fritz (1992) writes about Tinos, "due to the recent finding of an *Emys orbicularis* on the neighbouring island (meaning Mykonos), however, the old record for Tinos deserves re-examination.". The wetland situation on Mykonos has deteriorated dramatically in the meantime, whereas a suitable habitat would be available on Tinos. From today's point of view, without further evidence, we must consider the 1985 finding as an introduction, as is now often the case for land tortoises.

Figure 3. Estuary of the Fonias-Brook on Samothrace with first record of *Emys orbicularis* in May 1988 (Photo: M.F. Broggi).

Figure 4. Shared habitat of *Emys orbicularis* and *Mauremys rivulata* on Lemnos (22.04.2016) (Photo: M.F. Broggi).

Rhodes? (1,401 km²)

Surprisingly, there is still no confirmed evidence for Rhodes. Helmdag (1993) visited the island and states: "It is possible that *Emys orbicularis*, the European Pond Turtle, also lives there. In any case, it seemed to me that an animal I saw belonged to this species but could only be seen once for a few seconds while taking a breath." Bader et al. (2009) cannot confirm the species in their herpetological article on Rhodes. Although several animal photographers have visited the island in recent years, there has been no confirmation from this side either. Thus, the species` presence on the island remains unconfirmed.

Samos (477 km²)

Calabresi (1923) and Ioannides et al. (1994) do not mention *Emys* for Samos in their herpetological contributions.

In 1975, I also failed to find any. In a popular article on the herpetofauna of some Greek islands, Buttle (1995) mentions *E. orbicularis* in a list for Samos, without commenting on this or identifying it as a first record. Meyer and Fritz (1996) succeeded in establishing a reliable record of *Emys* on Samos on 12.4.1996. A specimen was photographed on the reedy shore of an irrigation pond near the mouth of the Tourkomyla River between the villages of Mesokambos and Mykalis in Eastern Samos. The site was about 200 metres from the seashore. Clark (2000) also mentions one observation of *Emys*. Speybroeck et al. (2014), in turn, report seven observations in four grids on the south-east coast. They describe *Emys* as "extremely rare on Samos" and thus threatened with extinction.

Samothrace (178 km^2)

Neither Werner (1938), nor Wettstein (1953) mentions the species for Samothrace, nor later do Clark (1991), Buttle (1995), Cattaneo (2001) and Ochsenhofer (2012). The first record of the species was made on the north coast, east of the Fonias stream, on 26.5.1987, when two *Emys orbicularis* were found in a group of *Mauremys* (Broggi 1988, 1994). Again, on 3.6.1987, I found *Emys* among *Mauremys* in a residual pond on the same road. The island of Samothrace is rich in water, and there are several watercourses with syphons, as well as standing water on the island (Fig. 3). The numbers of *Emys* are clearly low if so many herpetologists have overlooked the species, and it has not been confirmed since. On the other hand, the existence of *Emys* has been cited several times since then, for example in the nomination application for a UNESCO biosphere reserve in September 2013.

Thassos (380 km^2)

Emys was also discovered late on Thassos. During my visit to the island in 1996 I did not see any *E. orbicularis* individuals. Clark (1999) also does not refer to *Emys*, though Fritz (2001) collected a specimen. In Fowles (2012) we read: "*Emys* was related as recorded from the island by Yann Horstink in July 2007 near Prinos. A single individual along with circa 20 *Mauremys* (was seen) in a small area of open water in a reed-filled ditch, and Lance Chilton photographed two *Emys* in May 2012." Berthomieu and Vermeer (2021) mention the presence of *E. orbicularis* on Thassos but without citing a source. So far, these are the only references to an occurrence on the island. Information on population sizes is also missing.

Tinos? (194.5 km^2)

Werner (1938) refers to the Morée expedition in 1832 for Tinos and adds a question mark to the statement. Beutler and Frör (1980) write: "St. Hilaire (after Bedriaga 1882) lists the European Pond Turtle, *Emys orbicularis*, for the River Eurotes on Tinos. However, a watercourse of that name does not exist on the island; the record probably refers to the Evrotes in Laconia, where the species was found by Cyren (1941)."Fritz (1992) notes the following on the history of Tinos: "1833 *Emys antiquorum* Valenciennes in Bory de Saint-Vincent (Eurotes estuary Peloponnese and Tinos) ". The river is recorded here for the Peloponnese, and Tinos is given independently. Fritz (1992) considers an earlier occurrence on Tinos (and also Mykonos) as possible. Regarding suitable habitats, the lagoons of Kolimpithra and Panormos on Tinos would be quite possible for *E. orbicularis*, according to my own visit in 2018, but did not find it there (Broggi 2019). Likewise, for both Andros and Mykonos, a confirmation is missing.

Zakynthos (406 km^2)

Werner (1938) first mentions *E. orbicularis* for the island with reference to W. Kühnelt without citing the source. Keymar (1988) mentioned *Emys* for Lake Keri, where he saw some juveniles. Podlouky and Fritz (1994) observed two specimens at Lake Keri and believed the site was threatened by desiccation. Extinction is feared. Wilson (2006a) mentions a marsh and watercourse at Limni Keri in the south-west of the island, where he observed six individuals and also noted drainage in the area. Wilson (2009) wrote from two excursions in May 2005 and June 2006 that there was only one occurrence on Zakynthos, at Lake Keri. He observed around 20 animals there in 2005 and 30 in 2006. Urosevic (2014) recorded an adult *Trachemys scripta scripta* in a canal near Lake Keri and later saw a juvenile *T. s. elegans* there. The other turtles observed there have been *Emys orbicularis* in larger numbers and some *Mauremys rivulata* (Urosevic 2014). Pafilis et al. (2015) points to a monitoring program as a case study for Zakynthos that also includes *E. orbicularis*. These references are all clearly about the same endangered site on Zakynthos, and thus this species must be assessed as threatened with extinction from the island.

Discussion

Emys orbicularis lives mainly in stagnant waters along the Greek mainland and on the larger Ionian Islands except Ithaca. It is absent from Kythera, Crete and the Cyclades. Its occurrences in the Ionian area and in the Aegean are restricted to the larger islands close to the mainland that have wetlands in their coastal areas. Viable populations exist on Lesbos and Corfu, but on other islands only small populations were found. The situation seems to be particularly critical on Kefalonia, Zakynthos, Thassos and Samos.

While the sympatric species *M. rivulata* occurs on 29 Greek and two Turkish islands (Broggi in press), *E. orbicularis* has so far been found on only 11 Greek islands and one Turkish. It has rather more specific habitat requirements than *Mauremys* (Fritz 2001). Occurrences on Rhodes and Chios have not yet been clarified, while for four other islands early reports have never been confirmed and are therefore questionable. Samothrace, with a land area of 178 km^2, is the smallest island with *Emys* sightings. It is obvious that only the larger islands allow large hydrological catchment areas, which can then form corresponding watercourses with estuaries.

Threats and protection

Most of the Greek islands have an arid climate and, therefore, have few wetlands due to a lack of annual precipitation (WWF-Greece 2014). According to my observations the habitats of swamp turtles in the estuaries of watercourses are often located in sandy areas and are characterized by the formation of beach walls. Sea currents can close the estuary to flowing waters, with falling water pressure then causing backwater in the form of lagoons or other smaller areas of standing water. These habitats are naturally rare in the Greek island world and tend to be small. Human influence is all the more detrimental to these hydrophilic species. A gene exchange between populations is hardly possible via land routes but only via drifting into the open sea after heavy rainfall. It is known that *Emys* from the Neretwa estuary in Croatia have drifted to the island of Korcula, 5km away (Jelič et al. 2012). However, the surviving animals did not find suitable habitats there. This observation supports the inter-island drift theory for the distribution of the species during the last millennia (Lymberakis and Poulakakis 2010). It is obvious that *Mauremys* is better adapted to this as it is more salt-resistant than *Emys*.

In its preferred habitat in the backwaters of sandy beaches, numerous conflicts of use arise. Tourism claims these sandy beaches and impairs or destroys habitats with its infrastructure. This affects not only the aquatic habitats but also the nesting sites needed. Intensified agriculture is also exploiting flat areas in the estuaries of watercourses. Agriculture is also involved in water loss by diverting water for field irrigation over many kilometers and thus drying out suitable habitats. It also extracts groundwater by pumping, causing watercourses to dry up more quickly. I have the impression that *Emys* proves to be a more sensitive species to pollution than *Mauremys*, which can still thrive in heavily eutrophic waters (Wischuf and Busack 2001). Similarly, refugial habitats in streams are used by *Mauremys*, which has not been observed for *Emys*, at least on the islands. All *Emys* occurrences on islands are in low-lying areas, on islands they hardly penetrate the streams at higher elevations. A new threat is the introduction of invasive turtles. Cady and Joly (2004) find weight loss and high mortality of *E. orbicularis* in mixed groups, and their study calls for a halt to slider turtle introduction in all European wetlands. Likewise, ongoing climate change with drying tendencies may be another threat.

Emys orbicularis is a species in Greece protected by national legislation (Presidential Decree 67/1981). It is also protected and listed in Annex II of the EU Habitats Directive, as well as by the Bern Convention. It is believed that many wetlands on the islands have been destroyed in the past century (WWF-Greece 2014). WWF-Greece mapped 824 wetland structures on 76 islands in 2004–2013. Of these, 100 are in the Ionian Islands, 526 in the Aegean and 192 in Crete. The data is available at oikoskopio.gr/ygrotopio (WWF-Greece 2014). In a presidential Act in 2012, 350 properties on 58 islands were placed under protection. According to Paragamian et al. (2014), this is said to have been extended from 350 to 562, which corresponds to around 70% of all those in the inventory. Enforcement of protection will now have to be monitored; some of our own observations suggest these decrees are being disregarded.

The present study is a second report on the occurrence and status of *E. orbicularis* on the Greek islands. It is based on literature studies, although there are certainly gaps in our knowledge. In addition, the author and his colleagues were able to visit about 60 Greek islands, with local inspections, to get a picture of the prevailing habitat conditions. The assessment of threats to *Emys orbicularis* on the individual islands is not based on monitoring, as such data are lacking. It is my subjective expert assessment. This is mostly based on a systematic search of suitable habitats on the islands. However, such findings may date back many years and thus be outdated. The occurrences of *Emys orbicularis* on the islands are usually very isolated, their populations small and therefore vulnerable. Accordingly, the occurrences of *Emys orbicularis* are endangered and must be ensured through conservation measures. The report explicitly refers to open questions that need to be closed by further field herpetological work. The most obvious is the clarification of the occurrence of *E. orbicularis* on Chios and Rhodes.

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Snake shed skin consumed by *Kinosternon vogti*: a case of interspecific keratophagy

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Abstract

Some reptile species can consume their own shed skin or that of conspecifics; despite its prevalence, the benefits of eating shed skin remain poorly understood. Here, we report a field observation in which a Vallarta Mud Turtle (*Kinosternon vogti*) consumed a snake shed skin of *Masticophis lineatus* (Bocourt, 1890) in Bahía de Banderas, Nayarit, Mexico. This type of record could allow us to understand the keratophagous behaviour between and within reptile species.

Key Words

aquatic species, food habits, reptiles, urban wetland

Some animals consume their shed skin or that of a conspecific during ecdysis. This behaviour is commonly observed in lizards and snakes in natural habitats and captivity, but it appears less common in turtles and crocodilians (reviewed by Weldon et al. (1993); Mitchell et al. (2006)). Specifically, the reports on consumed shed skin by turtles are available for less than 2% of the 357-turtle species currently recognised (TTWG 2021) and include the families Chelidae, Emydidae and Testudinidae (reviewed by Weldon et al. (1993)). Herein, we report a snake shed skin consumed by the Vallarta Mud Turtle (*Kinosternon vogti* López-Luna, Cupul-Magaña, Escobedo-Galván, González-Hernández, Centenero-Alcalá, Rangel-Mendoza, Ramírez-Ramírez & Cazares-Hernández, 2018; Fig. 1A). This species is endemic to the Bahía de Banderas, situated on the western-central coast of Mexico. Vallarta Mud Turtles inhabit small streams and ponds in this region, although in low population densities (López-Luna et al. 2018; Cupul-Magaña et al. 2022).

We conducted surveys between October 2019 and February 2023, to collect individuals of *Kinosternon vogti* in Bahía de Banderas. The captured turtles were transported in a plastic box to the Biodiversity and Ecosystem Services Laboratory at the Centro Universitario de la Costa of the Universidad de Guadalajara, Mexico. Once in the laboratory, we measured meristic characters and collected their stomach contents using the stomach flushing technique (Legler 1977). Stomach contents were preserved in 70% ethanol for laboratory analysis. Turtles were under observation between 12 to 48 hours before being returned to the capture site and no deaths

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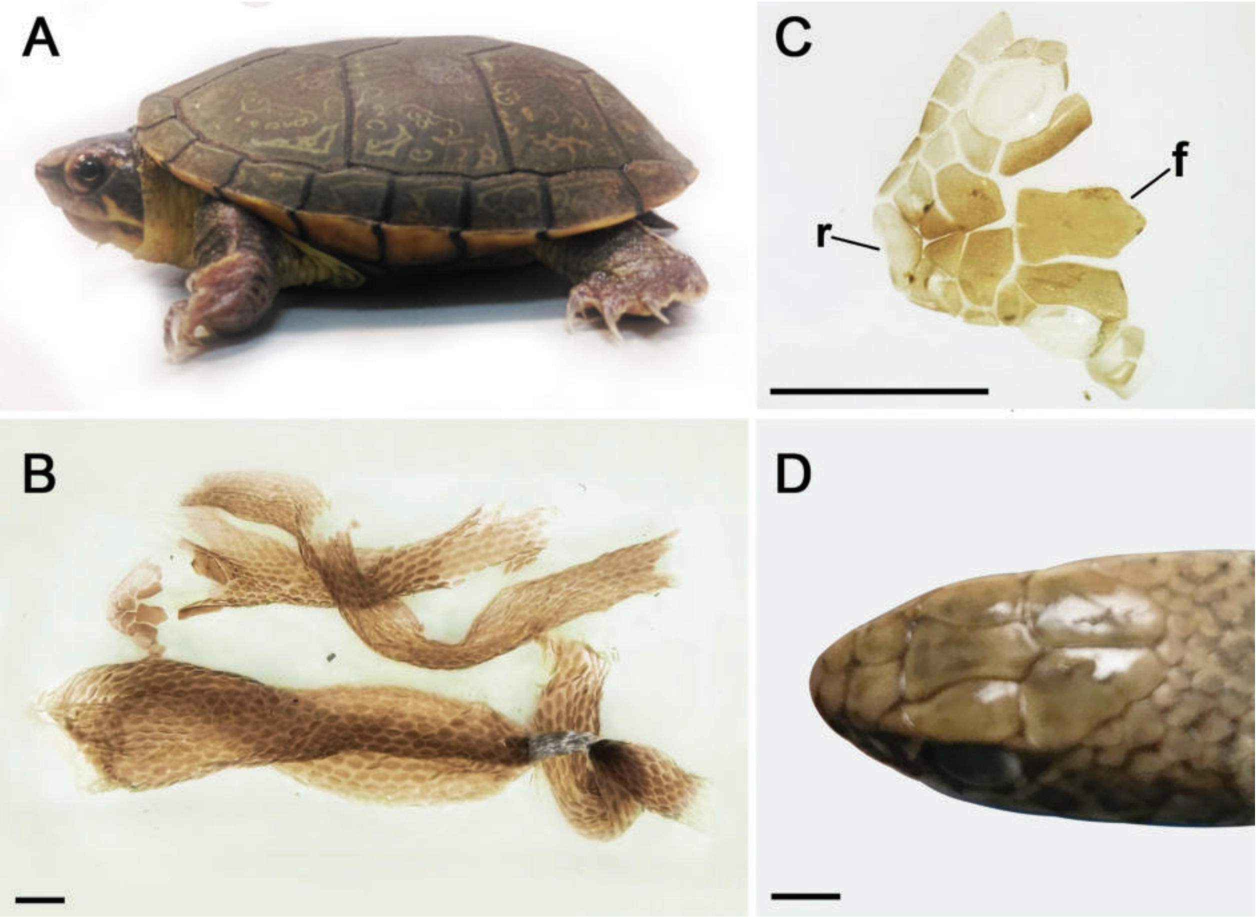

Figure 1. **A.** Lateral view of an adult female specimen of *Kinosternon vogti* from Puerto Vallarta, Jalisco, Mexico; **B.** Dorsal view of the shed skin of *Masticophis lineatus* obtained from *K. vogti* stomach content; **C.** Dorsal view of the head shed of the skin of *M. lineatus*, r = rostral scale, f = frontal scale; **D.** Dorsal view of the head of *M. lineatus* specimen catalogue from Mayborn Museum Complex of Baylor University. Scale bars: 5 mm (**B–D**).

were observed due to manipulation. During the laboratory analyses, the stomach contents were separated using a Carl Zeiss Stemi DV4 stereoscopic microscope and were examined under an Olympus optical microscope. Prey items were identified to the lowest taxonomic level possible using specialised literature.

We captured a total of 24 individuals of *K. vogti* (17 females and 7 males). During the analysis of stomach contents, we found seeds and partially digested snake shed skin (total length < 28 cm; Fig. 1B) in one adult female *K. vogti* captured on 24 October 2022 (carapace length: 96.1 cm, plastron length: 76.6 cm and height: 35.5 cm). By examining the characteristics of the shed skin, particularly the frontal and rostral scales as described by Johnson (1977), we were able to identify the snakeskin as belonging to the species *Masticophis lineatus* (Bocourt, 1890) (for its current taxonomic status, see O'Connell and Smith (2018)). In addition, we also used a photograph of the *M. lineatus* specimen from the Mayborn Museum Complex at Baylor University for comparison of head squamation (R 14597, 67-566; Fig. 1C, D). *Masticophis lineatus* has been recorded in Arizona and New Mexico (USA), as well as from the western coast of Mexico, from southern Sonora to Colima, including islands in the Islas Marías Archipelago Biosphere Reserve (O'Connell and Smith 2018; Nolasco-Luna et al. 2022).

Our observation provides a field record of interspecific ingestion of shed skin involving a turtle and a snake. This record not only sheds light on the food habits of *K. vogti*, but also on its terrestrial foraging strategy. Although the specific food habits of *K. vogti* are still unknown, Ramírez-Ramírez et al. (2019) classified it as an omnivorous species, based on the presence of unidentified plants, isopods and hemipterans in the stomach contents. That *K. vogti* consumed opportunistically the snake shed skin confirms that this species will forage terrestrially and occasionally consume the items it encounters. The Vallarta Mud Turtle shares its habitat with other turtle species such as *Kinosternon integrum* and *Trachemys ornata* and two non-native species, *Trachemys scripta* and *Staurotypus triporcatus* (Cupul-Magaña et al. 2022). Assessing the dietary habits of these species could provide us with valuable insights into whether the consumption of snake shed skin is a shared behaviour and how competition amongst species could expand the food habits of turtle species. However, this idea must be studied in more detail.

The benefits of consuming shed skin remain unexplained. However, hypotheses primarily based on lizards and snakes have been proposed to explain this behaviour (see Mitchell et al. (2006)). Briefly, the consumption of shed skin has been related to ectoparasite load. According to Watkins and Blouin-Demers (2019), larger lizard individuals tend to have a higher ectoparasite load due to increased surface area available for parasites to inhabit. Consequently, the consumption of their own skin or that of conspecifics might offer advantages in social interactions. On the other hand, dry environmental conditions and low prey availability may explain the ingestion of shed skin (Barraza-Soltero and Escobedo-Galván 2020; Rojas-Carranza and Anderson 2021). Another angle to consider is the nutritional aspect. The hypothesis proposes that this behaviour allows the acquisition of epidermal proteins, such as alfa keratin and beta keratin present in shed skin (Weldon et al. 1993; Fabrício-Neto et al. 2016; Pough et al. 2016). Herein, we found a snake shed skin in the stomach contents of an adult female *K. vogti* during the reproductive season (Montaño-Ruvalcaba et al. 2020; Rosales-Martínez et al. 2022), which may be related to the nutritional hypothesis. For instance, Vacheva (2018) observed that of the 17 cases of keratophagy in the European common lizard *Zootoca vivipara*, with 76.5% of the cases occurring in subadults and adult females. The author suggests that this behaviour could be associated with the nutritional requirements for gravid females. In the case of kinosternid turtles, Macip-Ríos et al. (2010) observed that females shift food resources seasonally unlike males, favouring this type of opportunistic ingestion. On the other hand, an anonymous reviewer suggests that our observation could be influenced by odour cues. Given that *Masticophis lineatus* is a terrestrial species and that female turtles exhibit greater mobility than males in their search for food, if the shed skin were fresh, it could have contained chemosensory cues leading the turtle to mistake it for a food item. In recent years, there has been an increased publication on keratophagous behaviour (e.g. Vacheva and Naumov (2022); Sandoval-Ponce et al. (2023)); therefore, the records of this type of behaviour offer us a window into understanding the reasons for shed skin ingestion between and within reptile species.

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Sexual dimorphism in postcloacal scales in the northern caiman lizard (*Dracaena guianensis*)

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Abstract

Morphological differences between males and females are common among reptiles. A particularly interesting sexually dimorphic feature whose function is largely unknown is the number and pattern of specific scales. Several lizard species possess an arrangement of centered scales near the cloacal region that differ between the sexes and can be used for sex determination. The presence of postcloacal buttons, sexually dimorphic postcloacal scales on both sides of the body, is an exclusive trait in the subfamily Tupinambinae and is only poorly documented. Here, we investigate postcloacal scales in northern caiman lizards (*Dracaena guianensis*) housed at the Vienna Zoo. For a period of two years, we documented scale patterns and performed morphometric measurements of individuals of different age classes. Caiman lizards were CT scanned to confirm the sexes. Males exhibit three raised postcloacal scales in a row behind the left and right leg, while females possess one or two large scales surrounded by several smaller scales. The study provides the first evidence that these scales can function as a reliable trait to distinguish the sexes regardless of age or reproductive status. The sexually dimorphic bilateral scale pattern is present immediately after hatching and does not change during development. Scales only increase in thickness and length during growth. We further demonstrate that sexual size dimorphism (SSD) exists in juveniles during ontogenetic development. Juvenile females had a larger SVL, body length, tail length and higher weight compared to juvenile males. This SSD could not be confirmed in adults, and sex determination based on SSD seems unreliable.

Key Words

computed tomography, postcloacal buttons, reptile, sexing, Teiidae

Introduction

Sexual dimorphism, the difference in morphology between male and female members of the same species (Andersson 1994), is common in the animal kingdom and particularly in reptiles (Butler and Losos 2002; Olsson et al. 2002). Several studies identified sexual size dimorphism (SSD) in reptiles by comparing morphological traits such as body length (i.e., snout-vent, carapace, or plastron length), as well as head width, head length, body length, and body mass (Olsson et al. 2002; Schwarzkopf, 2005; Cox et al. 2007; López Juri et al. 2018; Yang et al. 2019) between the sexes. Selection for such difference might pose an advantage in intrasexual mate competition (Salvador et al. 1995; Martin and Salvador 1997; Cox et al. 2003; Naretto et al. 2014) or provide a fecundity advantage (Olsson et al. 2002; Cox et al. 2003; López Juri et al. 2018) to store larger energy reserves or more eggs/embryos (Du and Lu 2009).

The widespread biological phenomenon in which traits of one sex are characteristically larger than those of the opposite sex for a given population or species (Cox et al. 2003) differs greatly among lizard species. For example, male-biased SSD reaches extremes of over 50% longer snout-vent length of males compared to females in anoles (*Anolis* spp.) (Butler et al. 2000), Neotropical ground lizards (*Tropidurus* spp.) (Pinto et al. 2005), marine iguanas (*Amblyrhynchus cristatus*) (Wikelski and Trillmich 1997), and monitor lizards (*Varanus* spp.) (Cox et al. 2007). By contrast, female's snout-vent length (SVL) exceeds that of males by as much as 20% in bush anoles (*Polychrus* spp.) (Cox et al. 2007), common sun skink (*Eutropis multifasciata*) (Shrma 2022), and legless lizards (*Aprasia* spp.) (Cox et al. 2007). Females have longer SVL even in horned lizards (*Phrynosoma* spp.) (Zamudio 1998) and South African dwarf chameleons (*Bradypodion* spp.) (Stuart-Fox 2009).

Several other morphological traits differing among sexes are ornamentations like dewlaps (Nicholson et al. 2007), horns (Amarasinghe et al. 2009; Wikramanayake et al. 2021), femoral pores (Avila-Pires 1995) or hidden characters as the number of vertebrae (Arnold 1973; Kaliontzopoulou et al. 2015). A particularly interesting sexually dimorphic feature whose function is largely unknown is the number and dimension of specific scales. For example, preanal scales and preanal plates, the scales situated in front of the cloaca of four-lined ameiva (*Holcosus quadrilineatus*), are dimorphic traits (Harvey et al. 2012). In males, a large anterior preanal plate projects posteriorly separating two small preanal plates, whereas small granular scales surround a single large preanal plate in females (Harvey et al. 2012). Males of this species also possess two enlarged postanal/postcloacal scales, also called postanal plates, situated immediately posterior to the postanal ridge and separated by 2–4 granular scales (Pietruszka 1981; Harvey et al. 2012). These scales are absent in most South American tegus but are present in western and Central American jungle-runners (*Ameiva* spp.), ameivas (*Holcosus* spp.), whiptail lizards (*Aspidoscelis* spp.), and some species of racerunners (*Cnemidophorus* spp.) (Pietruszka 1981; Ashton 2003; Harvey et al. 2012). Enlarged postanal scales are even present in males of anoles iguanian lizards (*Anolis* spp.) (Malhotra and Thorpe 1997; Lovern et al. 2004), common Indian monitor (*Varanus bengalensis*) (Deraniyagala 1958), spiny lizards (*Sceloporus* spp.) (Ballinger et al. 1996; Mueller and Moore 1969; Weintraub 1969), horned lizards (*Phrynosoma* spp.) (Whiting and Dixon 1996) and side-blotched lizard (*Uta* spp.) (Stejneger 1895; Mayhew and Tinkle 1968). In all the above-mentioned taxa, postanal scales are a dimorphic trait already present in juveniles.

A further scale dimorphism is the presence of a small cluster of 2–3 slightly raised and enlarged rounded scales behind the vent of males, so-called postcloacal buttons. This character is not well documented and was only briefly described in the 16 species of the subfamily Tupinambinae (Fitzgerald et al. 1991; Harvey et al. 2012; Silva et al. 2018; Borczyk and Skawiński 2019). The only available picture of postcloacal buttons was documented in one male of dwarf tegu (*Callopistes maculatus*) (Harvey et al. 2012, fig. 30, p. 35). Limited information is available about the presence of similar scales in the northern caiman lizard: *Dracaena guianensis* Daudin, 1802. Individuals with three enlarged and raised scales in a row behind the vent are considered males. However, sexual scale dimorphism is only known empirically; the precise structure and variation has never been described.

Northern caiman lizards can grow up to a meter long and are among the largest lizards in South America (Vanzolini and Valencia 1965; Avila-Pires 1995). Captive individuals can reach up to 412 mm in snout-vent length (SVL) (Duellman 1978), while males found in the wild ranged from 300–355 mm SVL and are larger than females ranging from 236–278 mm SVL (Mesquita et al. 2006). Similarly, two males housed at Prague Zoo are larger and heavier than one female kept in the same facility (Rehak 1999). However, no SSD in body size and head size correlation were found (Mesquita et al. 2006), even if males appeared to be bigger than females (Rehak 1999; Mesquita et al. 2006; Frýdlová and Frynta 2015).

The Vienna Zoo houses *D. guianensis* since 2007, and some individuals exhibit three scales arranged in a row, while others have one or two larger scales surrounded by several small scales to the right and left of the cloaca (Fig. 1). These potentially sexually dimorphic postcloacal buttons are present instantly after hatching. To investigate this idea, we documented scales of eight juveniles for a period of almost two years starting at the age of two months, and eventually identified the corresponding sex with computed tomography scans. Similarly, we examined postcloacal scales of adult *D. guianensis* housed at the Vienna Zoo and tested SSD by conducting continuous measurements of body weight, SVL, head-, body- and tail length on every individual. As such we determined if individuals can be sexed immediately after hatching and how SSD supports discrimination between the sexes.

Methods

Study species and location

The study was conducted with a captive population of *Dracaena guianensis* at the Vienna Zoo (Vienna, Austria). The population consisted of 15 individuals at the start of this study and currently nine individuals are housed in the Terrarium House in Vienna while six individuals were transferred to other Zoos. Individuals were pair- or single-housed in large terraria (245 × 170 × 190 cm or 100 × 75 × 100 cm), with a water area (respectively 100 × 150 × 20 cm and 100 × 75 × 15 cm). All terrariums were equipped with rocks, big branches, plants, and coco peat as a substrate. Individuals were housed under 12-hour light and 12-hour dark cycles. During the 12-hour light period illumination was provided by a combination of metal-halide lamps as well as heating lamps (250 W)

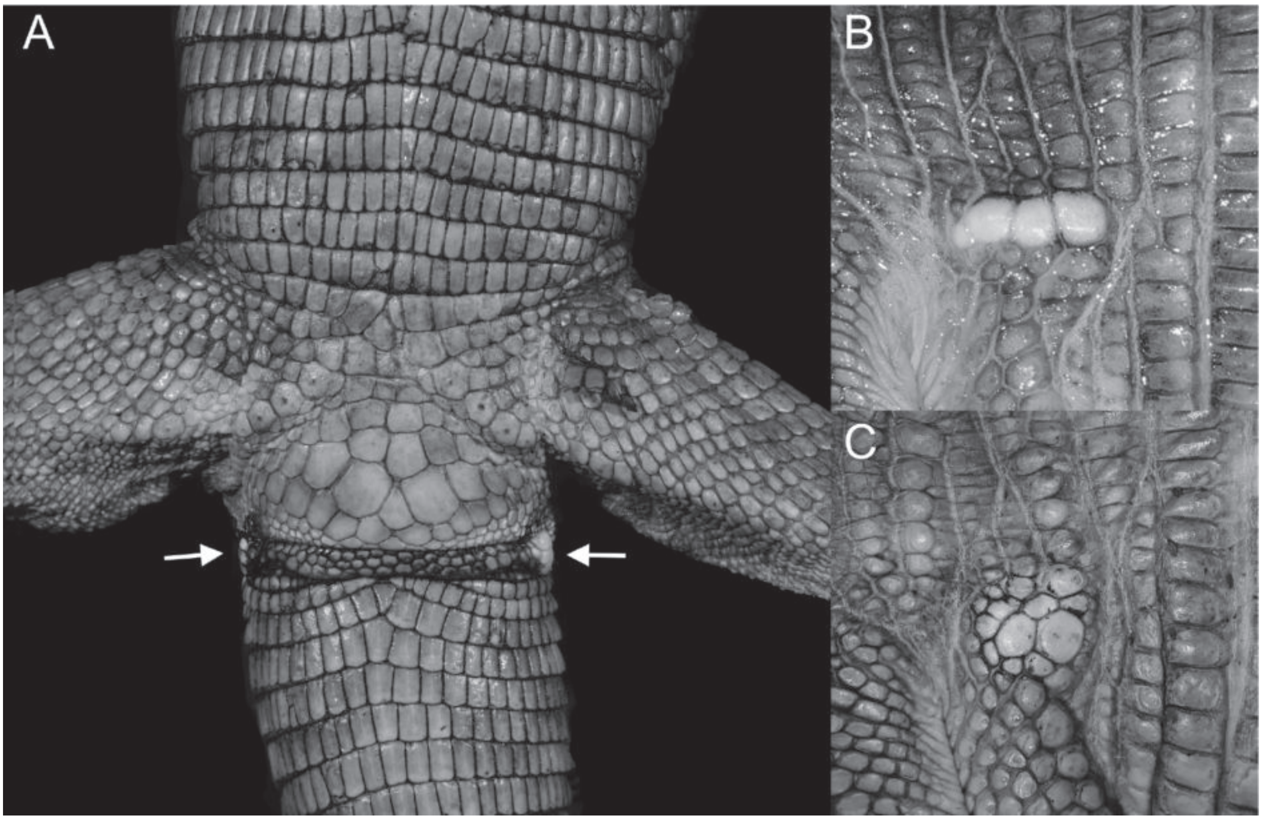

Figure 1. Overview of postcloacal scales of *Dracaena guianensis*. **A.** Arrows show location of sex dimorphic scales left and right of the cloaca; **B.** Three male postcloacal buttons in a row and **C.** One large female scale surrounded by several smaller and one larger scale in a circular pattern.

shining for 6 hours per day and UVB lamps shining for 9 hours per day (150 W for small terrariums, 300W for large terrariums). The air temperature was approximately 30.1 °C (SE ± 0.1; range: 27.8–32.7), the water temperature was 27.9 °C (SE ± 0.1; range: 25.8–33.6) and relative humidity reached 66.3% (SE ± 0.7; range: 47.4–96.3). Individuals were fed three times a week with either snails (*Achatina* spp., *Helyx* spp.) without shells, or freshwater fish fillet (*Salmo trutta* ssp.) dusted with Spirulina powder.

Table 1. Summary of *Dracaena guianensis* study population and methods used to determine sex. Individual identification number (ID).

ID	Birth year	Age class	Sex	Sex-determination Method		
				Scales	CT scan	Reproduction
930	2018	juvenile	female	x	x	
925	2018	juvenile	male	x	x	
927	2018	juvenile	female	x	x	
928	2018	juvenile	male	x	x	
142	2016	adult	male	x	x	x
370	2016	adult	female	x	x	x
358	2005	adult	female	x	x	x
361	2005	adult	male	x	x	x
366	2015	adult	female	x	x	
368	2016	adult	female	x		
369	2016	adult	male	x		
929	2018	juvenile	female	x		
923	2018	juvenile	female	x		x
924	2018	juvenile	male	x		
926	2018	juvenile	male	x		

Data collection

Monthly morphometric measurements were taken from October 2018 to September 2020 on a total of 15 individuals of *D. guianensis* of different age classes (Table 1). As no information is available when individuals sexually mature, hence reach adulthood, we determined individuals older than two years as adults according to a single documented incident of a female (ID 923) born in the Vienna Zoo, that laid 6 eggs at the age of 2 years and 4 months at the Basel Zoo. Accordingly, seven individuals were classified as adults at the start of the measurements. Two adult individuals were born in 2005 in Peru and were transferred to the Vienna Zoo in 2007. The remaining five adult individuals were bred and raised at the Vienna Zoo in 2015 and 2016. Eight individuals were juveniles that hatched in October 2018. One week after hatching, the first morphometric measurements were taken. To determine SSD, we took monthly head length, body length, tail length, and weight measurements from October 2018 to July 2020 ($N = 21$) for juvenile individuals and additionally from January to September 2020 ($N = 9$) for adult individuals. Depending on the size of the individuals we used a dial caliper or measuring tape to determine length. The head was measured from the tip of the snout to the posterior end of the parietal scale, the body length from the posterior end of the parietal scale until the cloaca, and from the cloaca to the tip of the tail was considered as tail length. SVL was calculated as the sum of head length and body length. Postcloacal scales of juveniles were photographed from December 2018, while monthly measurements and photo documentation of scales of all 15 individuals were performed from June 2019 – July 2020 ($N = 14$). We used a dial caliper to measure postcloacal scales length to the nearest 0.01 mm. Depending on the visual appearance of scales we either measured the length of three same-sized scales arranged in a row or the diameter of one large scale on the left body side of the individuals (Fig. 1).

In August 2022 nine *D. guianensis* underwent a health check and were sexed with the help of computed tomography (CT) at the University of Veterinary Medicine, Vienna. Six individuals (2 adults and 4 juveniles) of the study group were transferred to Liberec and Basel Zoo before the CT scans and were not included in the analysis. All examinations were performed in awake animals positioned in a box in sternal recumbency with a dual energy 128-slice helical CT (Siemens Somatom X.cite, Vienna, Austria), using 80–100 mAs, 130 kV, rotation time 1.5 s, pitch 0.8, and slice thickness 0.5 to 0.75 mm. The scans were reformatted with an ultra-sharp bony and a soft tissue kernel, FOV 55 × 55 mm, matrix size 512 × 512, increment 0.6 mm, and then evaluated in a bony and soft tissue window. Image interpretation was done with multiplanar reconstruction with JIVEX, Version 5.3.0.2 RC01 (Visus Health IT GmbH, Bochum, Germany). Contrast-enhanced images were gained using intravenous iodine (Optiray(R) 300 mgJ/ml, Guerbet, France) with a dosage of 2 ml/kg BW.

Statistical analysis

To test SSD, we compared morphometric parameters (SVL, head size, body size, tail length, and weight) of either all adults or all juveniles between the sexes using generalized linear mixed models (GLMMs) with normal distribution, identity link function and Student's t statistic for post hoc comparisons. The sex of individuals transferred to other zoos, that could not be confirmed by CT scans or a reproductive event (juvenile: 924,926, 929; adult: 368, 369) was assigned according to the visual appearance of scales. The morphometric parameters were entered as dependent variables, with sex as predictor variables and individual and point of measurement as random variables to correct for repeated measurements of the same individual. Statistical analyses were performed with the program SPSS 26 (IBM SPSS Statistics, USA).

Results

Dracaena guianensis possess distinct postcloacal scales behind their left and right hind legs (Fig. 1A). Male individuals exhibit three same-sized and raised scales in a row (postcloacal buttons) and females have a single large center scale bordered in some cases by a second larger scale and 4–7 not raised smaller scales (Fig. 1B, C). Female scales are arranged in a circular or curved pattern and never form a linear row. The sexual dimorphic scales are similar on both sides of the body and visible immediately after hatching. The form of the scales remains consistent but increases in size with increasing age (Fig. 2). Male buttons length averaged 6.70 mm (range 5.21–8.13; $N = 3$) in adults and ranged from 2.89 to 5.72 mm in juveniles ($N = 4$) during the age of 8–21 month. The single center scale of adult females averaged 3.57 mm (range 2.18–3.47; $N = 4$) and ranged from 1.43 to 2.62 mm in 8–21 month-old juveniles ($N = 4$).

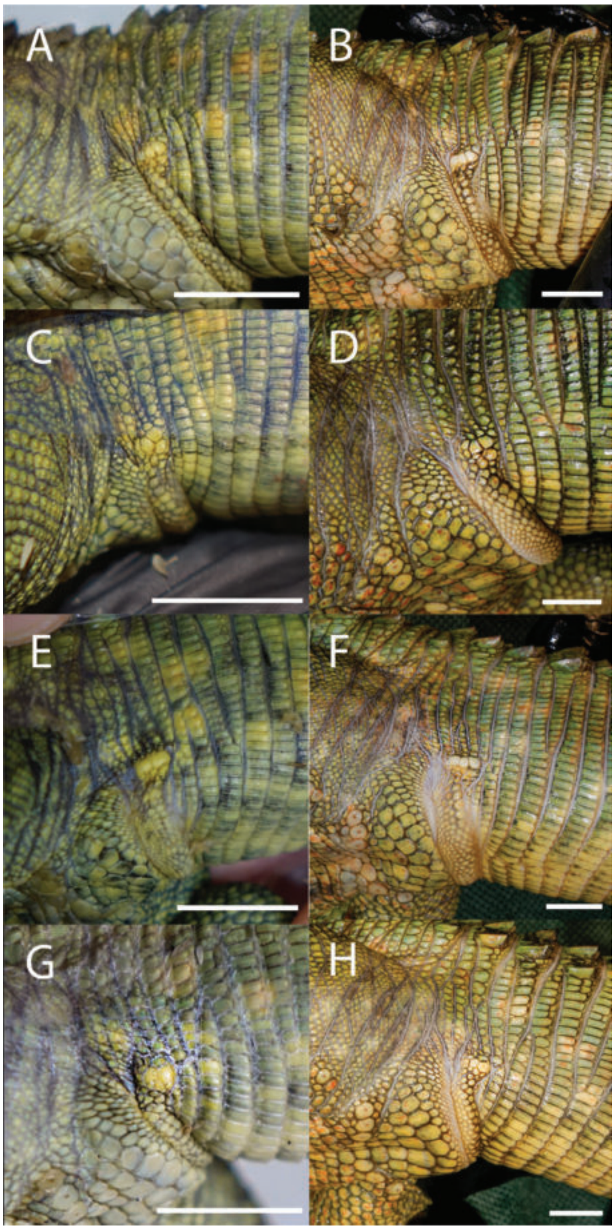

Figure 2. Scale comparison between 3 month (left side) and 3 years and 10 month (right side) old *Dracaena guianensis* individuals from the Vienna Zoo. **A, B.** Male ID 925; **C, D.** Female ID 927; **E, F.** Male ID 928; **G, H.** Female ID 930. Pictures taken in January 2019 (age: 3 month) and in August 2022 (age: 3 years and 10 month). Scale bar: 1 cm.

Out of 15 individuals (8 juveniles and 7 adults) included in the current study, the sex of nine individuals (4 juveniles and 5 adults) could be determined by computed tomography scans (Fig. 3). All individuals could be identified by their gonads and scans showed visible testis and active ovary. The resulting sex corresponded to the above-described respective male or female scale pattern. In addition to scale pattern, the sex of one juvenile (ID 923) could be confirmed as female by a reproductive event in which the individual deposited six eggs at Basel Zoo (Table 1). The sex of the remaining five individuals (2 adults and 3 juveniles) that were not CT scanned was exclusively classified according to scale patterns based on the results of this study and included in SSD analysis.

Sexual size differences

Male and female juveniles differed in body length (GLMM: $F_{1,166}$ = 4.992, P = 0.027; Fig. 4A), SVL (GLMM: $F_{1,166}$ = 4.162, P = 0.043; Fig. 4B), tail length (GLMM: $F_{1,166}$ = 6.577, P = 0.011; Fig. 4D), and weight (GLMM: $F_{1,166}$ = 6.025, P = 0.015; Fig. 4E) during the first 21 months after hatching. We found no difference in head length (GLMM: $F_{1,166}$ = 2.183, P = 0.141; Fig. 4C). Female juveniles had a longer SVL (GLMM: pairwise comparison, female vs. male: β = 14.208, SE = 6.964, t = 2.040, P = 0.043), body (GLMM: pairwise comparison, female vs. male: β = 11.310, SE = 5.062, t = 2.234, P = 0.027), tail (GLMM: pairwise comparison, female vs. male: β = 38.583, SE = 15.045, t = 2.565, P = 0.011), and were heavier (GLMM: pairwise comparison, female vs. male: β = 96.845, SE = 39.456, t = 2.455, P = 0.015) compared to male juveniles. Contrary to the juveniles, the adult individuals showed no SSD in SVL, head-, body-, tail-length, or weight (GLMM: $P > 0.05$ for all parameters; Table 2). The above mentioned differences remain consistent when removing individuals whose sex could not be confirmed by CT scans or a reproductive event from the respective SSD analysis (juveniles: 924, 926 and 929, or adults: 368 and 369, Suppl. material 1).

Discussion

The *Dracaena guianensis* population at the Vienna Zoo has sexually dimorphic scales behind their left and right hind legs, at the end of the cloacal opening. Males exhibit three raised postcloacal scales in a row, termed postcloacal buttons, while females possess one or two large scales surrounded by several smaller scales in a circular pattern. The scales to the right and left of the cloaca are already present after hatching (personal observation by the authors) and provide a reliable sexual characteristic that can be used to easily identify the sex of an individual regardless of age or reproductive status. The scale pattern does not change during development, merely the thickness of the buttons and the length of the scales of both males and females are altered during growth. In juveniles, differences in the pattern are visible between the sexes, but buttons and scales are flat and level with surrounding body scales.

Table 2. Body measurements for *Dracaena guianensis* individuals from the Vienna Zoo. Data are estimated means ± standard error (SE) of generalized linear mixed models. 21 measurements were performed for juveniles, nine for adults. Sex was assigned according to subsequent classification (see Table 1); sample sizes in parentheses.

Class	Sex	SVL (mm)	Head length (mm)	Body length (mm)	Tail length (mm)	Weight (g)
Juvenile	Male (4)	196.42±11.11	52.37±2.56	144.05±8.62	340.02±22.23	327.02±49.35
	Female (4)	210.63±11.11	55.27±2.56	155.36±8.62	378.61±22.23	423.87±49.35
Adult	Male (3)	336.61±20.70	83.35±4.32	253.26±16.64	621.33±30.25	1,585.30±295.94
	Female (4)	355.94±17.94	83.35±3.75	272.58±14.42	578.56±26.22	1,910.83±256.43

Scutellations around the anal region play an important role in identifying the sex of several lizard species and occur in various types among the suborder Lacertilia. Preanal and postanal scales are described in both sexes, situated before or after the cloaca, usually in a central position. Such scales were observed in males of several families among the suborder Lacertilia, for example, family Dactyloidae (Malhotra and Thorpe 1997; Lovern et al. 2004), Varanidae (Deraniyagala 1958), Liolaemidae (Fernando et al. 2019), Phrynosomatidae (Mayhew and Tinkle 1968; Ballinger et al. 1996; Whiting and Dixon 1996), Xantusiidae (Davis and Leavitt 2007) and Teiidae (*Ameiva* spp., *Holcosus* spp., *Aspidoscelis* spp., and *Cnemidophorus* spp. (Ashton 2003; Pietruszka 1981; Harvey et al. 2012)), but were reported as not present in its subfamily Tupinambinae (Pietruszka 1981; Ashton 2003; Harvey et al. 2012).

The only visual representation of particular bilateral scales, postcloacal buttons, in Tupinambinae, comes from a male dwarf tegu (*Callopistes maculatus*) (Harvey et al. 2012, fig. 30, p. 35). *Dracaena guianensis* studied in the present work show sex dimorphic scales located laterally on both sides of the body immediately after the cloaca (Fig. 1) corresponding to the few descriptions of postcloacal buttons (Harvey et al. 2012). Although both enlarged postanal plates and postcloacal buttons, indicate the sex in lizards, it is significant that buttons have only been observed in the subfamily Tupinambinae, while enlarged postanal plates were absent in the studied animals. Further studies should investigate the hereby suggested divergent evolution of scales in species of the family Teiidae and take a closer look at postanal/cloacal scales of species among the subfamily Tupinambinae, and the particular interesting congeneric species

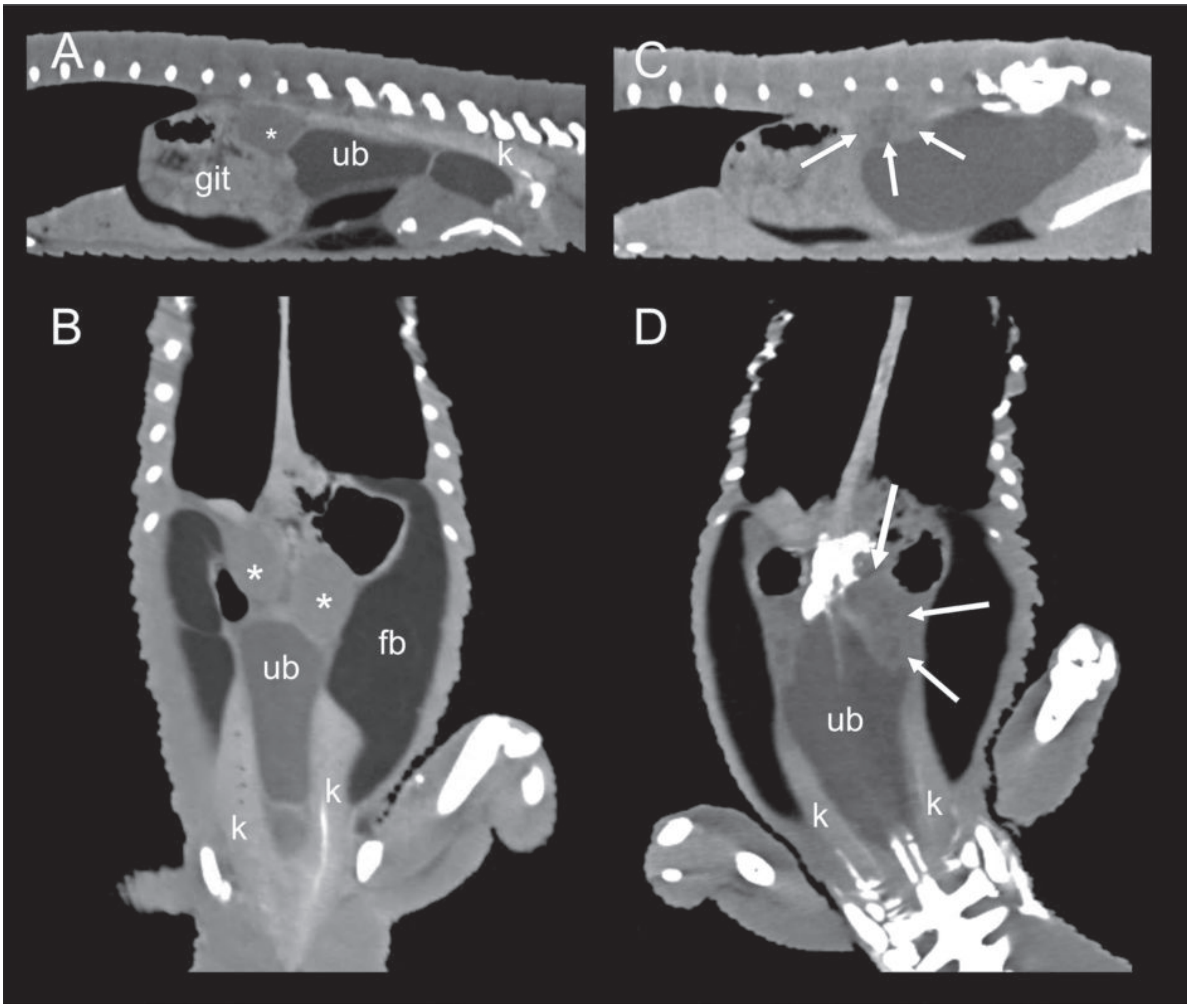

Figure 3. Sagittal (**A, C**) and coronal (**B, D**) contrast enhanced CT in adapted soft tissue windows of **A, B.** A male (ID 142) and **C, D.** A female (ID 366) *Dracaena guianensis*. The testis (asterisk) of the male individual appear as soft tissue dense (mildly hypodense to muscle tissue) homogeneous ovoid structures in the dorsal half of the mid-coelom. The ovaries (arrows) consist of multiple, grape-like positioned, small nodular hypodense structures surrounded with a contrast enhanced hyperdense wall or rim. fb - fat body, k - kidney, ub - urinary bladder-like structure, git - gastrointestinal tract.

D. paraguayensis. Likewise, the origin of these sexually dimorphic scales is still unknown despite their presence in a great number of species. (Lovern et al. 2004; Harvey et al. 2012) and so is their function. How could lizards benefit from displaying sex-dimorphic characteristics? Morphological traits or ornaments differing among sexes are usually used for courtship, agonistic behavior or communication in lizard species (Watkins 1998; Iraeta et al. 2011; Johnston et al. 2012). Prominent examples come from male anoles (*Anolis* spp.) using colored dewlaps in conjunction with head bobbing displays during courtship, whereas females rarely or never perform this behavior (Jenssen et al. 2000; Lovern et al. 2004). Similarly, rostral appendages or horns occurring in only a few lizard groups (Johnston et al. 2012) are suggested to provide information about male quality for both sexes (Whiting et al. 2015), mate or rival recognition (Rand 1961; Johnston et al. 2012), and are used in males fighting in territorial species (Čerňanský et al. 2014; Whiting et al. 2022). Concerning the comparatively inconspicuous position and size of scales in *D. guianensis*, we suggest that none of the above-mentioned signal characteristics can be affirmed for the scales in our study species. It is unlikely that conspecifics detect scale differences in juveniles as they mostly blend into the appearances of surrounding scales. The visual detection in adult male scales can, however, not be fully neglected. During basking, the postcloacal male buttons of adults are recognizable to human observers and potentially also to conspecifics. Adult male scales also show some degree of reflectance when observed under UV light (personal observation by the authors). Scales could also play a role in pheromonally mediated behaviors or serve as scent glands. We did not test visual or chemical signal function of scales in our current study, but it is something that could be looked at in future studies.

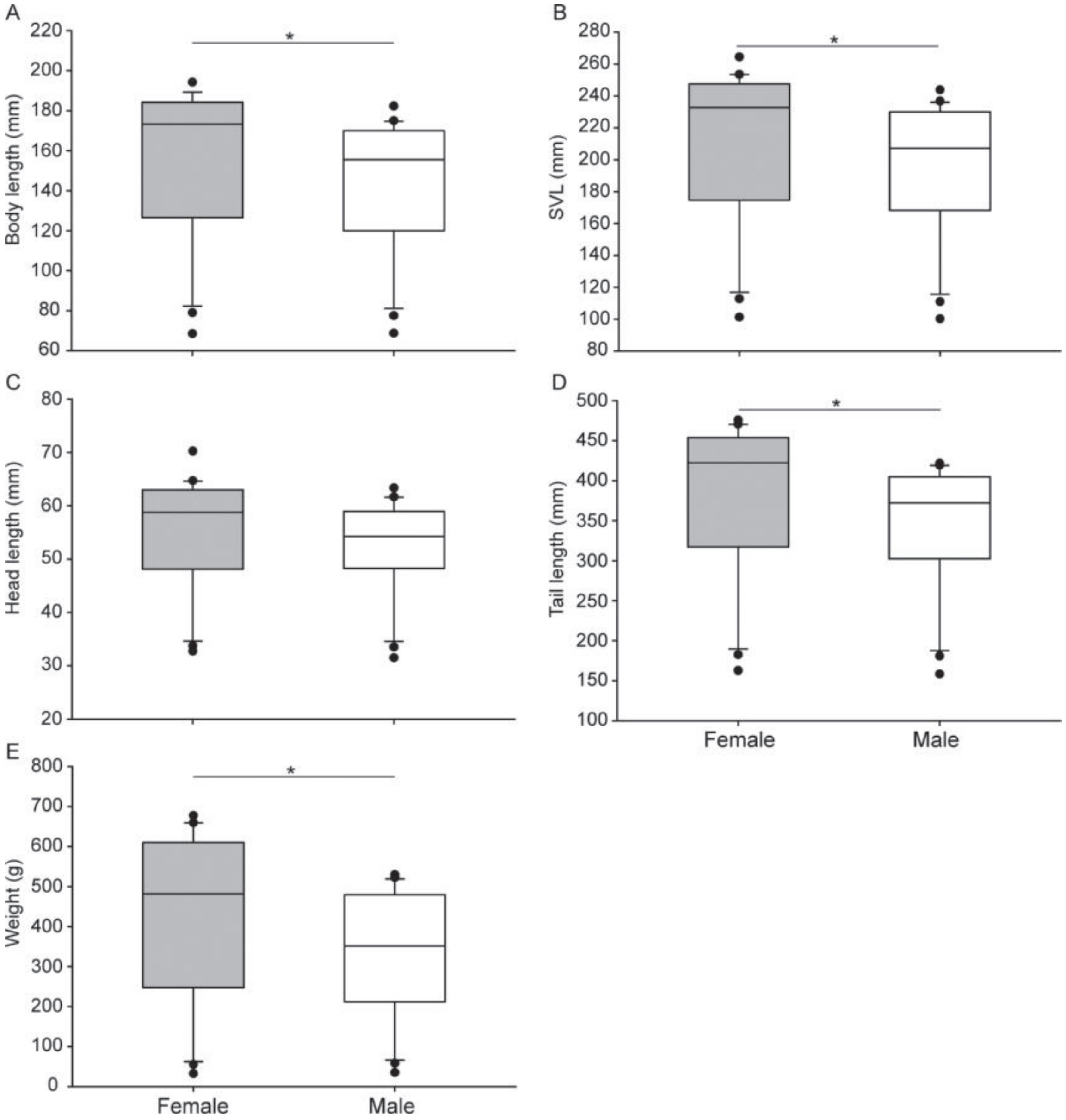

Figure 4. Size and weight differences of *Dracaena guianensis* juveniles. Boxplots show mean individual values of female (n=4) and male (n=4) sexed according to CT-scans and scales for a period of 21 month after hatching, with interquartile range, minimum and maximum values. Points designate outliners. Asterisk denote p-values from GLMMs.

Contrary to other studies where males were bigger than females (Rehak 1999; Mesquita et al. 2006; Frýdlová and Frynta 2015), we found no significant differences in SVL or other morphological parameters between adult males and females of the northern caiman lizard. Overall, lizards in the Teiidae family show a male-biased SSD (Anderson and Vitt 1990; Santana et al. 2010). Males of tegus (*Tupinambis* spp.) even show an enlarged jaw musculature during the reproductive season (Fitzgerald et al. 1991; Naretto et al. 2014) increasing bite performance, with the benefit of a stronger grip on a female to copulate, or dominating fights with other males (Naretto et al. 2014). Individuals at the Vienna Zoo rarely display dominant behavior. Males do not fight or behave aggressively if held together in a terrarium. Agonistic behavior has been reported in females from the Prague Zoo, but dominance patterns between conspecifics are scarce. When sexual selection on body mass is low or absent, males may benefit by maintaining a relatively light body, allowing them to be more mobile, and spend more time and energy on searching for mates instead of food (Trivers 1976). This is typical in lizard populations where densities are low and females are widely dispersed, thereby male mating success could depend on the number of females encountered rather than on competitive advantages over other males (Zamudio 1998).

In *D. guianensis*, we found morphometric differences between juveniles according to their sex classified by the sex-dimorphic scales. Female juveniles had a larger SVL, body length, tail length, and higher weight. In several species of lizards, skinks, and geckos the tail is also considered energetic storage and correlates with fat reserves

(Clark 1971; Roig and Carretero 2000; Sanggaard et al. 2012; Cardozo et al. 2015). The longer SVL in juvenile females resulted from longer body length, as head length did not differ compared to males. Female lizards might invest more energy in mass and length which in turn could increase their chances to breed earlier and reach relatively high fecundity (Yang et al. 2019), considering a pressure for fecundity selection (Olsson et al. 2002; Cox et al. 2003; López Juri et al. 2018), where large female body size allows the production of larger clutch size (Winck and Rocha 2012).

Conclusion

The current study provides the first evidence that juveniles can be sexed by sexually dimorphic bilateral scales, providing a non-invasive method to sex individuals rapidly at any life stage. We further show that SSD exists in juveniles during ontogenetic development, however, this SSD disappeared in adults. Hence studying differences and similarities of morphometric parameters between the sexes during development and in correlation with behavior, clutch size, and associated reproductive success might help to understand selection factors promoting SSD in different life stages.

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Supplementary material 1

Body measurement comparison for *Dracaena guianensis* individuals from the Vienna Zoo

Authors: Riccardo Antonini, Rupert Kainradl, Michaela Gumpenberger, Anton Weissenbacher, Doris Preininger

Data type: docx

Explanation note: Data are estimated means ± standard error (SE) and P-value of generalized linear mixed models (GLMM). 21 measurements for juveniles, nine for adults. Sex was assigned according to CT scans or a reproductive event (see Table 1) excluding juveniles: 924, 926 and 929, or adults: 368 and 369; sample sizes in parentheses.

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Advertisement calls of *Leptobrachella suiyangensis* and *Leptobrachella bashaensis* (Anura, Megophryidae)

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https://zoobank.org/CA5BB859-6436-4187-AE1A-9EBA21AAFB97

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Abstract

In this study, the advertisement calls of *Leptobrachella suiyangensis* and *Leptobrachella bashaensis* are described. The advertisement call of *L. suiyangensis* includes simple and complex calls, with four different call types and a dominant frequency ranging 4.13–4.82 kHz. The advertisement call of *L. bashaensis* consists of a single note, with a dominant frequency 6.03–6.46 kHz. We compare the advertisement calls with other species in the genus *Leptobrachella*, and discuss the definitions of primary advertisement calls and secondary advertisement calls. Our results provide basic data for further acoustic, taxonomic and ecological studies in the genus *Leptobrachella*.

Key Words

acoustic differences, bioacoustics, frogs, southern China

Introduction

In anurans, acoustic communication is the most important form of communication at the interspecific and intraspecific levels, playing an important role in species reproduction, evolution and interspecific identification (Cunningham and Birkhead 1998; Brenowitz and Rose 1999; Kelley 2004). For further understanding the relationship between the behaviors and vocal communication in frogs, researchers have divided frog calls into the following four types: reproductive calls, aggressive calls, defensive calls, and feeding calls. Reproductive calls include advertisement calls, courtship calls, amplectant calls, release calls, post-oviposition male release calls, and rain calls (Toledo et al. 2015; Köhler et al. 2017). Advertisement calls are not only the main vocal type of frogs, but also vary greatly among different species, so they can be used as a basis for systematic classification and identification of cryptic species (Sullivan et al. 1996; Rowley et al. 2015).

The Asian leaf litter toads of the genus *Leptobrachella* (Smith, 1925) are a group of forest-dependent species, widely distributed in Southeast Asia, southern China, and northeast India (AmphibiaChina 2023; Frost 2023). *Leptobrachella* often inhabits the rocks on the stream banks during the breeding season. Several *Leptobrachella* species are threatened with extinction, 22.2% of them are listed as critically endangered (CR) or endangered (EN) in the IUCN Red Species List, such as the critically endangered *L. botsfordi* and *L. kecil* (IUCN 2021). High levels of morphological similarity and rampant homoplasy appear to have misled estimates of diversity and evolutionary relationships (Chen et al. 2018). Thirty-seven species of *Leptobrachella* have been described in the last five years, representing 37.8% of the total number in this genus (AmphibiaChina 2023; Frost 2023). Despite

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this considerable number of discoveries and publications, the vocalizations of many *Leptobrachella* species remain unknown (Yeung et al. 2021). Both *L. suiyangensis* and *L. bashaensis* were described in 2020 (Luo et al. 2020; Lyu et al. 2020), but their advertisement calls have not been reported so far. In this study, we describe for the first time advertisement calls of *L. suiyangensis* and *L. bashaensis*. Furthermore, we also compare these calls with other species in the genus *Leptobrachella* from literature (Suppl. material 1), in order to provide basic data for further acoustic, taxonomic and ecological studies in the genus *Leptobrachella*.

Materials and methods

Call recordings

Our experimental procedures complied with the applicable laws on animal welfare and research in China and were approved by the Subcommittee on Experimental Animal Ethics of Guizhou University (Permit No. EAE-GZU 2023-E013).

The advertisement calls of *L. suiyangensis* and *L. bashaensis* were both recorded from their type localities Suiyang County and Congjiang County, Guizhou Province, China. A total of 322 calls were recorded from four individuals of *L. suiyangensis*, collected from Huoqiuba Nature Reserve (107.08°E, 28.47°N, ca. 1450 m elev., 15.7 °C air temperature, 93% ambient humidity) on April 27, 2022, between 19:00–23:00 h, and 100 calls from three *L. bashaensis* individuals collected from Basha Nature Reserve (25.63°N, 108.39°E, ca. 980 m elev., 16.3 °C air temperature, 83% ambient humidity) on May 1, 2022, between 19:00–20:00 h. Calls of each individual were obtained using a digital recorder, 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. Recorded calls were always of isolated individuals and never from a mixed chorus. The recordings were saved as WAV files. The recordings are publicly available in Figshare at https://doi.org/10.6084/m9.figshare.24147255. Snout vent lengths (SVLs) of all recorded males were measured in situ using a precision digital calliper to the nearest 0.1 mm. One *L. suiyangensis* (specimen number: SY20220427003) and one *L. bashaensis* (specimen number: CJ20220501001) were collected for species identification and the others were released to their original habitat after measurement. After taking photographs, they were euthanized using isoflurane and then the specimens were fixed in 10% buffered formalin. Tissue samples were taken and preserved separately in 95% ethanol before fixation. Specimens were deposited in the Forestry College of Guizhou University, China. Mitochondrial 16S rRNA genes were extracted and amplified from muscle samples of all 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. For the morphological identification, the procedure described by Luo et al. (2020) and Lyu et al. (2020) was followed.

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 "note-centered" terminology as summarized by Köhler et al. (2017), in which the fundamental unit was defined as a "note" and each "call" contains a single "note" or "note series". We measured all parameters and characteristics following the procedure described by Köhler et al. (2017) and Yeung et al. (2021) including (1) call duration (ms), CD (2) inter-call intervals, CI (3) call rate (calls/minute), CR (4) note per call, NPC (5) first note duration, first ND (6) second note duration, second ND (7) inter-note intervals, NI (8) note rate (notes/s) (9) first note pulse number, first NP (10) second note pulse number, second NP (11) pulse rate (pulses/s), PR (12) dominant frequency (kHz), DF (Table 1). Oscillograms, spectrograms, and power spectra 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 highlight whether the different types of advertisement calls of *L. suiyangensis* were separated in space.

Table 1. Descriptions of acoustic parameters measured.

Parameter (units)	Description
Call duration (ms)	The time between onset of first pulse and offset of the last pulse in a call.
Inter-call intervals (ms)	The time interval between two adjacent calls.
Call (repetition) rate	Instantaneous call rate. Number of calls emitted in a defined period.
Notes per call	The number of notes contained in a call.
First note duration (ms)	The duration of the first note in a complex call.
Second note duration (ms)	The duration of the second note in a complex call.
Inter-note intervals (ms)	The time interval between two adjacent notes.
Note (repetition) rate	Number of notes repeated within a defined period within a call or note series.
First note pulse number	The number of pulses contained in the first note of a complex call.
Second note pulse number	The number of pulses contained in the second note of a complex call.
Pulse (repetition) rate	Instantaneous pulse rate. Number of pulses repeated in a defined period within a note.
Dominant frequency (kHz)	Maximum frequency using Raven's selection spectrum function throughout the entire call.

Results

Leptobrachella suiyangensis Luo, Xiao, Gao & Zhou, 2020 (Fig. 1A)

The recorded males were calling on rocks in streams, with shrubs and bamboo forests growing nearby. After molecular identification, we determined that the species captured in the Huoqiuba Nature Reserve was *L. suiyangensis*. The 16S rRNA of the GenBank accession number is OR398777 (specimen number: SY20220427003). The SVL of *L. suiyangensis* ranged from 27.6 mm to 31.6 mm (Table 2). Based on parameters such as call duration, call structure and pulse structure of the advertisement calls of *L. suiyangensis*, we divide its advertisement calls into four types (Fig. 2; Table 3). In the PCA for the *L. suiyangensis* advertisement call type, the total variation of the first two principal components was 82.55%. The PC1 axis explained 59.83% of the total variation, mostly by the number of pulses per call (factor value = 0.987) and the pulse rate (factor value = 0.939); the PC2 axis was explained by the dominant frequency (factor value = 0.946), which explained 22.72% of the variance (Table 4). On the plot of PC1 vs. PC2, the four types of advertisement calls can be distinguished (Fig. 3). Although there is some overlap between call type C and type D, they can be defined by the difference in pulse numbers per note. Both type A (n = 3) and type B (n = 136) advertisement calls contained a single note, while type C (n = 138) and type D (n = 45) advertisement calls contained note series, and each note series contained two notes (Fig. 2). Among the four types of advertisement calls, the type D calls had the longest call duration, while the type A calls had the shortest call duration (Table 3). Type A, type B and type C advertisement calls had the same dominant frequency range in a call series. The dominant frequency of type D was slightly higher than the other three call types (Table 3).

Leptobrachella bashaensis Lyu, Dai, Wei, He, Yuan, Shi, Zhou, Ran, Kuang, Guo, Wei & Yuan, 2020 (Fig. 1B)

Recorded males perched on shrubs 0.5–1 m above the ground or were calling on rocks in streams. After molecular identification, we determined that the species captured in the Basha Nature Reserve is the *L. bashaensis*. The 16S rRNA of GenBank accession number is OR398776 (specimen number: CJ20220501001). The SVL of *L. bashaensis* ranged from 25.2 mm to 26.2 mm (Table 2). The advertisement call of *L. bashaensis* comprised a single note with a mean call duration of 66.01 ± 6.86 ms (Fig. 4; Table 5). The mean inter-call interval was 334.59 ± 65.61 ms. The call rate was 153.43 ± 9.16 calls/minute. The mean pulse number was 3.00 ± 1.00, with a mean pulse rate of 34.40 ± 4.46 pulses/second. The mean dominant frequency was 6.16 ± 0.08 kHz, and no obvious harmonics were found.

Table 2. Measurement of SVL of samples of *Leptobrachella suiyangensis* and *Leptobrachella bashaensis*.

Species	Vouchers	SVL (mm)
Leptobrachella suiyangensis	SY20220427000	29.0
L. suiyangensis	SY20220427003	31.6
L. suiyangensis	SY20220427004	27.6
L. suiyangensis	SY20220427005	27.7
L. bashaensis	CJ20220501001	25.2
L. bashaensis	CJ20220501003	26.2
L. bashaensis	CJ20220501005	25.3

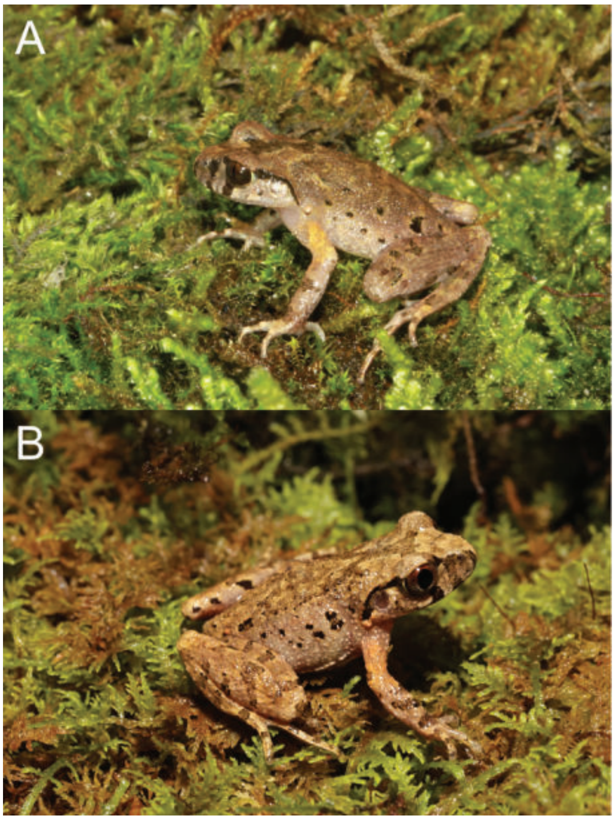

Figure 1. Photograph of the specimen. **A.** Adult male of *Leptobrachella suiyangensis* (SY20220427003, SVL 31.6 mm) from Huoqiuba Nature Reserve, Guizhou Province, China; **B.** Adult male of *Leptobrachella bashaensis* (CJ20220501001, SVL 25.2 mm) from Basha Nature Reserve, Guizhou Province, China.

Discussion

We describe for the first time the spectral and temporal parameters of the advertisement call of *L. suiyangensis* and *L. bashaensis*. The advertisement calls of *L. bashaensis* were simpler, with fewer pulse numbers. In addition, the dominant frequency in *L. suiyangensis* was significantly lower than that of *L. bashaensis*.

Among cryptic species, the use of acoustic diagnostic features for identification could be a potential alternative to morphometric and molecular diagnosis (Köhler et al. 2005; Vences and Köhler 2008). Despite their morphological similarities, *L. suiyangensis* and *L. bashaensis* exhibit different acoustic features in their calls, supporting the species-specificity of their acoustic signals. However, the extent of cryptic diversity and the characteristics of their advertisement calls remain largely unexplored.

Related studies have shown that the evolution of advertisement call traits may be explained by historical (phylogeny), intrinsic (body size, exclusively male parental care), and extrinsic (calling site) factors (Bosch and De la Riva 2004; Mclean et al. 2013; Escalona et al. 2018). From the perspective of phylogenetic relationships, *L. suiyangensis* was assigned to the *L. oshanensis* species group (Luo et al. 2020; Liu et al. 2023). *Leptobrachella bashaensis* is nested in the *L. liui* species group. Previous studies have shown that the species in the *L. oshanensis* species group have more complex advertisement calls (Rowley et al. 2013; Wang et al. 2019; Shi et al. 2021; Suppl. material 1). In contrast, the advertisement call types of *L. liui* and *L. bashaensis* are simpler (Ding et al. 2019; Suppl. material 1). The genetic distance between the *L. bashaensis* and *L. oshanensis* species group is large, and they are not in the same branch (Liu et al. 2023).

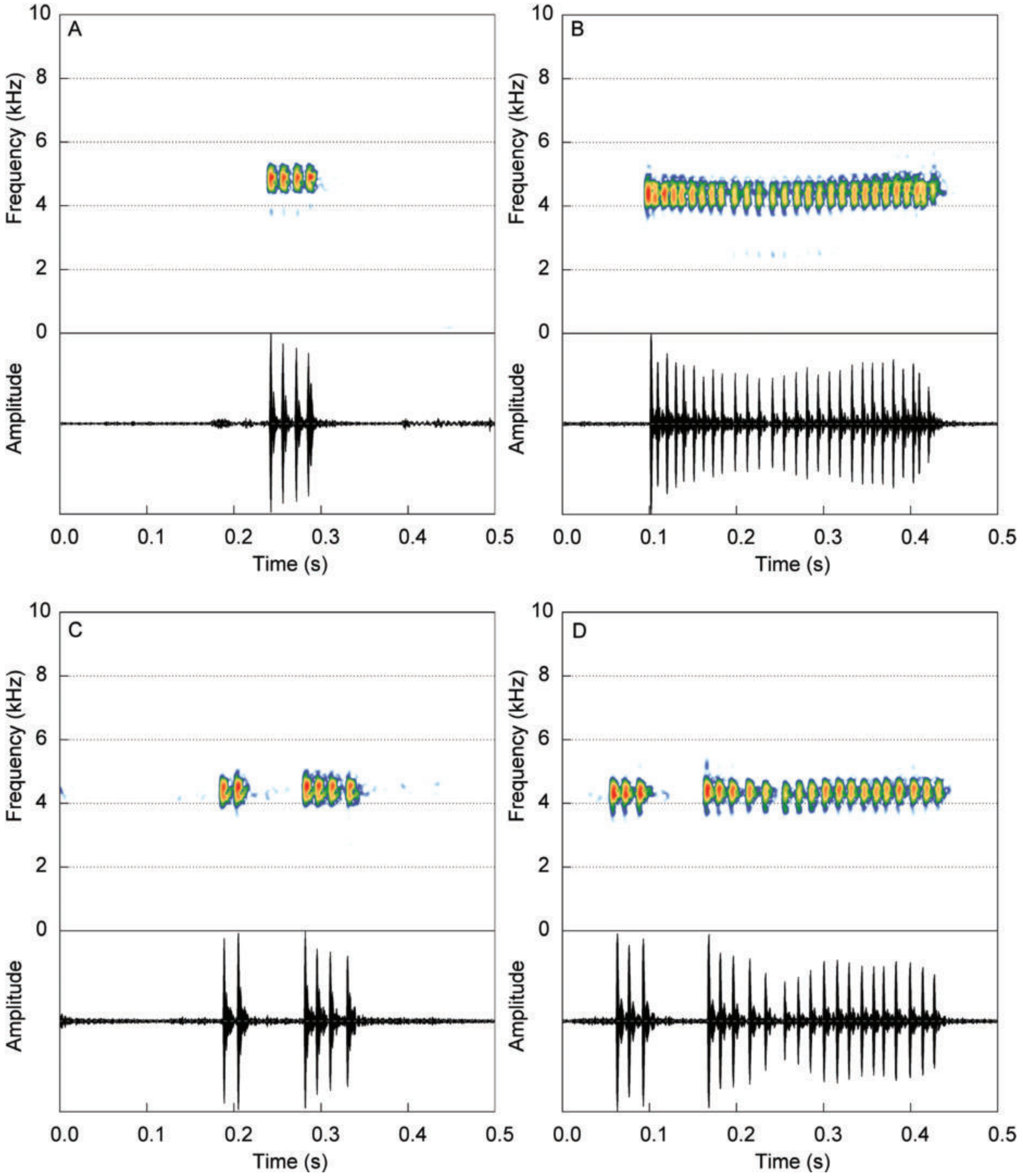

Figure 2. Oscillogram, spectrogram of four types of advertisement call of the *Leptobrachella suiyangensis* (SY20220427003, SVL 31.6 mm, 15.7 °C air temperature, 93% ambient humidity). **A.** 0.5 second type A advertisement call; **B.** 0.5 second type B advertisement call; **C.** 0.5 second type C advertisement call; **D.** 0.5 second type D advertisement call.

Table 3. Descriptive statistics for acoustic characteristics of advertisement calls of *Leptobrachella suiyangensis*. NA = not applicable.

11.75 mm	Vouchers	SY20220427000	SY20220427003	SY20220427004	SY20220427005	All Individuals
Type A	Calls analyzed	Not recorded	2	1	Not recorded	3
	Call duration (ms)	/	25.30–64.70	52.7	/	25.30–64.70
	Inter-call intervals (ms)	/	NA	NA	/	NA
	Call rate	/	NA	NA	/	NA
	Note per call	/	1	1	/	1
	Pulse number	/	2.00–4.00	4	/	2.00–4.00
	Pulse rate	/	39.53–46.37	56.93	/	39.53–56.93
	Dominant frequency (kHz)	/	4.13–4.48	4.82	/	4.13–4.82
Type B	Calls analyzed	33	43	30	30	136
	Call duration (ms)	315.84 ± 35.11 229.30–382.70	289.86 ± 35.05 209.10–343.30	284.39 ± 16.94 251.20–331.90	274.06 ± 13.22 256.40–309.90	291.47 ± 31.59 209.10–382.70
	Inter-call intervals (ms)	411.15 ± 103.38 297.74–716.47	420.35 ± 193.01 182.85–975.99	388.39 ± 99.26 245.01–768.02	470.82 ± 174.18 269.19–973.05	422.64 ± 154.88 182.85–975.99
	Call rate	NA	86.33	90.93	82.28	82.28–90.93
	Note per call	1	1	1	1	1
	Pulse number	18.00[a] ± 1.50[b] 13.00–21.00	24.00 ± 3.50 15.00–34.00	25.00 ± 0.50 20.00–29.00	25.00 ± 1.13 21.00–28.00	24.00 ± 2.50 (13.00–34.00)
	Pulse rate	53.89 ± 4.53 46.40–64.05	79.03 ± 10.74 59.80–105.19	83.39 ± 6.09 70.29–99.04	87.43 ± 6.02 75.34–97.93	75.75 ± 14.83 46.40–105.19
	Dominant frequency (kHz)	4.38 ± 0.19 4.13–4.65	4.45 ± 0.09 4.13–4.48	4.55 ± 0.14 4.48–4.82	4.62 ± 0.07 4.48–4.65	4.49 ± 0.15 4.13–4.82
Type C	Calls analyzed	31	63	3	41	138
	Call duration (ms)	191.85 ± 38.02 152.70–277.30	155.22 ± 8.11 138.30–172.10	159.20–192.30	207.83 ± 41.95 154.50–284.60	179.49 ± 37.56 138.30–284.60
	Inter-call intervals (ms)	176.10 ± 28.97 136.97–260.22	144.92 ± 8.19 128.20–164.87	NA	187.96 ± 15.02 162.39–230.23	164.43 ± 25.60 128.20–260.22
	Call rate	174.87	201.46	NA	157.13	157.13–201.46
	Note per call	2	2	2	2	2
	First note duration (ms)	40.35 ± 17.21 24.54–73.37	30.39 ± 9.19 23.36–62.11	24.85–35.78	62.09 ± 19.47 31.51–100.96	42.00 ± 20.04 23.36–100.96
	Second note duration (ms)	65.32 ± 12.67 47.43–92.41	55.05 ± 10.24 25.96–81.40	47.22–70.97	52.57 ± 5.03 40.94–61.03	56.76 ± 10.77 25.96–92.41
	Inter-note intervals (ms)	86.19 ± 31.72 45.20–159.90	69.78 ± 13.43 36.20–99.20	63.60–101.50	93.18 ± 29.46 52.70–157.00	80.73 ± 25.87 36.20–159.90
	Note rate	10.77	12.92	11.57	9.99	9.99–12.92
	First note pulse number	2.00 ± 0.50 2.00–4.00	2..00 ± 0.00 2.00–3.00	2.00–3.00	4.00 ± 0.50 3.00–6.00	2.00 ± 1.00 2.00–6.00
	Second note pulse number	4.00 ± 1.00 3.00–5.00	4.00 ± 0.50 2.00–5.00	3.00–5.00	4.00 ± 0.50 3.00–5.00	4.00 ± 0.50 2.00–5.00
	Pulse rate	29.15 ± 6.40 18.83–39.22	31.53 ± 4.32 19.34–43.86	25.13–41.10	36.91 ± 6.46 26.61–48.08	32.58 ± 6.29 18.83–48.08
	Dominant frequency (kHz)	4.27 ± 0.07 4.13–4.31	4.48	4.48–4.82	4.58 ± 0.09 4.48–4.65	4.47 ± 0.13 4.13–4.82
Type D	Calls analyzed	Not recorded	12	28	5	45
	Call duration (ms)	/	276.86 ± 63.23 220.50–442.00	320.00 ± 40.88 253.20–402.70	242.60–282.90	302.22 ± 50.97 220.50–442.00
	Inter-call intervals (ms)	/	NA	NA	NA	NA
	Call rate	/	NA	NA	NA	NA
	Note per call	/	2	2	2	2
	First note duration (ms)	/	27.92 ± 7.66 13.22–45.19	51.38 ± 20.10 18.24–80.09	19.03–39.48	42.75 ± 19.93 13.22–80.09
	Second note duration (ms)	/	194.04 ± 40.14 148.19–262.35	225.17 ± 42.37 156.69–299.04	182.55–214.70	213.97 ± 41.71 148.19–299.04
	Inter-note intervals (ms)	/	54.88 ± 27.51 26.80–137.10	43.45 ± 12.49 24.70–72.20	29.00–40.30	45.49 ± 18.10 24.70–137.10
	Note rate	/	7.50	6.35	7.61	6.35–7.61
	First note pulse number	/	2.00 ± 0.00 1.00–3.00	4.00 ± 1.00 2.00–6.00	2.00–3.00	3.00 ± 1.50 1.00–6.00
	Second note pulse number	/	10.50 ± 2.50 9.00–21.00	17.00 ± 2.50 9.00–21.00	14.00–17.00	16.00 ± 3.00 9.00–21.00
	Pulse rate	/	49.40 ± 11.45 27.15–71.75	60.09 ± 5.95 38.36–67.57	61.83–73.27	57.89 ± 9.31 27.15–73.27
	Dominant frequency (kHz)	/	4.48	4.76 ± 0.13 4.48–4.82	4.65	4.67 ± 0.16 4.48–4.82

[a] Median instead of mean.

[b] Interquartile range instead of SD.

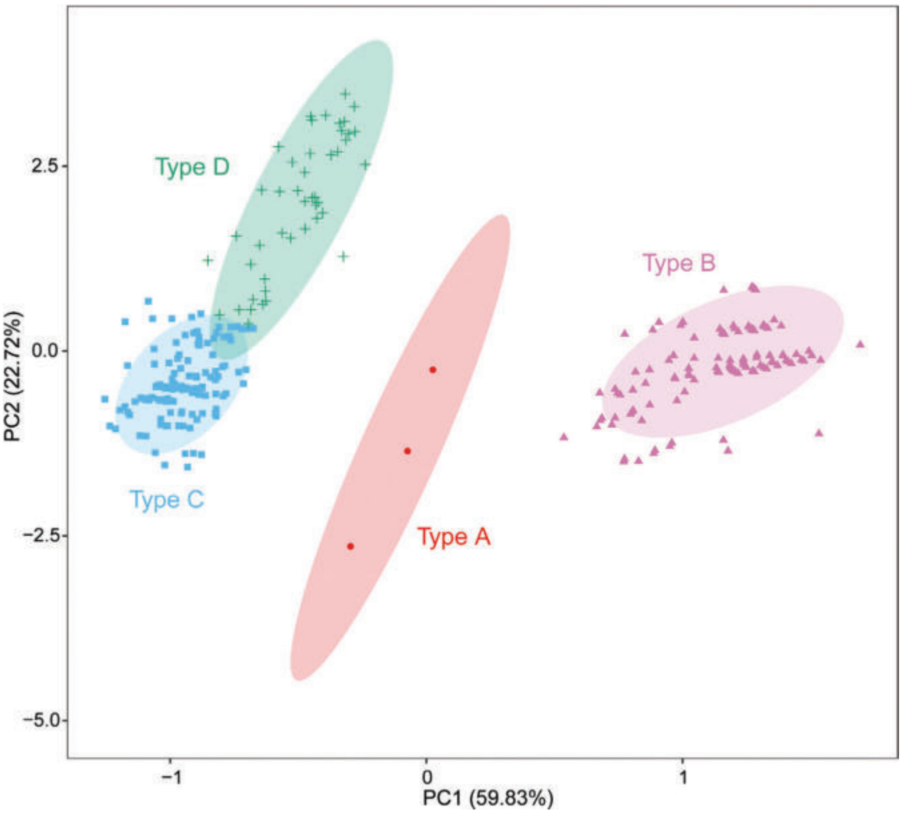

Figure 3. Plots of the first principal component (PC1) versus the second (PC2) for four types of advertisement calls of *Leptobrachella suiyangensis* from a principal component analysis.

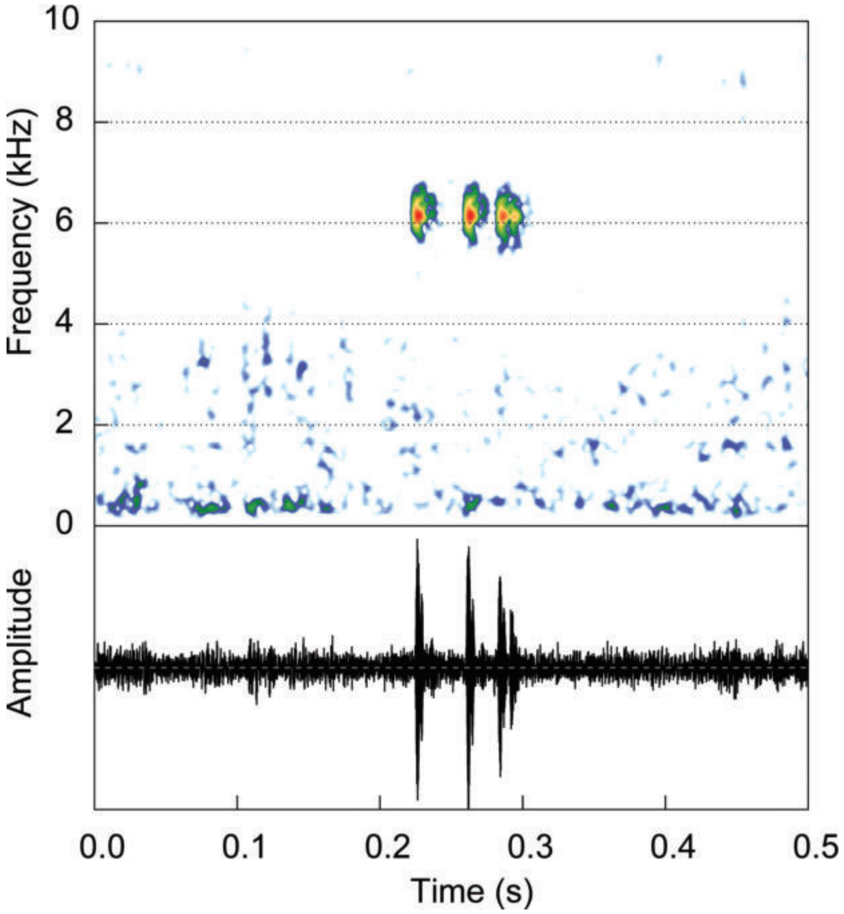

Figure 4. Oscillogram, spectrogram of the advertisement call of the *Leptobrachella bashaensis* (CJ20220501001, SVL 25.2 mm, 16.3 °C air temperature, 83% ambient humidity).

Table 4. Factor loadings of the Principal Component Analysis (PCA) on the advertisement call parameters of the *Leptobrachella suiyangensis*.

Call parameters	Principal components	
	1	2
Call duration	0.821	0.092
Dominant frequency	0.258	0.946
Note per call	-0.836	0.386
Note pulse number	0.987	0.02
Pulse rate	0.939	-0.019

Therefore, the significant differences in the call structure and type between *L. bashaensis* and *L. oshanensis* species groups may be related to their genetic distance.

Due to the diverse types of advertisement calls in *Leptobrachella*, Rowley et al. (2013) defined the primary advertisement call (PAC) and secondary advertisement call (SAC). These terms have been used in subsequent research on advertisement calls of *L. petrops*, *L. puhoatensis* and *L. tengchongensis*, etc (Rowley et al. 2017a, b; Yeung et al. 2021). Rowley et al. (2013) consider that the more frequent and higher amplitude clicking calls are referred to here as “primary calls”, and low amplitude “rasps” are referred to as “secondary calls”. Secondary calls were of less amplitude than primary calls, and not strongly amplitude modulated. But there is a similar dominant frequency between the “primary calls” and “secondary calls”. However, the terms PAC and SAC do not apply to the advertisement calls of *L. suiyangensis*, as the advertisement calls of *L. suiyangensis* are more complex. Hence, we refer to Shi et al. (2021) to divide the advertisement calls of *L. suiyangensis* into four types. The difference in the structure of advertisement calls may be related to the phylogeny of the *Leptobrachella*, the *L. oshanensis* species group belongs to lineage A1 of the genus, while *L. petrops* belongs to lineage A3 and *L. botsfordi* belongs to lineage C (Chen et al. 2018). The differentiation of advertisement calls in *Leptobrachella* can serve as evidence for their differentiation in phylogenetic relationships.

Table 5. Descriptive statistics for acoustic characteristics of advertisement calls of *Leptobrachella bashaensis*. NA = not applicable.

Vouchers	CJ20220501001	CJ20220501003	CJ20220501005	All Individuals
Calls analyzed	40	30	30	100
Call duration (ms)	66.68 ± 6.60	65.78 ± 6.24	65.34 ± 7.87	66.01 ± 6.86
	51.20–79.80	51.30–78.80	48.00–78.50	48.00–79.80
Inter-call intervals (ms)	318.73 ± 52.10	323.61 ± 54.01	366.89 ± 81.25	334.59 ± 65.61
	257.69–468.88	264.25–468.42	259.61–538.39	257.69–538.39
Call rate	158.96	158.48	142.86	142.86–158.96
Note per call	1	1	1	1
Note rate	NA	NA	NA	NA
Note pulse number	3.00 ± 0.38	3.00 ± 0.00	3.40 ± 0.50	3.00 ± 0.50
	3.00–4.00	3.00–4.00	3.00–4.00	3.00–4.00
Pulse rate	33.55 ± 3.82	33.37 ± 4.36	36.55 ± 4.72	34.40 ± 4.46
	29.28–40.65	28.78–46.08	30.08–47.69	28.78–47.69
Dominant frequency (kHz)	6.18 ± 0.07	6.20 ± 0.06	6.10 ± 0.08	6.16 ± 0.08
	6.03–6.29	6.12–6.29	6.03–6.46	6.03–6.46

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Supplementary material 1

Summary of male advertisement call parameters of *Leptobrachella suiyangensis*, *Leptobrachella bashaensis* and 16 other species

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Exploring the dietary niche of *Atlantolacerta andreanskyi* (Lacertidae) using DNA metabarcoding

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Abstract

Determining the dietary niche is an essential part of any conservation strategy, and for modeling the community responses to climate change. DNA metabarcoding methods are revolutionizing such approaches, allowing higher taxonomic resolution than typically possible using microscopy. However, few studies have compared directly the approaches to evaluate the differences in methodology. Here we assess the dietary niches of two genetically diverse populations of *Atlantolacerta andreanskyi*, a lizard endemic to the Atlas Mountains, Morocco, using DNA metabarcoding of faecal samples, and for one of these we compared the results to two previously published assessments of diets obtained using microscopy of pellets and stomach contents respectively. While results at the Order level were similar, the higher taxonomic resolution obtained in this study provided new insights into the dietary niche of this species. Comparisons between the two populations further highlighted how ecologically distinct these are.

Key Words

16S rRNA, dDNA, lizards, metabarcoding, Morocco

Introduction

Species interactions form the backbone of any ecosystem, and as these relationships underpin food webs, it is essential to elucidate them in order to predict how ecosystems respond to disturbances. Dietary studies have traditionally relied on visual identification of prey, either from stomach contents or fecal matter, but such approaches are time consuming, require substantial taxonomic expertise – especially when dietary ranges are broad – and may fail to detect more easily digested prey (Taberlet et al. 2012). High-Throughput-Sequencing (HTS) of diet contents provides a powerful alternative tool to provide rapid assessments of large numbers of samples, as well as greater taxonomic resolution, but has its own limitations (reviewed in Sousa et al. 2019) and potential errors such as risk of contamination (Furlan et al. 2020). Nevertheless, dietary studies based on HTS are becoming routine in studies focusing on single species, as well as in broad-scale studies, aiming to illuminate levels of ecosystem resilience to climatic change (e.g. Berry et al. 2019).

In the specific case of ectothermic species, more studies providing baseline dietary data are urgently needed, for example in cases of species restricted to remote high mountain areas, where predator-prey dynamics are more susceptible to climate change (Manes et al. 2021). In lizards, few studies have employed HTS approaches for dietary assessments, despite early works demonstrating the potential

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advantages of the methodology (e.g. Brown et al. 2012). Recent appraisals of island specialists have highlighted how greater prey diversity is apparent when using HTS, due to the higher taxonomic resolution obtained, while also identifying the need for further dietary studies to evaluate across-season changes (Gil et al. 2020; Tercel et al. 2022). A recent assessment of generalist lizards from North Africa highlighted differences between results obtained from HTS and microscopy (Pereira et al. 2019), as did an assessment of a ground-dwelling gecko (Kurita and Toda 2022) but there are still too few comparative studies to generalize regarding differences between the approaches.

There are multiple reasons why dietary assessments would be particularly enlightening for *Atlantolacerta andreanskyi*, a lizard species endemic to the Atlas Mountains of Morocco (Martínez del Mármol et al. 2019). Firstly, global biodiversity assessments show there is a bias towards research on other vertebrates, such as birds, with lizards receiving disproportionately less attention. This is particularly problematic, because lizard biodiversity hotspots demonstrate limited overlap with other taxa (Roll et al. 2017), while catastrophic vertebrate population declines are driven by clusters primarily among amphibians and reptiles (Leung et al. 2020). At a more local level, climate change is predicted to particularly impact Moroccan endemic reptiles, with montane forms expected to be especially affected (Martínez-Freiría et al. 2013). Furthermore, *A. andreanskyi* appears to constitute a cryptic species complex, encompassing deeply divergent genetic lineages with major clades separated since the Miocene and multiple potential species occupying distinct montane massifs since the Plio-Pleistocene (Barata et al. 2012). Additionally, while different populations of *A. andreanskyi* are morphologically similar, some are significantly larger than others, and the degree of sexual dimorphism varies appreciably, with females typically being larger than males (Barata et al. 2015). Dietary breadth is often correlated with body size, so variation in prey diversity between populations could be associated with this aspect, even if larger lizards may optimize prey-capture strategies by targeting larger, more profitable prey items, and thus counter-intuitively decrease dietary niche breadth (Costa et al. 2008). Although dietary patterns have not been elucidated in most *A. andreanskyi* lineages, two assessments using microscopy have been performed on the population at Oukaimeden in the High Atlas Mountains, where females were found to be bigger than males (Busack 1987; Carretero et al. 2006). However, results were not consistent; Busack (1987) analyzing stomach contents reported that males consumed mainly Coleoptera (30% of dietary items identified) and females Hymenoptera (42%), Carretero et al. (2006) analyzing pellets identified primarily Hemiptera (40%) and then Coleoptera (19%) as common prey, with no differences between males or females. Thus, while in both studies sampling was carried out in the spring (May and June in Busack 1987; and April in Carretero et al. 2006), results were quite different.

In the present study we performed an assessment of the diet of *A. andreanskyi* from two locations, Oukaimeden and Ait Bouguemez, employing an HTS approach. These populations correspond to different genetic lineages that also differ in average body size (Barata et al. 2012, 2015). Our main objectives are to: 1) compare the results with those of previous diet studies based on microscopy analysis; 2) determine whether the diets of these genetic lineages differ; 3) assess diet variability across seasons; 4) test whether increased taxonomic resolution will enable us to identify dietary differences between sexes. Finally, these data will be essential for proposing conservation strategies for these endemic lizards, but will also give further baseline information regarding the prey found in these unique habitats, which are under ever-increasing anthropogenic pressures.

Methods

Eighty-five adult specimens of *A. andreanskyi* were captured by hand from two localities in the High Atlas Mountains in Morocco, 1) Ait Bouguemez (Central High Atlas; 31°45'45.8"N, 6°16'50.6"W, altitude: 2,723 m), and 2) Oukaimeden (South limit of Central High Atlas; 31°12'09.5"N, 7°51'24.4"W, altitude: 2,600 m), which belong to different lineages of the *A. andreanskyi* complex (Barata et al. 2012, where the first population is called "Jebel Azourki"). Both localities feature a mountain climate, with cold winters, and were characterized by vegetation predominantly composed of xerophilous thorny bushes, especially *Alyssum spinosum*, which provide refuge against predators for these lizards. It has been previously shown that specimens from Ait Bouguemez are significantly larger on average than those from Oukaimeden (Barata et al. 2015). Only adult specimens were considered, with those with snout-vent length (SVL) over 36 mm considered adults following Schleich et al. (1996). Sex was determined by the presence of developed femoral pores and head robustness in males (Barata et al. 2015). Faecal samples were collected by gentle massaging of the individual's abdomen when necessary, and were stored in 96% ethanol. All specimens were then released at the collection sites. The populations at Oukaimeden were sampled in both 2016 and 2017 (25 and 36 individuals respectively), while the population at Ait Bouguemez was sampled only in 2017 (24 individuals). Considering seasonal variation, Ait Bouguemez was sampled in summer and autumn, while Oukaimeden was sampled in autumn 2016, and spring and summer 2017. Full details are given in Suppl. material 1: table S1.

DNA extraction was performed in a dedicated positive controlled pressure room designed to prevent contamination. Prior to extraction, samples were dehydrated at 37 °C overnight. Extraction was performed using the PureLink^{TM} Genomic DNA kit (Thermo Fisher Scientific) following the manufacturer's protocol, but with triplication of lysis buffer and proteinase K quantities. All samples were vortexed to disrupt the faecal mass and digested overnight. Extracted DNA was stored at -20 °C prior to library preparation.

A two-step PCR (Polymerase Chain Reaction) approach was performed. For the first PCR, a 16S rRNA fragment was amplified using the primers Ins16S_1F and Ins16S_1R (Clarke et al. 2014) at 55 °C annealing temperature for 35 cycles. Platinum Taq Polymerase (Invitrogen) (2U/μL) was mixed in a 10 μL reaction volume with 0.5 μL of DNA. PCR reactions contained BSA (Bovine Serum Albumin) 25 mM and 2.5 mM $MgCl_2$ concentration. PCRs were run in triplicate for each sample with a negative control (blank). Amplified fragments were examined in a 2% agarose gel and replicate PCRs were pooled. PCR purification was performed using the Agencourt AMPure XP (Beckman Coulter) system with a proportion of 0.8 μL of magnetic beads to 1 μL of PCR product. To attach a unique tag to each sample, a second PCR was performed in a 10 μL reaction volume using the same conditions as the first PCR for 10 cycles. A final purification was performed using the ratio of 1.2 μL of beads to 1 μL of PCR. Quant-iT PicoGreen dsDNA Assay Kit (Thermo Fisher Scientific) was used to quantify amplicon concentration and all samples were normalized to 5 nM and then pooled, with 2 μL of each sample. Sequencing was outsourced to a commercial company (Genewiz, Germany) and conducted using an Illumina MiSeq sequencer, with 2×150bp paired-end configuration and ≤30% of PhiX spiked-in to increase sequencing diversity.

Samples were de-multiplexed and adaptors removed. Fastq files were analyzed using USEARCH v11.0.667 (Edgar 2010), to merge, quality filter and cluster reads into Zero-radius Operational Taxonomic Units (ZOTUs). After overall sequencing quality control, primers were removed and paired-end reads were merged using the command -fastq_mergepairs. Samples were filtered by quality with -fastq_filter, which was first set to discard reads shorter than 100 bp (Yu et al. 2012) and then for overall quality score filtering. Maximum expected sequencing errors was set to 1 due to the high quality of the sequences (Edgar and Flyvbjerg 2015), and replicated reads and singletons (unique reads present across the entire dataset) were discarded with –fastx_uniques. Unique sequences were de-noised (sequencing errors were corrected and chimeras removed) and then clustered into ZOTUs using –unoise3. Finally, -otutab was used to establish a ZOTU table showing the frequency of all ZOTUs per sample.

Sequences were compared against the GenBank database using the BLAST algorithm (Altschup et al. 1990). Sequences were identified to order, family, genus or species when similarities were ≥85%, 90%, 98%, and 99% respectively, following Pereira et al. (2019). When equal similarity to two or more taxa was found, sequences were identified to the higher taxonomic level that included both taxa. When a match-up to the order level was not found, ZOTUs were considered unidentified. Sequences that were 1) present in extraction or PCR blanks; 2) classified as the host; or 3) not considered a prey item (e.g. bacteria, fungi) were removed. Additionally, samples with fewer than 100 sequences were removed and ZOTUs representing less than 0.1% of the total number of reads of each sample were not considered for that sample (Deagle et al. 2019).

Possible differences in SVL between sexes in each population (i.e. Oukaimeden 2016; Oukaimeden 2017 and Ait Bouguemez 2017) were tested with the Kruskal-Wallis test due to the non-normality of the data. For the populations sampled in several seasons, the non-significant effect of season in SVL was first confirmed to avoid misleading results regarding differences between sexes. Differences in diet between sexes and seasons (for the samples collected in 2017) at each locality were estimated with a Permutation Multivariate Analysis of Variance (PERMANOVA) using the adonis function implemented in the vegan package in R software, Oksanen et al. 2008) and the Jaccard dissimilarity index calculated both at ZOTU and family levels. Samples from Oukaimeden 2016 were not included in direct comparisons between localities because of the possible variation in diet between 2016 and 2017 and because those samples were sequenced in a different Miseq run from the rest of the samples. Given that there were significant differences associated with season (see results), to assess differences in prey at the family level between the two populations sampled 2017, the PERMANOVA was performed with the factor season nested within locality and using the strata option for season (diet composition ~ locality/season, strata = season). All sequences were deposited in the National Center for Biotechnology Information (NCBI) Short Read Archive (PRJNA 1019328).

Results

Of the 85 samples collected, 84 gave informative sequences – 61 from Oukaimeden (25 from 2016 and 36 from 2017) and 23 from Ait Bouguemez. One sample from Ait Bouguemez was discarded because it had less than 100 sequences after filtering. Nine orders of prey were detected, Coleoptera, Lepidoptera, Hymenoptera, Hemiptera, Orthoptera, Diptera, Dermaptera, Plecoptera and Thysanoptera. Six orders were identified in both localities, while Dermaptera was only identified in Ait Bouguemez, and Plecoptera and Thysanoptera only in Oukaimeden (only in 2017) (Fig. 1). Within these orders, at least 16 prey families were identified at Ait Bouguemez, 11 in Oukaimeden 2016 and 32 in Oukaimeden 2017, but potentially more were present, since some prey items were only identified to order level (Suppl. material 1: table S2). In total, 22 prey items were identified to the species level (Suppl. material 1: table S2). There were significant differences in diet composition between individuals sampled in the two localities in 2017 when considering prey families (PERMANOVA: $F_{57}= 2.66$; $p = 0.001$). There were also differences between seasons in both localities when comparing ZOTUs, as well as seasonal differences

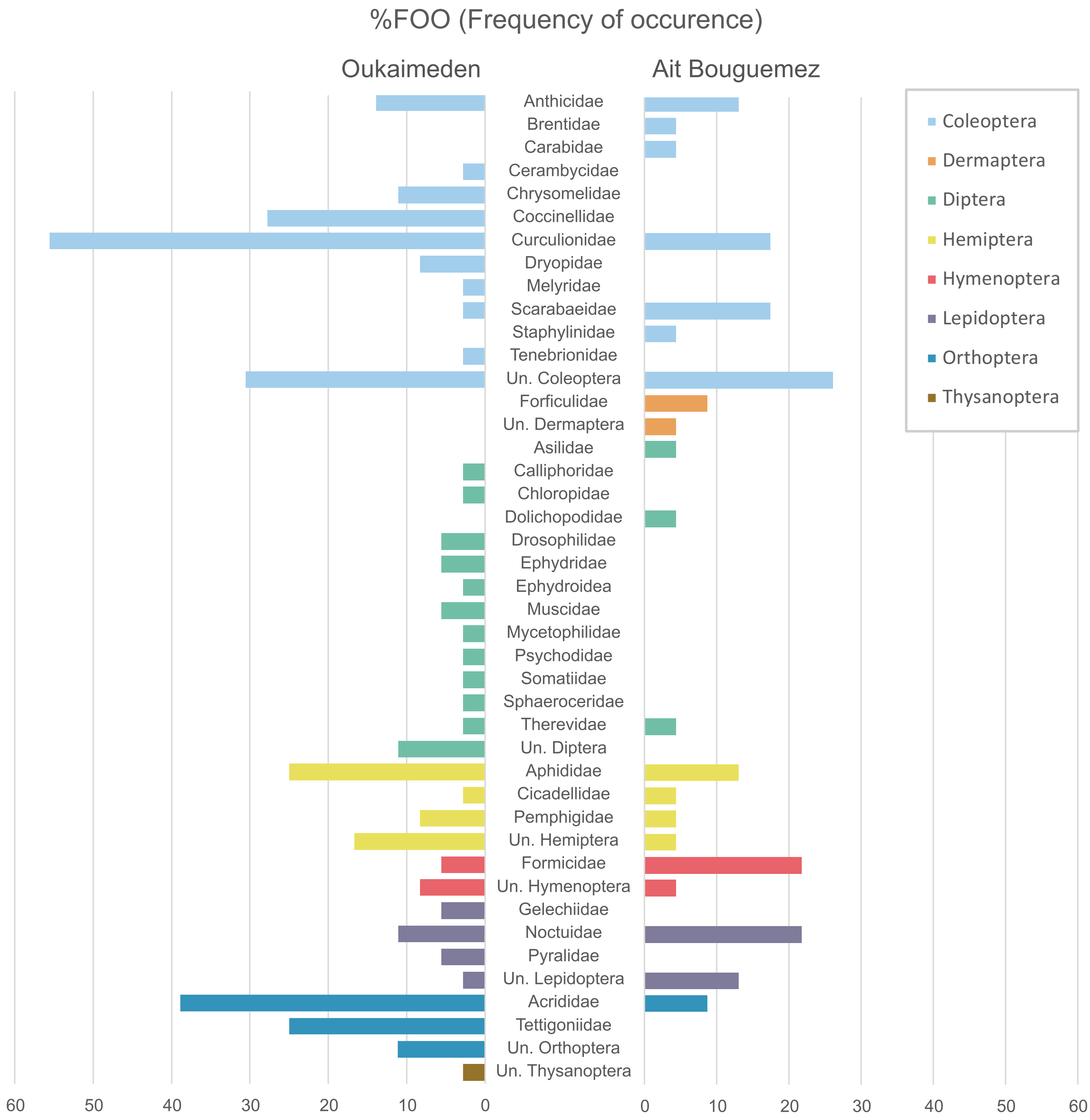

Figure 1. Comparison of the percentage of Frequency of Occurrence (%FOO – the number of samples that contain a given food item, expressed as a percentage, following Deagle et al. 2019) of prey taxonomic families between the samples collected in Oukaimeden and Ait Bouguemez in 2017. Families belonging to the same order are represented in the same color.

at the level of prey families in Ait Bouguemez (Table 1). There were no differences in diet between males and females, either when considering ZOTUs, or when comparing prey taxonomic families (Table 1). For the size comparison between males and females, SVL was obtained from 61 adults at Oukaimeden (26 females, 35 males) and 32 at Ait Bouguemez (14 females and 18 males). In both populations there was no significant difference in SVL between sexes ($\chi^2 < 3.22$; $p > 0.07$), while the population at Ait Bouguemez was substantially larger on average than the one from Oukaimeden (average SVL 48.90 mm versus 43.05 mm respectively).

Discussion

There were clear similarities in the identity of insect prey items at the order level between the earlier studies based on microscopy of pellets (Carretero et al. 2006) and stomach contents (Busack 1987) and the present study using HTS at Oukaimeden. All studies recorded Hemiptera, Lepidoptera, Coleoptera, Hymenoptera and Diptera as the prey of *A. andreanskyi*. Orthoptera were also recorded by Busack (1987) and the present study, but not in Carretero et al (2006). Busack (1987) further reported Odonata and Neuroptera, although notably these were recorded in low numbers (between 1 and 5 records each), none of

Table 1. Differences in diet composition between sexes and seasons at each locality. Differences in SVL between sexes were tested with a linear model (significance obtained with ANOVA). Differences in diet composition were estimated with a PERMANOVA of the Jaccard dissimilarity index with the presence/absence matrix of each OTU and also with the presence/absence matrix of prey families. Significant values are in bold.

		Oukaimeden 2016	**Oukaimeden 2017**	**Ait Bouguemez 2017**
		χ^2/F (p-value)	**χ^2/F (p-value)**	**χ^2/F (p-value)**
SVL	Sex	3.22 (0.07)	0.07 (0.79)	0.92 (0.34)
Diet (ZOTUs)	Sex	1.08 (0.32)	1.14 (0.11)	0.90 (0.69)
	Season	–	**1.46 (0.003)**	**1.62 (0.005)**
	Sex*Season	–	0.94 (0.68)	1.04 (0.36)
Diet (prey families)	Sex	1.41 (0.14)	0.83 (0.67)	0.54 (0.93)
	Season	–	1.28 (0.17)	**2.90 (0.002)**
	Sex*Season	–	0.91 (0.58)	0.41 (0.99)

which were detected in the present study, while Thysanoptera was only recorded in the present study. Most of these differences are unlikely to represent methodological artifacts, since for example Carretero et al. (2006) identified Orthoptera in pellets of *Podarcis vaucheri* from Oukaimeden but these were absent from the pellets of *A. andreanskyi*. Rather, given the low numbers of prey items from these orders, dissimilarities likely reflect differences in prey availability or sampling effects. The percentage of occurrence of prey items from the different orders varied greatly between studies, with Busack (1987) identifying predominantly Hymenoptera (particularly in females), Carretero et al. (2006) Hemiptera (within the suborder Homoptera), and in this study the order with the greatest number of ZOTUs identified was Coleoptera. Again, these differences may reflect seasonal differences in availability of prey, this being supported by the significant variation in prey item ZOTUs found across seasons in both populations examined in the present study. On the other hand, the presence of thrips (Thysanoptera) and aphids (Hemiptera) could potentially be due to secondary predation, since ladybirds (Coccinellidae) – which were also detected – are known to feed on them. Identification of DNA via secondary predation has been demonstrated experimentally in dietary metabarcoding studies, but it is difficult to set rules that are not arbitrary for eliminating this potential source of detected DNA (Ando et al. 2020). In this case, many thrips and aphids are large enough to have been preyed on directly by lizards, but still small enough to have possibly been overlooked in previous diet assessments based on microscopy. However, based on the current evidence, the identification of such DNA in the pellets due to secondary predation cannot be excluded, and the possible inflationary effect on the proposed diet should not be disregarded.

While two previous dietary assessments of *A. andreanskyi* have been performed at Oukaimeden, the present study is the first to assess the population at Ait Bouguemez. At the order level the results are very similar to those from Oukaimeden, with six orders found in both populations. Only Plecoptera and Thysanoptera were found at Oukaimeden but not at Ait Bouguemez, and Dermaptera at Ait Bouguemez but not at Oukaimeden. However, the composition of diets at the family level was significantly different between the populations, with prey items belonging to 32 families identified in Oukaimeden 2017, and only 21 at Ait Bouguemez, despite the specimens from the latter population being larger on average. It is clear that the dietary niches are different between these two - genetically distinct but morphologically similar - populations of *A. andreanskyi*. Furthermore, these results highlight again how the higher taxonomic resolution achieved using a DNA metabarcoding approach relative to microscopy analysis allows additional inferences to be made. However, the notable differences between the results from 2016 and 2017 from Oukaimeden highlight the effects of season and the need for caution when interpreting differences between populations.

Interestingly, we found no difference in SVL between males and females in either population, or any significant differences in diet. In the assessment of Carretero et al. (2006) at Oukaimeden, females were larger than males although the significance level was marginal (Student T-test, t_{23}=-2.14, p=0.04), while Busack (1987) reported similar average SVLs for males and females, although the maximum size of females was larger. Thus, it seems that these populations have minimal sexual dimorphism, and this is reflected by similarity in the diet of both sexes. Other populations of *A. andreanskyi*, such as those at Jbel Ayache in the Middle Atlas range, have much higher levels of sexual dimorphism (Barata et al. 2015). Hence, it would be useful to assess diet variation in these other populations, both to confirm that dietary niches change between genetic lineages, but also to determine how sexual dimorphism and diet are related.

The use of dietary DNA as part of biodiversity assessments of prey items has been proposed before, especially in the case of remote and inaccessible regions (Boyer et al. 2015; Sousa et al. 2019). The primers used herein enable the identification of various insects to the species level (Pereira et al. 2019, 2021) allowing data collection on the distributions of these prey items. The data provided by the present study can be useful in this regard as well. For example, the cross-backed grasshopper of the genus *Dociostaurus* includes various cryptic species, so the identification of *D. jagoi* in Oukaimeden adds to the understanding of the distribution of these species (González-Serna et al. 2017).

Our findings provide important information for conservation of the focal lizard. *Atlantolacerta andreanskyi* is the only representative of its genus, and considered as Near Threatened in the Red List of IUCN due to its high habitat specificity and the small and fragmented geographic range (Geniez 2006). Furthermore, since the genetic lineages are potentially distinct species, some could be considered endangered due to the small known ranges. The observed niche conservatism in the thermal ecophysiology of the species complex reduces the expected evolutionary response to fast environmental shifts (S'khifa et al. 2022), and these authors highlight how a nuanced approach including water availability and microhabitat will be needed to better model the impact of climate change. Our data, which highlight the dietary differences between populations, also underline the complexity of the relevant food web dynamics, that in a changing environment need to be understood for effective management and conservation (Roslin and Majaneva 2016).

Dietary studies are inevitably point estimates of a more complex continuum, varying with season and size of the organisms. It is therefore not surprising that different studies made at different times will produce somewhat different results. This work highlights how, in *A. andreanskyi*, diet varies extensively along the year, and between years, likely reflecting prey availability, and the same is almost certainly true for other lizards from these localities. Dietary niche also differed between the two populations, despite both being high montane habitats, separated by only a little over 50km in a straight line. This study provides additional evidence for the cryptic differences within this apparent species complex, and with recent assessments of ecophysiological differences (S'Khifa et al. 2022) demonstrate how distinct these populations actually are. Assessment of additional populations, and years, is needed to determine how much these reflect consistent differences. On the other hand, even with greater taxonomic resolution of prey items, differences between sexes were not identified. Finally, the study demonstrates again how the identification of the prey items can be useful not just for determining the diet of these endemic lizards, but also for assessing the distribution of the insect prey species themselves.

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Supplementary material 1

Information for the samples and prey item identification

Authors: Abderrahim S'Khifa, Ana Pereira, Mohamed Amine Samlali, Tahar Slimani, D. James Harris, Raquel Xavier

Data type: docx

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Notes on the pattern of head size in the Philippine Spotted Flying Lizard, *Draco spilopterus* (Weigmann, 1834), within Luzon Biogeographic Region, Philippines

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https://zoobank.org/1F062A82-B948-4B08-813F-F8398D676D14

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Abstract

Sexual head dimorphism is a condition where head morphology of male and female of the same species differ, which provides an additional way to recognise sex for species identification. Fifty four adult Philippine Spotted Flying Lizards, *Draco spilopterus*, were examined from four populations in the Luzon Island, Philippines to assess whether head morphometry can drive sexual dimorphism. We identified the sex and measured five morphometric measurements of the head (jaw length, jaw width, head height, head length, head width) and snout-vent length. A univariate analysis of the five morphometric measurements showed clear sexual dimorphism. Considering all individuals, female heads are significantly larger than those of males (all p-values < 0.05). We also revealed major differences in head size amongst the four populations due to their geographical location and habitat differences.

Key Words

head size, flying lizard, Luzon Island, morphological variation, reptile

Introduction

In the traditional taxonomic classification of vertebrates, morphological characteristics are widely used for species identification. In lizards, morphological traits, associated with performance and dominance in competition and territoriality, are well-characterised (Wegener et al. 2019). For example, larger males of *Crotaphytus collaris* and *Anolis carolinensis* tend to be dominant over smaller ones and occupy larger territories (Jenssen et al. 2005; Lappin and Husak 2005). When males encounter equally-sized males of *Anolis cristatellus*, head shape and body size are the key predictors of the dominant behaviour (Perry et al. 2004).

Moreover, morphological traits are also examined to determine possible differences between males and females of species (sexual dimorphism), including the body size and shape, skin colouration and scale patterns (Cooper and Greenberg 1992; Adriana et al. 2005). Sexual dimorphism is commonly found within iguanid and agamid lizards (Stamps 1993). In some agamid *Draco* species, sexual dimorphism has been identified in dewlap size, patagial colouration and body size, wherein males have brighter and longer dewlaps, brighter and larger patagiums than females (Muster 1983; Mori and Hikida 1994; Shine et al. 1998), while females usually exhibit larger body size (Mori and Hikida 1994; McGuire and Alcala 2000). In contrast, Srichairat et al. (2016) revealed that males of *Draco maculatus* are larger than females with males having elongated and pointed dewlap. These two phenomena in sexual dimorphism can be attributed to varying levels of sexual differences related to evolutionary adaptations, such as sexual selection and natural selection (Stuart-Fox and Ord 2004; Srichairat et al. 2016).

More specifically, sexual head dimorphism is a condition where head morphology of male and female of the same species differ. Several studies were conducted in lizards to assess this condition (e.g. Scharf and Meiri 2013; Meyer et al. 2019; Cruz-Elizalde et al. 2020; Wang et al. 2020; Liang et al. 2022). Such research is limited in agamid *Draco* species (e.g. Srichairat et al. 2016), particularly to those that are endemic and widely distributed to the Philippines. Although the Philippine Spotted Flying Lizard (*Draco spilopterus*) has the widest geographic distribution, occurring on the Philippine Islands of Luzon, Marinduque, Polillo, Negros, Panay and Tablas (McGuire and Alcala 2000), little is known about potential sexual dimorphism within the species and amongst populations. Thus, the purpose of this study was to evaluate the head morphometric differences between males and females of *D. spilopterus* in the Island of Luzon in order to gain insight into their foraging behaviour and dietary pattern. Different populations of the flying lizard were also compared using the head morphometry as key predictor.

Materials and methods

Study species

The flying lizards of the genus *Draco* Linnaeus, 1758 (Family Agamidae, Subfamily Draconinae) are a unique group of agamid lizards, well-known for their ability to glide, utilising wing-like patagial membrane attached to their specialised thoracic ribs (McGuire and Alcala 2000; McGuire and Heang 2001; McGuire and Dudley 2011). *Draco spilopterus* can be diagnosed from other congenerics in the Philippines by unique characteristics, such as lateral orientation of the nostrils, large size with a maximum snout-vent lengths of 85 mm and 97 mm in males and females, respectively, dorsal scales that are variable in size and often keeled or rugose, six ribs for patagial support, absence of lacrimal bone, absence of black postrictal ocellus, males with orange-yellow dorsal patagium and females with dark brown dorsal patagium with pale yellow mottling, ventral surface of patagium yellow for males, ventral surface of patagium brown for females, males with triangular lemon-yellow dewlap and both sexes having pale brown dorsal colouration (McGuire and Alcala 2000). However, there were no data on head morphometry that can be used in the diagnosis of *D. spilopterus* and in identifying the sex. Meanwhile, *D. spilopterus* feeds predominantly on a variety of insects and non-chitinous invertebrates, particularly ants, insect larvae and termites (Tabug et al. 2018; Tabug et al. 2020) which may drive variation in head morphometry.

Study sites

We conducted field sampling on Luzon Island, within the eponymous faunal region, a major biogeographic region in the Philippines, one of the centres of endemism in the country and amongst the oldest and the largest oceanic regions of the Philippine Archipelago. Luzon Island has an area of 104,688 km^2 and a roughly rectangular shape orientated in its longest axis north to south from 18°32'N to 12°31'N (Salita 1974; Vallejo 2014) (Fig. 1). This Island had a complex geological history and is considered as a product of the accretion of four paleo-islands (major mountains) during the Pleistocene (Hashimoto 1981; Auffenberg 1988; Hall 1998; Vallejo 2014). To the north are the Northern Sierra Madre and Central Cordillera mountain ranges, with Cagayan Valley situated in between and to the south and south-east lie the Southern Sierra Madre and Bicol Peninsula trending southeast for about 150 km and to the west large central plains and Zambales mountain ranges. Luzon Island has been surrounded by deep water and was never connected to other islands (Heaney et al. 2016). Due to a high degree of endemism across taxa, Luzon has been extensively studied for mammals (Heaney et al. 2016) and herpetofauna (Brown 1996; Diesmos 1998; Brown et al. 2012; Brown et al. 2013) and patterns of island biogeography (e.g. Brown and Alcala 1970; Heaney 2000). In addition, Luzon was suggested to have multiple lineages of widely-distributed flying lizards due to its geographic features and history (McGuire and Alcala 2000).

We selected four sites, based on museum locality data and published articles on the distribution of the Philippine Spotted Flying Lizard on Luzon (Brown 1996; Diesmos 1998; Brown et al. 2012; Brown et al. 2013). These sites were Ilocos, Laguna, Cavite and Bicol and were represented by their localities, namely Ilocos Norte Watershed, Mt. Banahaw, Mt. Palay-palay and Tapayas, respectively (Fig. 1).

Ilocos Norte Watershed (18°22'18"N, 120°38'49"E, datum WGS 84, 365 m elevation) lies at the northern edge of the Cordillera Central mountain range. This area is a human-modified environment dominated by commercially-important trees, such as mango, manila palm, coconut, star apple, gmelina tree and shrubs.

Mt. Banahaw (14°7'23"N, 121°28'8"E, datum WGS 84, 447 m elevation) is located in the southern part of Luzon Central Valley Basin. The Basin was formed between Southern Sierra Madre and Zambales Range which is composed of volcanic materials and sediments (Ku et al. 2009). In this area, *Draco* samples were collected within Barangay Bukal, Nagcarlan, Laguna. This barangay is dominated by Lanzones trees, coconuts, shrubs, mahogany and cultivated crops (banana). Ground cover included tall grasses, small ferns and leaf litters.

Mt. Palay-palay (14°13'54"N, 120°39'26"E, datum WGS 84, 311 m elevation) is situated within the Municipality of Nasugbu Batangas Province and Maragondon and Ternate, Cavite Province in a 4,000–hectare mountain

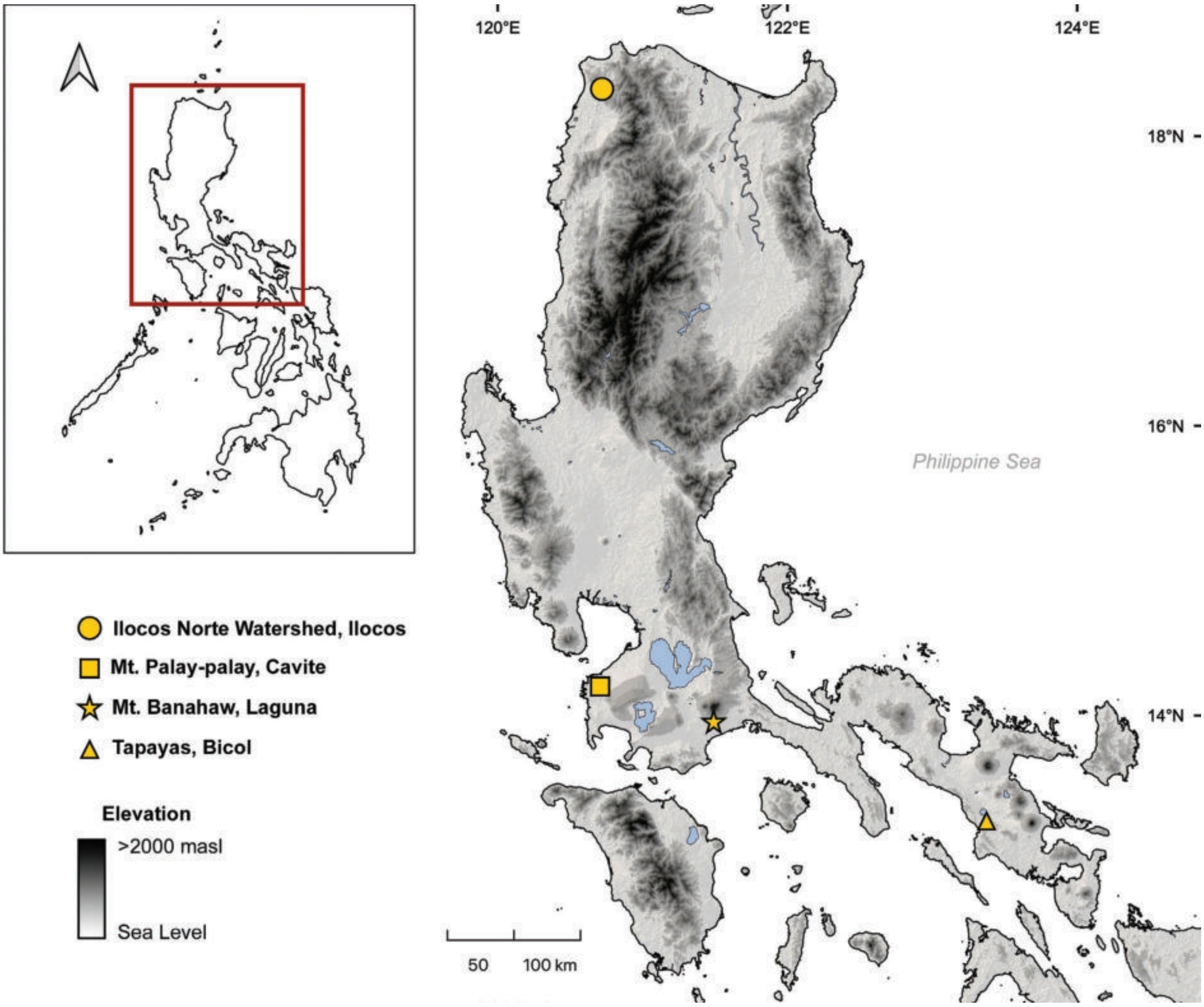

Figure 1. Sampling sites for *Draco spilopterus* on Luzon Island and general location of the island (mapped by YLC del Prado).

range (Luyon and Salibay 2007). The *Draco* samples were collected along the trails which are best-conserved forest. Bamboos are common along with the different trees, shrubs, leaf litters and fallen logs.

Tapayas (13°22'36"N, 123°16'8"E, datum WGS 84, 48 m elevation) is a village in the Municipality of Balatan, Camarines Sur Province in Bicol Peninsula. This area is located beside the rice field and creek and dominated by coconut trees. Mahogany and mango trees were also common along with small shrubs.

Sampling

We collected 54 adult individuals of *D. spilopterus* from four sites between June 2017 and March 2019. Collection of specimens was done 0700–1100 hr and 1400–1700 hr, which were the observed optimum periods of their foraging activity. In each study site, flying lizards were located by carefully searching each tree with the aid of binoculars (Mori and Hikida 1994). In capturing specimens, the technique described by Alcala (1967) and Mori and Hikida (1994) was adapted wherein a 7-m long pole with a pliable tip was used to tap on the tree trunk at a point just above the perched lizard, in order to induce them to glide down either to the ground or to more accessible parts of trees for capture. Alternatively, a blowpipe loaded with plastic pellets was also used to catch flying lizards. We euthanised the specimens by immersion in dissolved chloretone (McDiarmid 2011) immediately after capture. The following characters of the collected specimens were measured using a digital caliper (to the nearest 0.1 mm): snout-vent length (SVL; measured from the snout to the vent, jaw length (JL; measured from the back of the retro-articular process to the tip of the lower jaw), jaw width (JW; measured at the widest point of the jaw), head width (HW; measured at the widest part of the head), head length (HL; measured from the back of the parietal bone to the tip of the snout) and head height (HH; measured at the highest point of the head) (Fig. 2). We identified sex, based on the displayed patagial colouration on the live individuals and the dewlap: triangular lemon-yellow in males or small dark brown in females (Inger 1983; Musters 1983). The specimens were then fixed in 10% formalin and later preserved in 70% ethanol for long-term storage. All specimens (USTHRC vouchers ## 1311–1325,

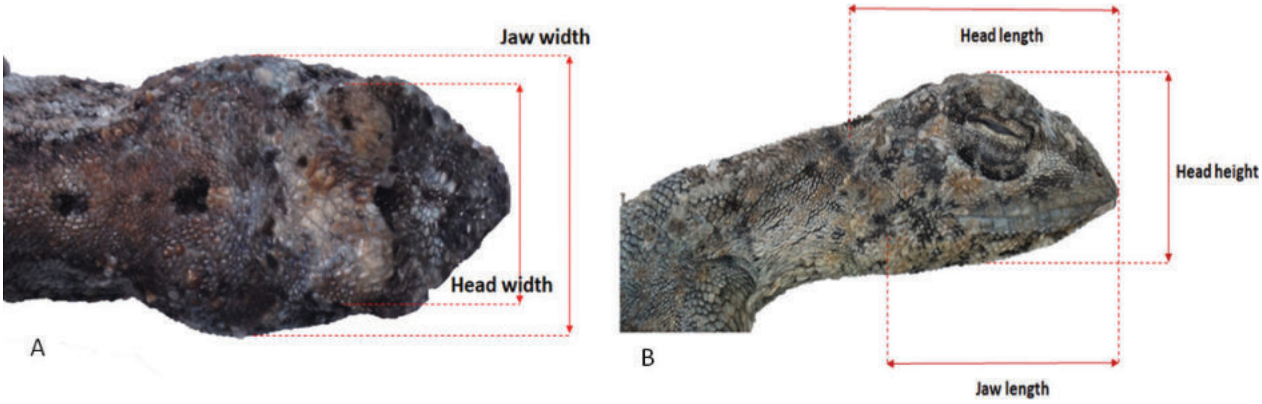

Figure 2. (**A**) Dorsal and (**B**) lateral view of the head of *Draco spilopterus* (Photo: LV Necesito).

1327, 1328, 1330–1366) were deposited at the Terrestrial Laboratory 707 of the Department of Biological Sciences at the University of Santo Tomas, Manila.

Head morphometric analysis

The SVL and head size variability between sexes and amongst *D. spilopterus* populations were quantified using morphometric characters. Log-transformed snout-vent length and head size variables were compared between sexes using t-test. One-way Analysis of Covariance (ANCOVA) was performed using log SVL (covariate) and log-transformed head-size variables (dependent variable) to determine the shared allometries amongst populations. When there is violation in the homogeneity of slope ($p < 0.05$), that is, differences in the rate of growth are detected, head size variables were size-corrected to standardise each individual to the same SVL and adjust measurements amongst populations using the technique of normalisation by Thorpe and Leonart (Thorpe 1976; Leonart et al. 2000) with the formula: $Y^{*}=Yi(SVLm/SVLi)^{b}$, where *Y** is the size–corrected morphological features of the individual *i*, *Yi* is the individual measurement of the morphological feature *Y*, *SVLm* is the mean of snout-vent length of the population to which the individual *i* belongs, *SVLi* is the snout-vent length of the individual *i* and *b* is the slope of the linear regression between *SVL* and the morphological feature *Y* in the population (Van Kleeck et al. 2015). Principal component analysis in Paleontological Statistics (PAST) version 4.03 (Hammer et al. 2001) was performed on log-transformed size-corrected features to determine variables that contribute most to head size variation. Two-way ANOVAs were performed on principal component scores with highest eigenvalues to determine differences in head size in locality or sex. To further analyse the detected differences, Dunn's post-hoc tests were used. We did linear regression analyses to each of the head measurement variables with snout-vent length (log-transformed).

Results

Sampled *D. spilopterus* varied in size from 55.16 mm to 98.31 mm SVL. Morphometric variation presented in Table 1 provides the range and mean of each population. The population from Laguna exhibited the largest body size. Ilocos population showed wider head dimensions both in males and females, while the Cavite population had the smallest body size and head dimension.

Table 1. Morphometric variation (mm) in mean and standard deviation of the SVL and head dimensions in each site. t-Test and ANCOVA (with SVL as covariate) of head dimension between sexes (pooled data of all sites). P-values with asterisk (*) are significant. Snout-Vent Length (SVL), Jaw Length (JL), Jaw Width (JW), Head Length (HL), Head Width (HW), Head Height (HH). Numbers in brackets indicate sample size.

	Ilocos		**Laguna**		**Cavite**		**Bicol**		**Overall**		**t test**	**ANCOVA**
	Male (6)	**Female (3)**	**Male (11)**	**Female (4)**	**Male (15)**	**Female (3)**	**Male (8)**	**Female (4)**	**Male (40)**	**Female (15)**		
SVL	75.9±10.7	80.89±5.37	81.85±8.86	83.86±15.74	71.32±5.03	66.78±2.84	71.89±4.01	75.62±10.44	75.01±1.18	77.65±3.08	0.148	-
	55.16–84.22	74.69–84.23	73.94–92.27	59.9–98.31	58.23–78.19	64.65–70.00	65.19–77.56	62.30–86.60	55.16–92.27	59.9–98.31		
JL	15.19±1.31	16.29±2.16	14.99±2.22	17.05±3.33	14.40±1.08	14.33±1.01	13.86±1.36	15.29±2.19	14.69±0.23	15.88±0.64	0.002*	0.031*
	13.36–16.82	14.99–18.74	12.89–19.40	16.46–21.03	11.90–16.44	13.17–15.02	12.53–16.47	12.44–17.31	11.9–19.40	12.18–21.03		
JW	9.71±0.21	12.08±0.99	9.46±1.50	11.34±2.61	8.81±0.86	9.64±0.61	8.93±0.38	10.29±1.92	9.22±0.16	10.87±0.50	0.002*	0.001*
	8.38–10.65	10.94–12.70	8.67–12.90	10.01–13.66	6.96–10.00	9.07–10.28	8.24–9.41	7.78–11.80	6.96–12.92	7.56–13.69		
HL	13.36±1.25	15.04±1.39	14.67±1.55	16.35±2.89	13.82±0.82	13.15±0.40	13.23±1.07	14.46±2.86	13.94±0.19	14.94±0.63	0.017*	0.046*
	12.08–15.5	13.46–16.07	12.97–18.02	15.82–19.44	11.90–15.66	12.71–13.50	11.62–14.87	11.91–17.65	11.65–18.02	11.91–19.44		
HW	10.06±0.53	11.92±1.39	10.07–0.96	11.08±1.13	9.18±0.83	9.67±0.51	9.06±0.74	9.28±1.40	9.59±0.14	10.49±0.39	0.008*	0.009*
	9.34–10.95	10.88–13.50	9.66–11.84	10.25–12.50	8.01–11.09	9.26–10.24	7.99–10.17	7.53–10.9	7.99–11.84	7.53–13.5		
HH	8.42±0.56	9.66±1.03	8.58±0.95	9.14±1.63	7.97±0.90	8.08±0.60	7.68±0.47	8.29±0.60	8.17±0.14	8.81±0.31	0.015*	0.049*
	7.79–9.03	8.90–10.83	7.14–10.15	7.93–11.19	6.30–9.75	7.56–8.73	6.84–8.18	7.46–8.89	6.30–10.15	7.43–11.19		

Snout-vent length was not significantly different between sexes, pooled for all individuals (t-test: P = 0.148, df = 1). However, all head measurements in females was significantly larger than in males (t-test). ANCOVA, with SVL as covariate, supports these findings (all P-values < 0.05; Table 1).

Regression analyses of the head dimension variables strongly correlated with snout-vent length (log-transformed, all P < 0.001) (Fig. 3, Suppl. material 1). ANCOVAs revealed differences in the rate of growth in jaw length amongst populations, as indicated in lack of homogeneity in the slope (P < 0.05, F = 47.04, df = 3), which requires the size-correction method to normalise the variables.

The size-corrected log-transformed data were analysed using PCA to determine the sources of variation. Five principal components were generated, three of which (PC1, PC2, PC3) accounted for 92.9% of the total variation (Table 2). PC4 and PC5 were also included to ensure that results presented are conservative with the analyses, even though they made up 4.2% and 2.9% of the total variation. However, two-way ANOVA revealed that PC4 and PC5 were not statistically significant amongst populations and between sexes and no interaction was detected (all P-values > 0.05; Suppl. material 2). Of these, the first three components were used and discussed.

Table 2. Results of the Principal Component Analysis on morphological variables (adjusted for SVL) of *Draco spilopterus* from populations in Luzon Region. Values in bold are the morphological gradients that highly contribute to the percentage of variance.

Variables	PC1	PC2	PC3
Jaw length	0.357	0.169	**0.521**
Jaw width	**0.710**	**-0.638**	-0.243
Head length	0.303	0.069	**0.696**
Head width	**0.389**	0.360	-0.349
Head height	0.354	**0.656**	-0.252
% of Variance	**71.9**	**11.3**	**9.7**

The first component (71.9% of variance, all positive loadings) describes a morphological gradient, based on jaw width and head width (Fig. 4; Table 2). The second component (11.3% of variance) describes head height and jaw width. The third component (9.7% of variance) describes a gradient, based on jaw length and head length. A two-way ANOVA on scores of the first three principal components revealed significant

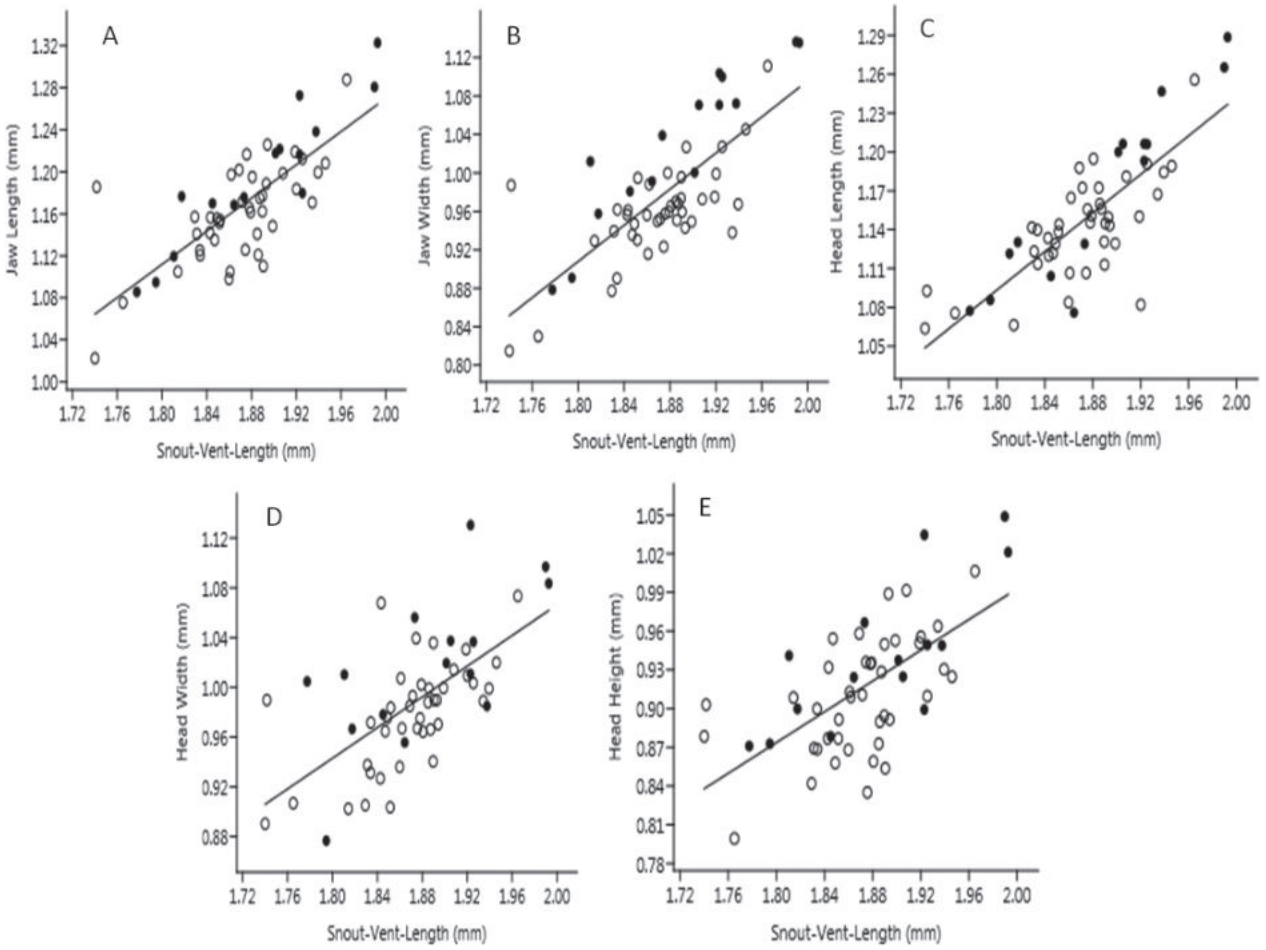

Figure 3. Dispersion diagrams from linear (Pearson's) correlations between **A.** Snout-vent length and jaw length (r = 0.776, P < 0.0001); **B.** Snout-vent length and jaw width (r = 0.750, P < 0.0001); **C.** Snout-vent length and head length (r = 0.797, P < 0.0001); **D.** Snout-vent length and head width (r = 0.644, P < 0.0001); **E.** Snout-vent length and head height (r = 0.639, P < 0.0001), of male (white dot) and female (black dot) of *Draco spilopterus*.

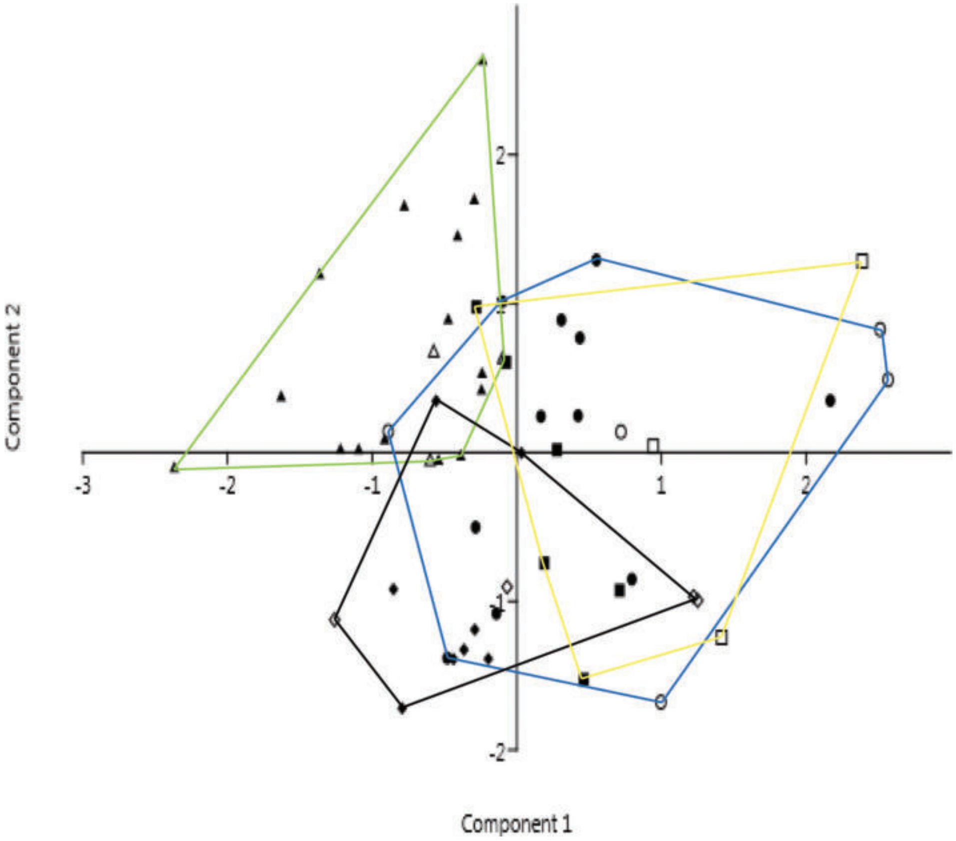

Figure 4. Principal Component Analysis scatter plot of head dimensions of *D. spilopterus* from four populations. (black dot) Mt. Banahaw, Laguna; (black diamond) Tapayas, Bicol; (black square) Ilocos Norte Watershed, Ilocos; (black triangle) Mt. Palay-palay, Cavite.

differences amongst populations (all P-values < 0.05; Suppl. material 2). Dunn's post-hoc tests were applied to determine the extent of the variation on each PC score. Ilocos and Laguna populations had higher PC1 scores than flying lizards from Cavite and Bicol (post-hoc P-values < 0.05; Suppl. material 3). Individuals from Bicol had lower PC2 scores than those from the other populations (post-hoc P-values < 0.05; Suppl. material 3). Populations from Cavite and Ilocos had the highest and lowest PC3 scores, respectively. Moreover, variation has been observed in plots of principal components (Fig. 4).

Discussion

In most agamid species, females have smaller body and head size than males, even when corrected for body size (Lappin et al. 2006). This was supported in *Draco maculatus*, wherein males were larger than females, based on the snout-vent length, head length, head depth, mouth length and dewlap length (Srichairat et al. 2016). However, our results revealed that males have smaller head size than females for *D. spilopterus*. Shine et al. (1998) emphasised that the small head size of male *Draco* lizards is an adaptive character related to the maximal extension of the large dewlap used for social displays. Moreover, Mori and Hikida (1994) and Shine et al. (1998) hypothesised that the larger body and head size of female *Draco* lizards is due to social and environmental interactions, such as less requirement for higher activity temperature, no territorial display and decreased gliding manoeuvrability.

Geographic variation in body size and head dimension in *Draco* has not been widely explored. As suggested by our results, even geographically close populations of a widespread lizard, *D. spilopterus*, can still vary morphologically. For instance, lizards from Cavite and Laguna, which are both located in south-central Luzon, show significant differences in head dimensions. Most interestingly, the lizards from Ilocos and Laguna, which are geographically distant populations, exhibit no significant differences in head dimension. Possibly, since the body size of lizards is tightly linked to their ecology (Miles 1994), the head variation amongst populations may be due to the effect of environmental factors that include diet composition, food availability and vegetation type (Boback 2006; Liang et al. 2022). The morphometric data presented in the study could be used as a supporting tool in studying other species of the remarkable group of flying lizards in the Philippines.

To further understand the sexual head dimorphism in *D. spilopterus* and head morphometric variation amongst its populations, we recommend an extensive assessment on the aspects of foraging behaviour, prey composition and size (e.g. natural selection) and bite force (e.g. sexual selection).

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Supplementary material 1

Graphs of ANCOVA

Authors: Michael A. Tabug, Levy V. Necesito, Arvin C. Diesmos

Data type: TIF file

Link: https://doi.org/10.3897/herpetozoa.36.e90412.suppl1

Supplementary material 2

Two-way analysis of variance of principal component scores

Authors: Michael A. Tabug, Levy V. Necesito, Arvin C. Diesmos

Data type: excel file

Link: https://doi.org/10.3897/herpetozoa.36.e90412.suppl2

Supplementary material 3

Dunn's Post Hoc of PC1, PC2 and PC3

Authors: Michael A. Tabug, Levy V. Necesito, Arvin C. Diesmos

Data type: excel file

Link: https://doi.org/10.3897/herpetozoa.36.e90412.suppl3

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Neotype designation and redescription of *Gloydius shedaoensis qianshanensis* Li, 1999 (Squamata, Serpentes, Viperidae)

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Abstract

The taxonomic status of *Gloydius shedaoensis qianshanensis* Li, 1999 has long been debated due to its narrow distribution range, low population, loss of type specimens, and lack of detailed descriptions. Neotypes were designated based on newly collected specimens in the Qianshan Mountain area, detailed comparisons between *G. s. shedaoensis*, and *G. s. qianshanensis* were recorded, while we redescribed *G. s. qianshanensis*. PCA results based on the external morphology of the two subspecies of *G. shedaoensis* demonstrate the inter-population distinction of *G. s. shedaoensis* and *G. s. qianshanensis*, including head length, and dorsoventral-postorbital stripe width. Additionally, the ecological data of *G. s. qianshanensis* were recorded as supplements while providing further conservation strategies.

Key Words

Gloydius shedaoensis, Liaodong Peninsula, Pit viper, Qianshan Mountain, Snake Island of China, subspecies

Introduction

Pallas (1776) first reported the Asian pit viper under the name *Coluber halys* Pallas, 1776. Later placed in the genus *Agkistrodon* Palisot de Beauvois, 1799. Hoge and Romano-Hoge (1978/1979 “1981”) distinguished the Asian species of pit vipers from *Agkistrodon* on the basis of morphological characteristics including bones, and scales, and described the new genus *Gloydius* Hoge & Romano Hoge, 1978. The genus *Gloydius* contains 24 known species and is mainly distributed in Asia. Based on morphological characters, the genus *Gloydius* contains three clades: the *G. blomhoffii* group, *G. halys-intermedius* group, and *G. strauchi* group (Orlov and Barabanov 1999; Zhao 2006; Shi et al. 2017, 2018, 2021).

Zhao (1979, 1980) described *Agkistrodon shedaoensis* Zhao, 1979 based on the ecological and morphological characteristics of the pit vipers from Snake Island,

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[*] These authors contributed equally to this work.

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Liaoning Province, China. This perspective was accepted by the following studies (Guo et al. 1999; Li 1999; Shi et al. 2016). Ji et al. (1987) first reported a pit viper species population distributed in the Qianshan Mountain Range of the Liaodong Peninsula with morphological similarities to *A. shedaoensis*, and placed it in the subspecific status of *A. saxatilis* (Emelianov, 1937) as *A. s. shedaoensis* Ji, 1987. With morphological comparisons and snake venom electrophoresis, Li (1999) considered the population from Qianshan Mountain as a subspecies of *Gloydius shedaoensis*, with the nomenclature *G. s. qianshanensis* Li, 1999. However, Li's taxonomic perspective was not accepted widely by peers. For example, the subspecific divergence of *G. shedaoensis* was not mentioned in Zhao (2006), but its geographical distribution was recorded as "The Snake Island near Lushun, Dalian; ? Qianshan Mountain, Anshan City". Shi et al. (2016) confirmed the validity of *G. s. qianshanensis* based on a sample from Wafangdian City, Liaodong Peninsula through morphological comparison and molecular systematics. Wen et al. (2021) considered the distribution of *G. shedaoensis* should not be limited to Snake Island, but the record of the *G. shedaoensis* population from the Liaodong Peninsula remains cryptic.

The taxonomic status of *Gloydius shedaoensis qianshanensis* is being debated due to the lack of detailed descriptions and image documents in the original description (Li 1999), and the whole type series are lost. Therefore, we collected new specimens of *G. s. qianshanensis* in Liaoyang City, Anshan City, and Wafangdian City of Liaodong Peninsula during field surveys. Based on the original description of *G. s. qianshanensis*, this study designates a neotype and re-describes *G. s. qianshanensis*.

Materials and methods

Sampling

Twenty-one specimens were collected in Liaoyang City, Anshan City, and Wafangdian City, Liaoning Province. After euthanasia, liver tissues were extracted from the specimens and preserved in 95% ethanol. All specimens were fixed in 75% ethanol and deposited in the Institute of Herpetology, Shenyang Normal University (SYNU).

Morphological analysis

Twenty samples of adult *Gloydius shedaoensis qianshanensis* were measured. The measurement includes a total of 16 morphological characteristics: Snout-vent length (SVL), tail length (TL), and total length (TTL) were measured to the nearest 1 mm. Other morphological measurements were examined with Vernier calipers to the nearest 0.1 mm: head length (HL, tip of snout to posterior margin of mandible), head width (HW, maximum head width), head depth (HD, maximum head depth), eye diameter (ED, measured as a horizontal distance), interorbital space (IOS, distance between the top margin of eyes), internasal space (INS, distance between nostrils), postorbital stripe width (PSW, postorbital stripe width on the largest temporal), temporal height (TH, distance between the upper and lower horn of the largest temporal) (Fig. 1). The numbers of supralabials (SPL), infralabials (IFL), dorsal scales (DS), ventral scales (V, including preventral scales), and subcaudal scales (SC) were counted (Table 1).

In addition, TTL, HL, HW, HD, ED, IOS, INS, PSW, and TH of 15 *Gloydius shedaoensis qianshanensis*, 11 *G. intermedius*, and 14 *G. changdaoensis* were measured (Table 2), and the difference between them and *G. s. qianshanensis* was analyzed by principal component analysis (PCA). The PCA is performed in the "prcomp" package in R 4.2.2.

CT-scanning and three-dimensional reconstructions

The CT scanning was carried out with Nano-computerized tomography. Specimens were scanned using a GE v|tome|x m dual tube 300/ 180 kV system in IVPP, CAS. The specimens were scanned with an energy beam of 80 kV and a flux of 80× µA using a 360◦ rotation and then reconstructed into the 4096 × 4096 matrices of 1536 slices. The final CT reconstructed skull images were exported with a minimum resolution of 6.10 µm. The skull images were exported from the virtual 3D model which was reconstructed by Volume Graphics Studio 3.4 (Volume Graphics GmbH, 2017). The dataset of the 3D models included in this study is available online in the repository (ADMorph, Shi et al. 2016; Hou et al. 2020).

Results

Taxonomic account

Gloydius shedaoensis qianshanensis Li, 1999

Justification of neotype designation. Li (1999) described *Gloydius shedaoensis qianshanensis* based on specimens collected from Qianshan Mountain, Anshan City, and Longtanshan Mountain, Wafangdian City, Liaoning Province. The specimens were preserved in the Snake Island Natural Museum in Lushun. However, the type specimens were lost during the relocation and expansion of the museum, including the holotype SB980563 collected from Qianshan Mountain, Anshan City, and paratypes collected from Longtanshan Mountain, Wafangdian City (interview to Jian-Li Li who described briefly and named this subspecies). Additionally, the original description only listed some numbers of ventral and subcaudal scales, with data on the length and weight of pregnant and juvenile snakes, but lacked detailed morphological descriptions and photos of specimens, which led to disagreements among later authors on the validity of this subspecies.

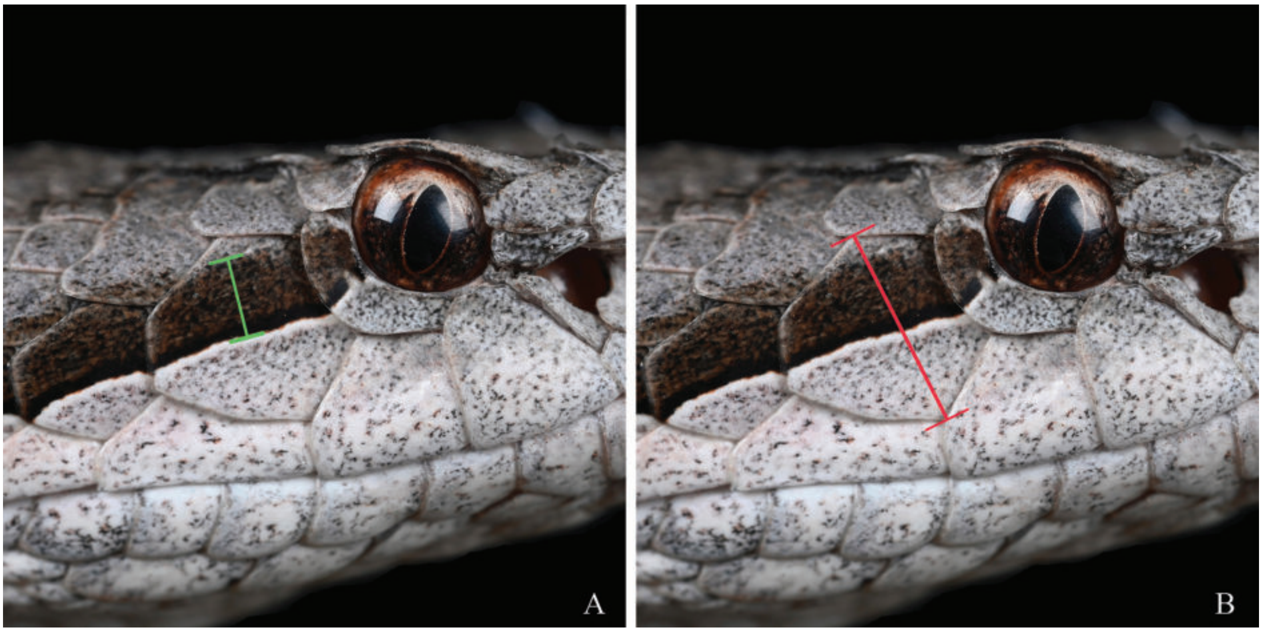

Figure 1. Illustration of measurement methods. **A.** Postorbital stripe width (PSW); **B.** temporal height (TH).

Table 1. Measurements (mm) and pholidosis data of adult *Gloydius shedaoensis qianshanensis*.

Voucher	Sex	SVL	TL	TTL	HL	HW	HD	ED	IOS	INS	PSW	TH	DS	V	SC	SPL	IFL	Location	Preserve
SYNU900701**	M	505	80	585	27.7	16.5	9.5	3.4	11.3	4.4	2.0	4.8	23-23-17	157	41	8/7	11/11	Liaoyang, Liaoning	SYNU
SYNU900702*	M	510	83	593	27.8	17.0	9.2	3.5	11.3	4.2	1.8	5.0	23-23-17	155	43	7/8	11/11	Liaoyang, Liaoning	SYNU
SYNU900703*	M	580	87	667	31.6	20.3	11.0	3.8	12.0	5.8	2.5	5.0	23-23-17	159	41	7/7	10/9	Liaoyang, Liaoning	SYNU
SYNU900704*	M	568	90	658	30.0	18.5	9.0	3.8	12.1	5.0	2.2	5.2	23-23-17	159	42	7/7	11/10	Liaoyang, Liaoning	SYNU
SYNU900705*	F	565	75	640	30.0	17.0	9.2	3.8	11.0	4.7	2.5	5.1	22-23-17	161	40	7/7	10/11	Liaoyang, Liaoning	SYNU
SYNU900706*	F	580	80	660	31.5	17.4	10.6	3.7	11.3	5.0	2.6	5.5	23-23-17	159	37	7/8	11/10	Liaoyang, Liaoning	SYNU
SYNU900707*	F	570	74	644	30.3	19.5	10.5	3.5	11.5	4.7	2.5	5.1	23-23-17	158	33	7/7	10/10	Liaoyang, Liaoning	SYNU
SYNU900708*	F	470	70	540	25.8	14.0	8.8	2.9	9.8	4.9	2.0	4.0	23-23-17	157	43	7/7	11/11	Liaoyang, Liaoning	SYNU
SYNU900709*	F	465	80	545	27.4	16.6	9.3	3.7	10.6	4.9	2.3	4.6	23-23-17	153	45	7/7	10/11	Liaoyang, Liaoning	SYNU
SYNU900710*	F	475	78	553	27.6	17.8	9.7	3.3	11.2	5.1	2.0	4.7	23-23-17	160	43	7/7	11/11	Liaoyang, Liaoning	SYNU
SYNU13090001*	M	591	89	680	30.8	19.6	11.1	3.7	12.3	5.6	2.6	5.5	23-23-17	159	43	7/8	11/11	Wafangdian, Liaoning	SYNU
SYNU900712*	F	514	89	603	29.1	16.7	10.5	3.6	11.3	5.2	2.4	4.5	23-23-17	158	48	7/7	10/11	Wafangdian, Liaoning	SYNU
SYNU900713*	M	558	96	654	29.1	16.3	10.1	3.1	10.6	4.7	2.6	4.9	23-23-17	154	45	8/7	12/12	Anshan, Liaoning	SYNU
220701	F	565	75	640	30.0	19.3	10.7	3.3	11.8	5.0	2.5	4.1	23-23-17	159	38	7/7	11/12	Liaoyang, Liaoning	/
220702	F	578	79	657	30.7	16.5	11.5	3.2	11.0	5.0	2.4	4.5	23-23-17	165	43	7/7	11/10	Wafangdian, Liaoning	/
220703	M	586	90	676	32.0	19.0	11.8	4.5	12.4	5.6	2.4	5.7	23-23-17	153	43	7/7	11/10	Liaoyang, Liaoning	/
220704	M	495	78	573	28.7	19.0	10.4	3.7	11.8	5.2	1.8	5.3	23-23-17	156	44	7/7	10/11	Liaoyang, Liaoning	/
220705	F	585	85	670	29.0	16.0	10.4	3.5	10.1	5.0	2.1	4.3	23-23-17	158	42	7/7	10/11	Liaoyang, Liaoning	/
220706	F	587	77	664	31.7	19.0	11.8	3.7	12.2	5.6	2.4	5.5	23-23-17	159	36	7/7	10/10	Liaoyang, Liaoning	/
220707	M	538	82	620	28.7	16.0	10.1	3.5	10.5	5.0	2.1	4.5	23-23-17	162	45	7/8	10/11	Wafangdian, Liaoning	/

Note: **, neotype; *, paraneotype; /, living body measured in the field.

Table 2. Measurements (mm) of *Gloydius shedaoensis shedaoensis*, *G. intermedius* and *G. changdaoensis* for PCA.

Voucher	Taxa	Sex	TTL	HL	HW	HD	ED	PSW	TH	IOS	INS	Location
/	*G. s. shedaoensis*	F	780	36.8	24.8	13.2	4.2	2.2	5.7	13.0	5.5	Snake Island, Liaoning
/	*G. s. shedaoensis*	F	743	36.9	24.0	12.3	4.4	2.1	4.7	14.2	6.5	Snake Island, Liaoning
/	*G. s. shedaoensis*	F	730	35.6	23.6	11.7	3.7	2.0	5.3	13.5	6.0	Snake Island, Liaoning
/	*G. s. shedaoensis*	M	745	35.0	21.4	12.0	4.2	1.8	6.0	14.3	6.0	Snake Island, Liaoning
/	*G. s. shedaoensis*	M	745	36.4	22.8	11.2	4.1	1.7	4.6	14.4	5.7	Snake Island, Liaoning
/	*G. s. shedaoensis*	F	700	35.6	20.0	11.2	3.8	1.9	5.5	12.9	5.5	Snake Island, Liaoning
/	*G. s. shedaoensis*	F	710	35.7	22.5	10.8	4.4	2.1	5.1	12.7	5.6	Snake Island, Liaoning
/	*G. s. shedaoensis*	F	745	36.4	25.5	11.7	4.1	2.0	5.8	14.3	6.1	Snake Island, Liaoning
/	*G. s. shedaoensis*	M	725	36.2	22.2	11.8	4.2	1.8	5.4	14.0	6.2	Snake Island, Liaoning
/	*G. s. shedaoensis*	F	730	35.9	23.9	11.5	4.4	2.2	5.5	13.4	5.6	Snake Island, Liaoning
/	*G. s. shedaoensis*	F	715	37.0	22.9	10.8	3.8	1.9	5.8	13.1	5.6	Snake Island, Liaoning
/	*G. s. shedaoensis*	F	730	33.3	22.5	12.5	3.7	1.8	5.3	13.1	5.2	Snake Island, Liaoning
/	*G. s. shedaoensis*	F	670	35.3	23.1	10.1	4.1	1.5	4.9	13.1	5.6	Snake Island, Liaoning
SYNU519001	*G. s. shedaoensis*	M	695	35.3	21.3	12.0	3.7	1.4	4.3	12.8	6.7	Snake Island, Liaoning
/	*G. s. shedaoensis*	M	815	40.4	24.6	11.0	4.3	2.2	5.2	14.4	6.0	Snake Island, Liaoning
/	*G. intermedius*	M	640	28.2	17.9	10.5	3.2	3.5	5.1	11.3	4.5	Changbai, Jilin
/	*G. intermedius*	F	770	35.9	22.1	12.6	3.6	3.9	4.9	13.2	6.1	Jilin
/	*G. intermedius*	M	720	33.1	18.9	11.3	3.5	3.7	5.4	6.0	6.0	Jilin
SYNU040271	*G. intermedius*	M	705	32.5	24.3	11.3	3.6	3.6	6.2	12.6	6.2	Kuandian, Liaoning
SYNU040272	*G. intermedius*	M	673	32.3	21.7	11.2	3.6	3.5	5.8	12.8	6.3	Kuandian, Liaoning
SYNU040273	*G. intermedius*	F	709	32.5	20.9	12.2	3.6	3.6	5.3	12.0	5.8	Kuandian, Liaoning
SYNU040274	*G. intermedius*	F	614	29.9	20.4	10.5	3.2	3.0	4.8	11.3	4.7	Kuandian, Liaoning
SYNU040275	*G. intermedius*	F	521	27.0	20.2	9.8	3.1	3.1	4.6	10.4	5.3	Kuandian, Liaoning
SYNU040276	*G. intermedius*	F	682	30.0	20.5	11.1	3.2	3.8	5.2	11.3	5.1	Kuandian, Liaoning
SYNU040277	*G. intermedius*	F	600	28.0	17.6	9.5	3.3	3.0	5.2	11.0	5.0	Kuandian, Liaoning
SYNU040278	*G. intermedius*	M	584	30.1	21.0	11.4	3.3	3.0	5.4	12.1	5.0	Kuandian, Liaoning
/	*G. changdaoensis*	F	617	32.5	20.8	13.0	3.9	3.4	6.0	11.4	5.4	Diaoyu Island, Shandong
/	*G. changdaoensis*	F	600	33.2	21.0	12.3	3.8	3.0	5.4	12.0	4.5	Diaoyu Island, Shandong
/	*G. changdaoensis*	F	645	34.2	19.8	11.8	4.0	3.6	5.4	12.5	5.7	Daheishan Island, Shandong
/	*G. changdaoensis*	F	610	31.3	21.0	11.6	3.4	3.5	5.5	11.3	4.8	Kunyu Mountain, Shandong
/	*G. changdaoensis*	F	558	35.2	21.2	11.1	4.2	3.1	5.3	12.2	5.3	Daheishan Island, Shandong
SYNU519002	*G. changdaoensis*	F	661	31.3	18.9	13.6	4.2	3.6	5.2	11.8	5.2	Rushan, Shandong
/	*G. changdaoensis*	M	680	33.7	23.3	12.5	4.2	3.9	7.4	12.4	6.3	Diaoyu Island, Shandong
/	*G. changdaoensis*	M	698	36.6	22.3	12.5	4.2	3.7	7.8	13.5	6.5	Diaoyu Island, Shandong
/	*G. changdaoensis*	M	670	35.7	24.8	12.5	4.0	3.3	6.5	12.6	6.0	Diaoyu Island, Shandong
/	*G. changdaoensis*	M	645	34.2	19.8	11.8	4.0	3.6	5.4	12.5	5.7	Daheishan Island, Shandong
/	*G. changdaoensis*	M	590	37.6	23.5	12.6	4.0	3.9	6.7	14.0	6.0	Daheishan Island, Shandong
/	*G. changdaoensis*	M	610	37.5	24.9	13.2	4.5	3.8	6.2	13.0	6.1	Daheishan Island, Shandong
/	*G. changdaoensis*	M	548	32.7	22.5	10.6	3.9	3.3	5.3	11.9	5.5	Daheishan Island, Shandong
/	*G. changdaoensis*	M	530	34.0	21.9	11.1	3.9	3.8	5.9	12.2	6.0	Daheishan Island, Shandong
/	*G. changdaoensis*	M	650	36.6	24.3	14.0	4.2	3.8	7.8	13.5	7.0	Daheishan Island, Shandong

Note: /, living body measured in the field.

Neotype. SYNU900701, adult male (Figs 2, 3), collected at Qianshan Mountain Area, Liaoyang City, Liaoning Province, China (40°53'54"N, 123°17'42"E; 385 m a.s.l.). Collected by Xian-Chun Qiu on 6 July 2020.

Paraneotypes. Twelve adult and eight juvenile specimens. Males SYNU900702–900704, females SYNU900705–900710, and juvenile SYNU900712 were collected from the same locality as the neotype by Xian-Chun Qiu between July and October 2020. Male SYNU13090001, female SYNU900712, and juveniles 1510184, 1510184, SYNU13090003, SYNU13090004, SYNU13090005, SYNU13090024 and SYNU1309006, collected by Jing-Song Shi during summer and autumn (2013–2015) from Wafangdian City, Liaoning province (39°56'20"N, 122°15'28"E; 450 m a.s.l.). Male SYNU900713, collected by Xian-Chun Qiu in July 2014 from Qianshan Mountain, Anshan City, Liaoning Province, China (40°59'36"N, 123°7'36"E; 406 m a.s.l.).

Etymology. This subspecies is named after its type locality, i.e., Qianshan Mountain, Liaoning Province, China.

Diagnosis. The morphological distinctions between *Gloydius shedaoensis qianshanensis* and other congeneric species and subspecies are as follows: (1) medium size, TTL up to 730 mm. (2) three palatine teeth. (3) dorsum light gray and covered with dark gray irregular X-shaped pattern; (4) black postorbital stripe with white lower margin; (5) postorbital stripe width smaller than eye diameter but larger than half of the eye diameter; (6) head flat and slender, slightly triangular; (7) mid-body dorsal scales 23; (8) ventral scales 153–170; (9) subcaudals 33–49.

Comparisons (Figs 4, 5): *Gloydius shedaoensis qianshanensis* belongs to the *G. halys*-*intermedius* group with 3 palatine teeth and 23-mid-body dorsal scale rows, which is different from the *G. strauchi* group with 21 rows of mid-body dorsal scales, including *G. strauchi* (Bedriaga, 1912), *G. himalayanus* (Günther, 1864), *G. qinlingensis* (Song & Chen, 1985), etc. It is different from the *G. blomhoffii* group with 4 palatine teeth, including *G. blomhoffii* (Boie, 1826), *G. brevicaudus* (Stejneger, 1907), *G. ussuriensis* (Emelianov, 1929), etc. The external morphology of *G. s. qianshanensis* is very similar to some taxa of the *G. halys-intermedius* group, including *G. s. shedaoensis*, *G. intermedius*, and *G. changdaoensis* Li, 1999. The results of PCA indicate that significant differences in head morphology exist between *G. s. qianshanensis*, *G. s. shedaoensis*, *G. intermedius*, and *G. changdaoensis* (Fig. 6). HL and PSW are the main indexes to distinguish the above species in PCA (Table 3). Furthermore, *G. s. qianshanensis* can also be distinguished by the following characters (Table 4): (1) light or dark gray dorsum (vs. brownish red or dark brown in *G. intermedius* and *G. changdaoensis*); (2) dark gray X-shaped spots appear on the dorsum (vs. dark and light cross striations interspersed in *G. intermedius*, rounded spots with light central color and dark margins on the lateral body in *G. changdaoensis*); (3) black postorbital stripe (vs. dark brown or brown in *G. intermedius* and *G. changdaoensis*); (4) postorbital stripe width less than the eye diameter but more than half of the eye diameter (vs. less than or approximately equal to half of the eye diameter in *G. s. shedaoensis*, approximately equal to eye diameter in *G. intermedius* and *G. changdaoensis*); (5) wider stripes cover 3–21 or 4–20 rows of mid-body dorsal scales (vs. stripes cover 5–19 rows of mid-body dorsal scales in *G. s. shedaoensis*).

Figure 2. Neotype SYNU900701 of *Gloydius shedaoensis qianshanensis*.

Figure 3. Head of *Gloydius shedaoensis qianshanensis* (neotype SYNU900701). **A.** Lateral view; **B.** dorsal view; **C.** ventral view. Scale bar: 10 mm.

Table 3. Variable loadings for principal components with eigenvalue.

	PC1	PC2	PC3	PC4	PC5	PC6	PC7	PC8	PC9
TTL	0.27	-0.37	-0.70	0.15	0.12	0.40	-0.15	0.22	-0.17
HL	0.41	-0.13	-0.03	0.00	-0.02	-0.17	-0.35	-0.11	0.81
HW	0.39	-0.01	0.08	-0.48	0.03	-0.03	-0.40	-0.49	-0.45
HD	0.35	0.23	-0.28	0.13	0.47	-0.35	0.55	-0.28	-0.02
ED	0.36	-0.12	0.39	0.67	0.01	-0.26	-0.23	0.19	-0.31
PSW	0.09	0.73	-0.18	-0.15	0.12	-0.12	-0.35	0.51	-0.04
TH	0.30	0.42	0.25	0.22	-0.07	0.74	0.14	-0.20	0.11
IOS	0.34	-0.28	0.39	-0.45	0.30	0.12	0.29	0.51	0.03
INS	0.36	0.05	-0.16	-0.11	-0.81	-0.18	0.33	0.14	-0.07
%Variance	0.57	0.18	0.07	0.04	0.04	0.04	0.03	0.02	0.01

The skull of *Gloydius shedaoensis qianshanensis* is quite similar to *G. s. shedaoensis* but differs in the following characteristics: (1) the postorbital processes of *G. s. shedaoensis* is more anteroposteriorly elongated than it in *G. s. qianshanensis*; (2) the anterodorsal edge of the postorbital of *G. s. qianshanensis* is in contact with the caudolateral edge of the frontal, while the anterodorsal edge of the postorbital is not in contact with the caudolateral edge of the frontal (separated from the frontal by the postorbital process of the parietal); (3) *G. s. qianshanensis* have less pterygoid teeth (9–10, n = 6) than *G. s. shedaoensis* (11–12, n = 3); the dentary teeth of *G. s. shedaoensis* are longer and slenderer relative to *G. s. qianshanensis*;

Figure 4. Lateral heads of *G. halys-intermedius* group from the Liaodong Peninsula and the Shandong Peninsula. **A.** Paraneotype SYNU900707 of *Gloydius shedaoensis qianshanensis* from Qianshan Mountain Area, Liaoning; **B.** G. s. shedaoensis from Snake Island, Liaoning; **C.** *G. intermedius* from Changbai Mountain, Jilin; **D.** *G. changdaoensis* from Kunyu Mountain, Shandong.

Figure 5. Dorsolateral view of *Gloydius halys-intermedius* group from Liaodong Peninsula and Shandong Peninsula. **A.** Paraneotype SYNU900702 of *G. shedaoensis qianshanensis* from Qianshan Mountain Area, Liaoning; **B.** *G. s. shedaoensis* from Snake Island, Liaoning; **C.** *G. intermedius* from Ji'an, Jilin; **D.** *G. changdaoensis* from Kunyu Mountain, Shandong.

Table 4. Morphological comparison of *Gloydius shedaoensis qianshanensis*, *G. s. shedaoensis*, *G. intermedius*, and *G. changdaoensis*.

Taxa	Dorsum color	Dorsum stripe	Postorbital stripe color	Postorbital stripe width
G. s. qianshanensis	Light gray	Dark gray X-shaped pattern	Black	PSW is smaller than ED but greater than half of ED
G. s. shedaoensis	Light gray	Dark gray X-shaped pattern	Black	PSW smaller than or approximately equal to half of ED
G. intermedius	Brownish red or dark brown	Dark and light cross striations	Dark brown or brown	PSW approximately equal to ED
G. changdaoensis	Brownish red or dark brown	Round spots with a light middle color and dark edge color on the lateral body	Dark brown or brown	PSW approximately equal to ED

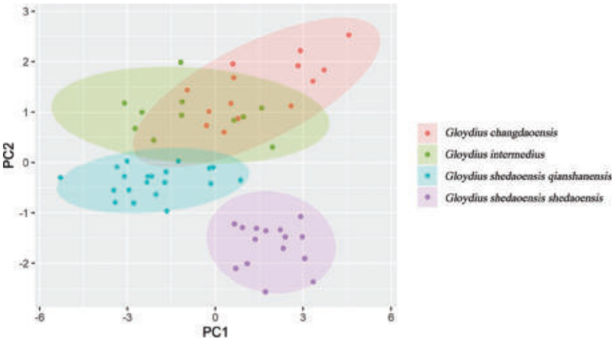

Figure 6. Plots of the first principal component (PC1) versus the second (PC2). *Gloydius changdaoensis* (red), *G. intermedius* (green), *G. s. qianshanensis* (blue), and *G. s. shedaoensis* (purple).

(4) *G. s. shedaoensis* has more curved palatine teeth than *G. s. qianshanensis*; (5) the dorsal edge of choanal process of the palatine in *G. s. qianshanensis* is significantly vaulted, rendering the palatine triangular shaped in lateral view, while the dorsal edge of choanal process of the palatine in *G. s. shedaoensis* is less vaulted and smooth (Figs 7, 8).

Description of neotype. SYNU900701, adult male, medium size, tail short (TTL 585 mm, TL 80 mm, TL/TTL 0.14). Head slightly triangular in dorsal view, slightly flat and slender, distinct from the neck (HL 27.7 mm, HW 16.5 mm, HD 9.5 mm, HW/HL 59.6%); snout blunt, slightly protruding from the lower jaw; eyes relatively small, pupil vertical (ED 3.4 mm, ED/HL 12.2%).

Scalation. Nine large scales are intact on the head. Rostral slightly trapezoidal, wide at bottom and narrow at top, the upper margin visible from dorsal view; nasal divided, anterior part larger, posterior margin of nostril tangent to the middle gap of nasal; two loreals, lower one forms the forward margin of pit; preoculars 3/3 (left/right), upper one turns up to dorsal head and extends to prefrontal, lower two slender and smaller, form the posterior margin of pit; postoculars 2/2, upper one small, lower one sickle-shaped and significantly longer, extending around the lower margin of eye to third supralabial; supralabials 8/7, first supralabial in contact with both parts of nasals, second supralabial smallest, third supralabial lagest and extending the bottom of orbit; temporals 2+3/2+3, largest temporal in contact with fourth and fifth supralabial on both sides; infralabials 11/11, first pair extends behind mental and connect, separate mental from chin shields; a pair of chin shields slightly rhombic, forming the mental groove in the middle; dorsal scales texture matte, keeled except for the row connecting with ventral scales; dorsal scale rows 23-23-17; ventral scales 157; anal plate single; subcaudals 41, in pairs.

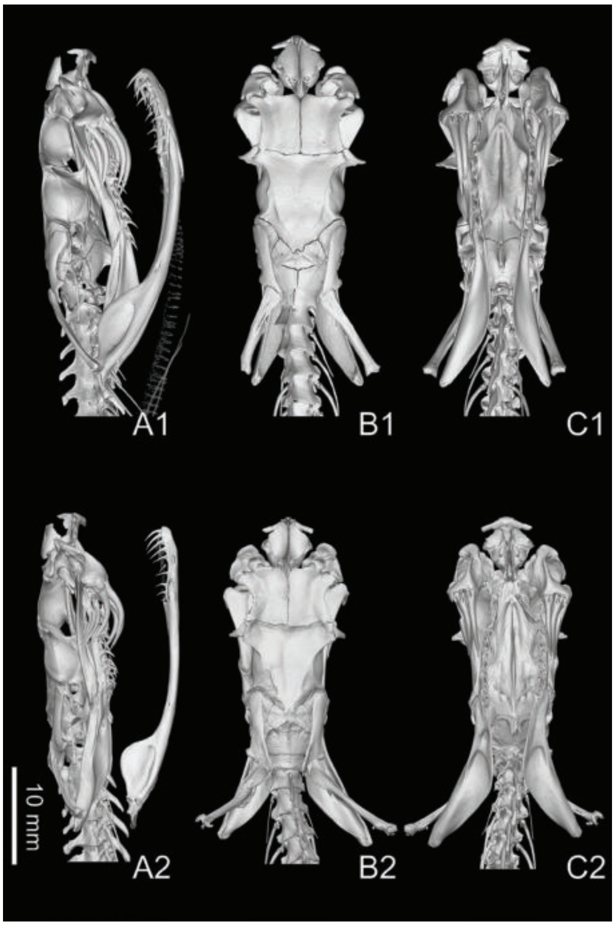

Figure 7. 3D reconstructed skull model of *Gloydius shedaoensis qianshanensis* (SYNU900701, A1–C1) and *G. s. shedaoensis* (SYNU519001, A2–C2). From left to right: lateral, dorsal, and ventral views.

Coloration. The background coloration of dorsal head is light gray, a pair of discontinuous black stripes on both sides extending backward from the outside of supraocular and parietal; eye rust red on the upper half while brown on the bottom half, pupil black with rust red margin; a black stripe appears behind eye and extends to the corner of mouth, black stripe slightly wider than half eye diameter, bottom of stripe is wavy with a very thin white margin; temporals, supralabials, and infralabials below black postorbital stripe are light gray and scattered with dark brown tiny blobs; a dark gray pincer shaped pattern with an opening toward the head appears on dorsal neck; dor-

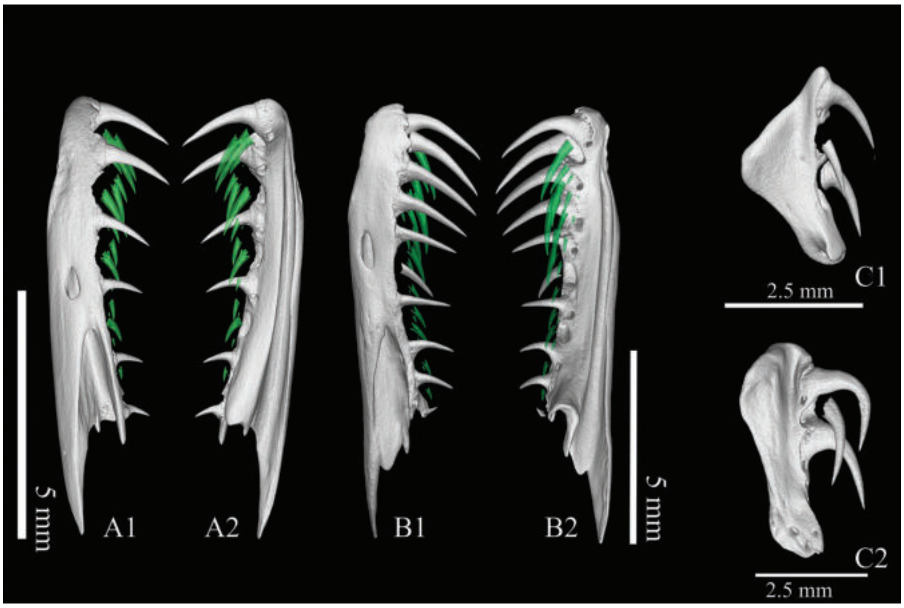

Figure 8. 3D reconstructed model of dentary and palatine of *Gloydius shedaoensis qianshanensis* and *G. s. shedaoensis*. **A1.** Labial view of the left dentary bone of *G. s. qianshanensis* (SYNU900701); **A2.** Lingual view of the left dentary bone of *G. s. qianshanensis* (SYNU900701); **B1.** Labial view of the left dentary bone of *G. s. shedaoensis* (SYNU519001); **B2.** Lingual view of the left dentary bone of *G. s. shedaoensis* (SYNU519001); **C1.** Labial view of the left palatine of *G. s. qianshanensis* (SYNU900701); **C2.** Labial view of the left palatine of *G. s. shedaoensis* (SYNU519001).

sum basically light gray with dark gray irregular X-shaped pattern, a light gray crossband composed of 1–2 scales is formed between two X-shaped pattern; a row of dark gray vaporous blobs separated from X-shaped pattern appear on lateral body; the end of tail is dark brown with grey crossbands; ventral surface of head grayish white, and the color gradually darkens from ventral neck to rear, the ventral surface of body from after neck to before tail is rust red, irregular vaporous black blobs appear near the gap of ventral scales; ventral tail gray and covered with dark brown spots.

Morphological variation. Morphometric data are summarized in Table 1. The dorsal background coloration of paraneotype SYNU900703 is close to earthy yellow. Postoculars 3/3 in paraneotype SYNU13090001.

Distribution and ecology. *Gloydius shedaoensis qianshanensis* is distributed in the Liaodong Peninsula mountainous area north to Liaoyang County, east to Xiuyan County, and south to Wafangdian City of Liaoning Province (Fig. 9).

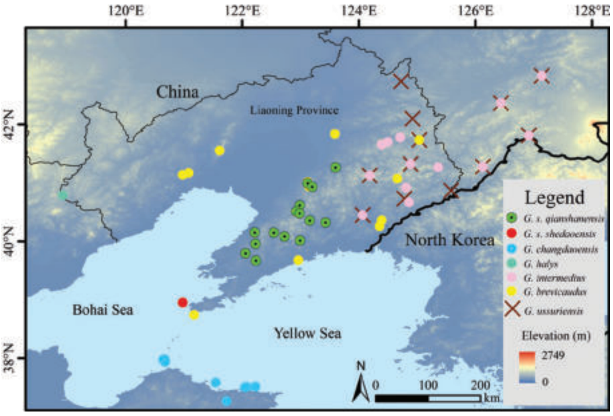

Figure 9. Collection localities of *Gloydius shedaoensis qianshanensis* (black center green circle) and some other congeneric species (subspecies) in Liaoning Province and surrounding areas.

Gloydius shedaoensis qianshanensis dwells in the deciduous broad-leaved forest in mountainous and hilly areas and the microhabitats contain forest edges, shrubs, grass, and gravel hillsides (Li 1999). Adults are easier to find in autumn because at that time they climb up branches to prey on avians, mainly Passerines (Fig. 10). The predation sites are usually selected on twigs above puddles or near the branches bearing berries, and other structures that attract avians. Sometimes two *G. s. qianshanensis* ambush on the same branch or adjacent branches in highly similar positions. In this study, two adult male *G. s. qianshanensis* were collected in Wafangdian City in early October 2019. One of them was collected from the ground bushes, and the other was collected from a branch not far away. It was established that both of their excrements had bird feathers that have not been completely digested. The excrements of adult individuals collected in Liaoyang City in October 2022 are mostly bird feathers, and only one sample excrement contains the hair of small mammals. *G. s. qianshanensis* do not estivate like *G. s. shedaoensis* on Snake Island. They prey on small mammals such as mice in midsummer. The main activity period of *G. s. qianshanensis* is from May to October of the year. They begin to hibernate in early November. The distribution of *Gloydius shedaoensis qianshanensis* is limited to the mountains of the Liaodong Peninsula, which may be related to their avian feeding habits. The East Asian-Australasian Flyway supports the greatest diversity and populations of migratory birds globally (Yong et al. 2015). The Liaodong Peninsula is located in the East Asian-Australasian Flyway and it is the necessary passage for some

Figure 10. A. The habitat of *Gloydius shedaoensis qianshanensis* in Wafangdian, Liaoning; **B.** Ambush state of *G. s. qianshanensis* on the branch, Xiang-Shu Meng photographed in Wafangdian, Liaoning.

south-north migratory passerine birds to cross the Bohai Strait to reach the southern part of the Chinese Mainland (Yong et al. 2021).

Mating occurs in spring and autumn, and hatch from late August to mid-September, with 3–16 offspring per adult. The total length of the baby snakes is 192–247 mm and they weigh 4.5–6.8 g (Li 1999). Two juveniles were collected in Wafangdian City in June 2013. One of the samples vomited a centipede, and found centipedes in the stomach of the other sample, indicating that centipedes are an important diet composition for juvenile *G. s. qianshanensis*. In addition, juveniles do not prey on avians, but on juvenile mice of *Mus musculus* Linnaeus, 1758 in our captivity observations.

Discussion

In previous decades, the population density of *Gloydius shedaoensis qianshanensis* was larger. People in some places call it “Tie shu pi (Stick on the bark)”, describing the snake’s posture of lurking on the branches to ambush avians. *G. s. qianshanensis* is described as “very common” in literature from the last century. Ji and Wen (1996) collected it every year during their internship with students from 1964 to 1994. In addition, Jian-Li Li also found a large number of *G. s. qianshanensis* during field surveys at the Liaodong Peninsula in the 1980s and 1990s (interview to Jian-Li Li). In recent years, the population density of *G. s. qianshanensis* has decreased rapidly in Qianshan Mountain, Wafangdian City, and other places. The main reasons include the destruction of the habitat caused by the development of local tourism and farmland. *G. s. qianshanensis* depend on primary forest to survive. Human activities have led to the continuous reduction of such environments, and the population of *G. s. qianshanensis* has thus declined. Additionally, people of the area hunt *G. s. qianshanensis* for reasons including the need for exotic dishes, and for traditional medicine. Therefore, conservation strength needs to be further enforced. Now, *G. shedaoensis* has been listed in category II of wild animals under the special state protection of China. The population on Snake Island has been well protected by establishing a national nature reserve. Therefore, we suggest establishing nature reserves where *G. s. qianshanensis* is concentrated.

The formation of Snake Island is closely related to the emergence of the Bohai Sea and geological changes. The geographical status of Snake Island was unstable during neotectonic movements, ice ages, and interglacials. The formation of insular in this case went through stages between being connected directly to the mainland, or disconnected, alternatively. Snake Island was last separated from the mainland 15,000 years ago, and such a short period of geographic isolation is insignificant for species formation (Zhou et al. 2000). Based on molecular phylogenetic analyses of ND4 and cytb genes, *Gloydius shedaoensis qianshanensis* constitutes a sister clade with *G. s. shedaoensis*. They form a monophyletic group with *G. intermedius*, and the three are closely related (Shi et al. 2016). However, *G. s. shedaoensis* adapted the insular climate, distinct from the mainland population. In both taxonomic traits (external morphology and skeletal morphology), and behaviors, they can be considered as ecospecies (Shi et al. 2016). According to O’Brien and Mayr (1991), a subspecies is characterized by having a unique geographic range or habitat, a group of phylogenetically concordant phenotypic characters, and a unique natural history relative to other subdivisions of the species (Auliya 2002). Therefore, this study supports Li’s (1999) subspecies delimitation of *G. shedaoensis*. Further research based on genomic information should be approached, in order to resolve the evolutionary relationships of *Gloydius* species around the Bohai Rim.

Acknowledgments

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Taxonomic status of *Lycodon subcinctus* sensu lato in China (Serpentes, Colubridae)

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https://zoobank.org/DA419ADA-E976-49B0-8CBD-949397BEE76F

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Abstract

The Malayan Banded Wolf Snake *Lycodon subcinctus* Boie, 1827 once included three subspecies, namely *L. s. subcinctus* Boie, 1827, *L. s. sealei* Leviton, 1955, and *L. s. maculatus* (Cope, 1985). Thereafter, *L. s. sealei* has been elevated to species level, and the taxonomic status of *L. s. maculatus* has not been resolved. We sequenced the mitochondrial cytochrome b (cytb) gene fragments of eight specimens of *L. s. maculatus* from China, including three from the adjacent areas of its type locality. Combining the sequences obtained from GenBank, we reconstructed a molecular phylogeny and reevaluated the taxonomic status of *L. s. maculatus*. Phylogenetic analysis revealed three highly divergent lineages within *L. subcinctus* sensu lato which correspond to *L. subcinctus* sensu stricto, *L. sealei*, and *L. s. maculatus*, respectively. Coupled with morphological comparison, we elevate *L. s. maculatus* to full species and redescribe it based on the type and freshly collected material.

Key Words

cytochrome b, morphology, phylogeny, subspecies, systematics, Wolf Snake

Introduction

Lycodon subcinctus Boie, 1827, a species originally described from Java, was subsequently considered to be widespread, ranging from almost the entire Southeast Asia to southern China and the Nicobar Islands of India (Boulenger 1893; Pope 1935; Smith 1943; Taylor 1965; Zhao 2006; Nguyen et al. 2009; Harikrishnan et al. 2010; Siler et al. 2013; Geissler et al. 2019; Reilly et al. 2019). Cope (1895) once dissected a snake specimen (Cat. No. 7339, U.S.N.M.) from Hong Kong, China, and gave it the name *Anoplophallus maculatus* Cope,

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1895. Stejneger (1926) considered the specimen dissected by Cope (1895) to belong to *L. subcinctus*, and therefore, the name *A. maculatus* was taken as a junior synonym of *L. subcinctus*. Leviton (1955) described a subspecies of *L. subcinctus* from the Philippines, namely *L. s. sealei* Leviton, 1955. Afterwards, Lanza (1999) recognized three subspecies within *L. subcinctus*: *L. s. subcinctus* Boie, 1827, mainly distributed in West Malaysia, Indonesia (Java, Sumatra), Singapore, Vietnam, Laos, Cambodia, and Thailand; *L. s. sealei*, mainly distributed in the Philippines, Brunei, and Borneo; and *L. s. maculatus* (Cope, 1895), mainly distributed in southern China. Currently, *L. s. sealei* has been elevated to full species (Leviton et al. 2018; Weinell et al. 2019) whereas *A. maculatus* is still treated as a junior synonym of *L. subcinctus* (e.g., Uetz et al. 2023) or by some authors, with a subspecific rank of *L. subcinctus* (e.g., Poyarkov et al. 2023).

In China, *Lycodon subcinctus* was recorded from Fujian, Guangdong, Hainan, Hunan, Sichuan, and Yunnan provinces, Guangxi Autonomous Region, and Hong Kong and Macao special administrative regions, and no subspecies has been recognized (Pope 1935; Zhao et al. 1998; Zhao 2006; Li et al. 2011; Francis 2021; Huang 2021; Wang 2021; Guo et al. 2022).

When studying *Lycodon* species in China, we found that there are some morphological differences between the snakes identified as *L. subcinctus* from China and *L. subcinctus* from the type locality in Java and the adjacent areas. In addition, molecular result revealed three strongly supported, highly divergent clades within *L. subcinctus* sensu lato, corresponding to the three subspecies previously considered, namely *L. s. subcinctus*, *L. s. sealei*, and *L. s. maculatus*. Since *L. sealei* has been treated as a separate species, *Anoplophallus maculatus* should also be regarded as a valid species, which we presently refer to as *Lycodon maculatus* comb. nov. (Cope, 1985).

Materials and methods

Total genomic DNA was extracted from liver tissue samples. A fragment of mitochondrial cytochrome b (cytb) gene was amplified using newly designed primer pairs SubF1: 5’–GCCAATATTGACTTAGCCTT–3’ and SubR1: 5’–ATTGAAAATGTTTGGGGTGA–3’. Polymerase Chain Reaction (PCR) amplification and sequencing were completed by Tsingke Biotechnology Co., Ltd. Sequences were edited and manually managed using SeqMan in Lasergene 7.1 (DNASTAR Inc., Madison, WI, USA). The new sequences have been deposited in GenBank, homologous sequences were downloaded from GenBank (Table 1). Sequences of *Boiga cynodon* (Boie, 1827) and *Dasypeltis atra* Sternfeld, 1912 were used as outgroups according to Wang et al. (2021). The technical computation methods for sequence alignment, genetic distance calculation, the best substitution model selection, and Bayesian inference (BI) and maximum likelihood (ML) phylogenetic analyses were the same as those in Liu et al. (2023).

Table 1. Cytochrome b (cytb) sequences used in the phylogenetic analysis.

Species	Voucher	Locality	GenBank
Lycodon albofuscus	LSUHC 3867	Tioman, Pahang, Malaysia	KX660500
Lycodon alcalai	KU 327847	Bataan, Philippines	KC010344
Lycodon anakradaya	SIEZC 20247	Song Giang, Khanh Hoa, Vietnam	OM674283
Lycodon aulicus	/	Jabalpur, Madhya Pradesh, India	HQ735416
Lycodon banksi	VNUF R.2015.20	Phou Hin Poun, Khammouane, Laos	MH669272
Lycodon bibonius	KU 304589	Cagayan, Philippines	KC010351
Lycodon butleri	LSUHC 9136	Bukit Larut, Perak, Malaysia	KC010353
Lycodon capucinus	LSUHC 9277	Nam Du, Kien Giang, Vietnam	KC010356
Lycodon carinatus	RAP 0447	Kanneliya, Galler, Sri Lanka	KC347486
Lycodon cathaya	SYS r001542	Huaping, Longsheng, Guangxi, China	MT602075
Lycodon cavernicolus	LSUHC 9985	Gua Wang Burma, Perlis, Malaysia	KJ607889
Lycodon chapaensis	VNUF R. 2017.23	Nam Dong, Thanh Hoa, Vietnam	MK585007
Lycodon chrysoprateros	KU 307720	Dalupiri, Cagayan, Philippines	KC010360
Lycodon davisonii	LSUHC 8479	O’Lakmeas, Pursat, Cambodia	KX660497
Lycodon deccanensis	BNHS 3610	Tumkur, Karnataka, India	MW006486
Lycodon dumerilli	KU 319989	Agusan del Sur, Mindanao, Philippines	KC010361
Lycodon effraenis	LSUHC 9670	Kedah, Malaysia	KC010376
Lycodon fasciatus	CAS 234957	Midat, Chin, Myanmar	KC010366
Lycodon flavicollis	/	Devarayanadurga, Karnataka, India	MW006488
Lycodon flavozonatus	SYS r000640	Huangganshan, Jiangxi, China	MK201413
Lycodon futsingensis	SYSr 000923	Guangdong, China	MK201432
Lycodon gongshan	KIZ 035112	Dulongjiang, Nujiang, Yunnan, China	MW353748
Lycodon jara	CAS 235387	Kachin, Myanmar	KC010367
Lycodon laoensis	FMNH 258659	Salavan, Laos	KC010368
Lycodon liuchengchaoi	JK 201704	Ningshan, Shaanxi, China	MK201563
Lycodon mackinnoni	ADR 197	Mussoorie, Uttarakhand, India	MW862977
Lycodon meridionalis	VNUF R.2017.54	Cuc Phuong, Ninh Binh, Vietnam	MH669268
Lycodon muelleri	DLSUD 031	Cavite, Luzon, Philippines	KC010373
Lycodon multizonatus	KIZ 01623	Luding, Sichuan, China	KF732926

Species	Voucher	Locality	GenBank
Lycodon nympha	RAP 0536	Kandalam, Matale, Sri Lanka	KC347476
Lycodon obvelatus	KIZ 040146	Panzhihua, Sichuan, China	MW353745
Lycodon pictus	IEBR 4166	Trung Khanh, Cao Bang, Vietnam	MT845093
Lycodon rosozonatus	SYS r001617	Jianfengling, Hainan, China	MK201531
Lycodon rufozonatus	SYS r001770	Taizhou, Zhejiang, China	MT625858
Lycodon ruhstrati	GP 285	Junlian, Sichuan, China	KC733195
Lycodon sealei	KU 327571	Barangay Estrella, Palawan, Philippines	KC010384
Lycodon sealei	KU 309447	Barangay Irawan, Palawan, Philippines	KC010385
Lycodon semicarinatus	/	Ryukyu, Japan	AB008539
Lycodon septentrionalis	CIB 117521	Medog, Nyinchi, Tibet, China	MW353736
Lycodon serratus	KIZ 038335	Deqin, Yunnan, China	MW353746
Lycodon sidiki	MZB 5980	Ache, Sumatra, Indonesia	KX822583
Lycodon stormi	JAM 7487	Air Terjun Moramo, Sulawesi, Indonesia	KC010380
Lycodon striatus	/	Savandurga, Karnataka, India	MW006489
Lycodon subannulatus	LSUHC 5576	Sibu, Johor, Malaysia	KX660499
Lycodon subcinctus	UTA-R 62972	Jawa Barat, Indonesia	KX822580
Lycodon subcinctus	MZB.Ophi.5398	Sumatera, Utara, Indonesia	KX822581
Lycodon subcinctus	UTA-R 62266	Sumatera, Utara, Indonesia	KX822579
Lycodon subcinctus	UTA-R 63046	Bengkulu, Indonesia	KX822582
Lycodon subcinctus	LSUHC 5016	Sungai Lembing, Pahang, Malaysia	KC010382
Lycodon subcinctus	MVZ291678	Lesser Sundas	MK844529
Lycodon subcinctus	MVZ291679	Lesser Sundas	MK844530
Lycodon subcinctus	MVZ291680	Lesser Sundas	MK844531
Lycodon subcinctus	MVZ291681	Lesser Sundas	MK844532
Lycodon subcinctus	MVZ291682	Lesser Sundas	MK844533
Lycodon subcinctus	MVZ291683	Lesser Sundas	MK844534
Lycodon subcinctus	MVZ291684	Lesser Sundas	MK844535
Lycodon subcinctus	MVZ291685	Lesser Sundas	MK844536
Lycodon synaptor	HS11006	Mengzi, Yunnan, China	MK201304
Lycodon tristrigatus	FMNH 269033	Bintulu, Sarawak, Malaysia	KX660474
Lycodon truongi	SIEZC 20249	Song Giang, Khanh Hoa, Vietnam	OM674282
Lycodon zawi	CAS 239944	Kaaukpyu, Rakhine, Myanmar	KC010386
Lycodon zayuensis	KIZ 032400	Chayu, Tibet, China	MW199792
Lycodon maculatus comb. nov.	SYS r001155	Shenzhen, Guangdong, China	MT625846
Lycodon maculatus comb. nov.	SYS r001943	Qingyuan, Guangdong, China	MT625859
Lycodon maculatus comb. nov.	SYS r001430	Guangdong, China	MK201493
Lycodon maculatus comb. nov.	HS15028	Fujian, Chima	MK201314
Lycodon maculatus comb. nov.	SYS r001621	Diaoluoshan, Hainan, China	MK201534
Lycodon maculatus comb. nov.	HS13005	Jiguanshan, Sichuan, China	MK201313
Lycodon maculatus comb. nov.	K1Z014158	Xishuangbanna, Yunnan, China	MK201560
Lycodon maculatus comb. nov.	KU 328531	Nakhon Ratchasima, Thailand	KC010383
Lycodon maculatus comb. nov.	KIZ2009061301	Hechi, Guangxi, China	OR823820
Lycodon maculatus comb. nov.	KIZ20190902	Hechi, Guangxi, China	OR823821
Lycodon maculatus comb. nov.	KIZ2023029	Guangzhou, Guangdong, China	OR823822
Lycodon maculatus comb. nov.	KIZ2023030	Putian, Fujian, China	OR823823
Lycodon maculatus comb. nov.	KIZ2023031	Xishuangbanna, Yunnan, China	OR823824
Lycodon maculatus comb. nov.	KIZ2023032	Shenzhen, Guangdong, China	OR823825
Lycodon maculatus comb. nov.	KIZ2023034	Xishuangbanna, Yunnan, China	OR823826
Lycodon maculatus comb. nov.	KIZ2023044	Dongguan, Guangdong, China	OR823827
Boiga cynodon	KU 324614	Negros Occidental, Philippines	KC010340
Dasypeltis atra	CAS 201641	Kabale, Uganda	AF471065

Measurements and scale counts were taken following Nguyen et al. (2022). SVL: snout-vent length, TaL: tail length, HL: head length, HW: head width, HH: head height, ED: eye diameter, SnL: snout length, EN: eye to narial distance, InD: internarial distance, SL: supralabials, IL: infralabials, SL-E: SL contacting the eye, LoR: loreals, LoR-E: LoR contacting the eye, PrO: preoculars, PtO: postoculars, aTMP: anterior temporals, pTMP: posterior temporals, DSR: dorsal scale rows at one head length posterior to the head, midbody, and one head length anterior to the vent, Ven: ventral scales, SC: subcaudals, Prec: cloacal plate, BB: light-colored body bands, TB: light-colored tail bands.

Results

The resulting topologies from BI and ML analyses are consistent (Fig. 1). All sequences of *Lycodon subcinctus* sensu lato formed a monophyletic lineage comprising three clades. Clade A included the sequence from the type locality of *L. subcinctus,* Java, and the sequences from Sumatra, Bengkulu, and the Lesser Sundas (Indonesia), and Pahang (West Malaysia); clade B included the sequences from the type locality of *L. sealei*, namely Palawan Island, the Philippines; and clade C included the sequences from the adjacent areas of the type locality of *Anoplophallus maculatus* in Guangdong, and the

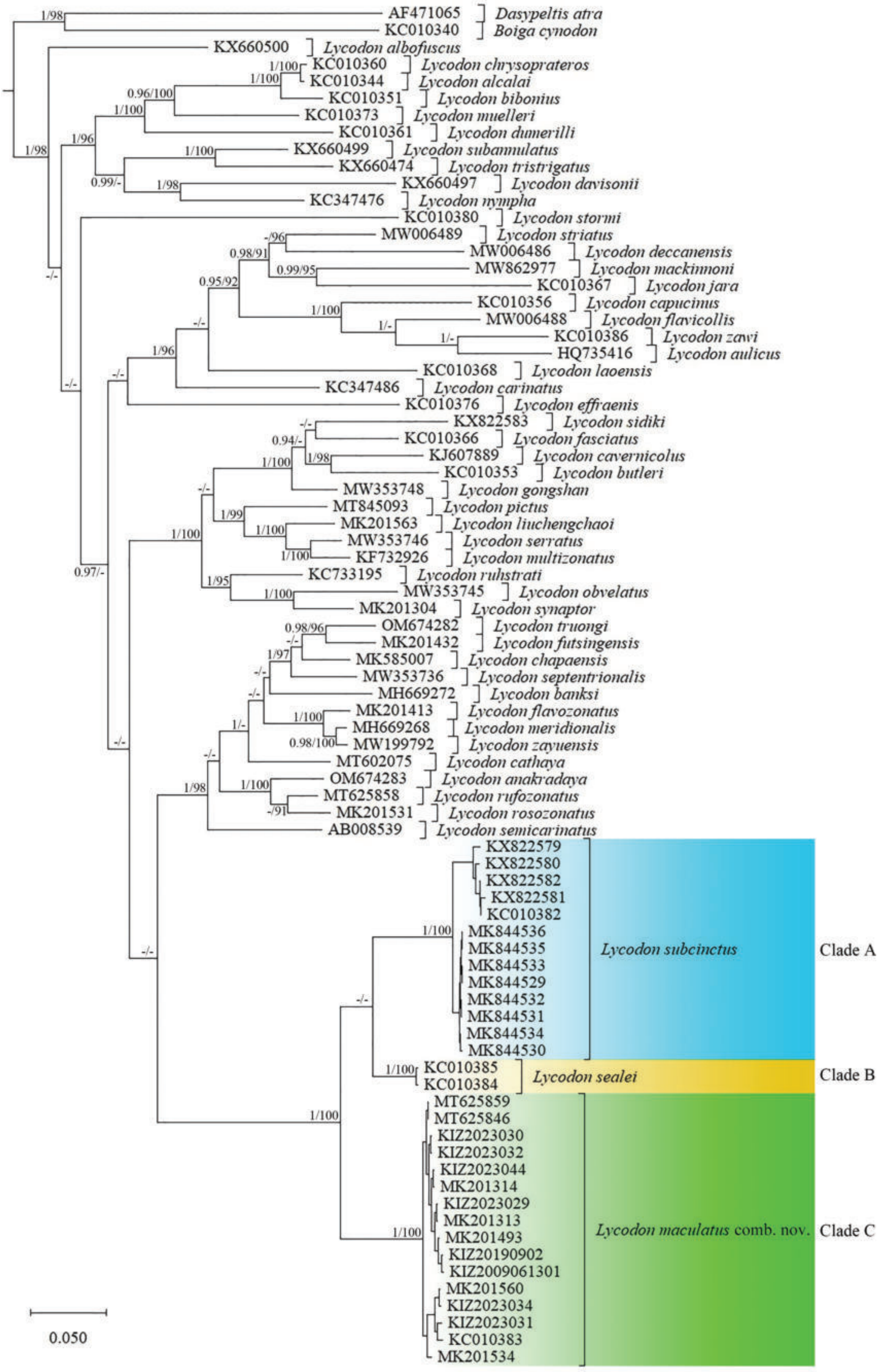

Figure 1. Bayesian phylogenetic tree of *Lycodon* inferred from the mitochondrial cytb sequences. Numbers before slashes indicate Bayesian posterior probabilities (>0.90) and numbers after slashes indicate ML bootstrap supports (>90).

sequences from Guangxi, Fujian, Sichuan, Hainan, and Yunnan, China, and Nakhon Ratchasima, Thailand. The average genetic distance (uncorrected p-distance) between clades A and B was 6.6%, the average genetic distance (uncorrected p-distance) between clades A and C was 8.8%, and the average genetic distance (uncorrected p-distance) between clades B and C was 8.9%. Therefore, we consider clade C, namely *A. maculatus*, to be a valid species instead of a strict synonym or a subspecies of *L. subcinctus*.

Lycodon maculatus comb. nov. (Cope, 1985)

Figs 2, 3

Type material. ***Holotype***. USNM 7339, adult male.

Type locality. Hong Kong Special Administrative Region, China.

Diagnosis. Body size relatively small, slender; 17-17-15 dorsal scale rows; eight supralabials, 3rd–5th or 3rd–6th contacting eye; 8–9 infralabials; no preocular; prefrontal contacting eye; two postoculars; one loreal contacting eye; one anterior temporal and two posterior temporals in most individuals; ventral scales less than 205; subcaudal scales more than 70, paired; cloacal plate divided; dorsal scale feebly keeled; anterior part of head dark grey or black; posterior lateral parts of head white in juveniles and dark gray or grayish black in adults; 20–27 distinct white bands on dorsal body and tail in juveniles; 5–8 grayish white bands gradually blur backward on anterior part of body in adults; no bands on posterior part of body and tail in adults.

Redescription of the holotype. Head flattened, somewhat elongate, HL 17.1 mm, HW 9.3 mm, HH 6.7 mm, HL/HW 1.84, HW/HH 1.39, distinct from the neck; snout relatively elongate, SnL 5.4 mm, SnL/HL 0.32, nostril closer to snout than to eye, internarial distance large, InD 4.2 mm, InD/HW 0.45; eye moderately sized, ED 2.0 mm, ED/HL 0.12, with a nearly rounded pupil; rostral approximately triangular, visible from above; two nearly triangular internasals; two large parallelogram-like prefrontals; single shield-shaped frontal; two large, elongate parietals; 1\1 nearly trapezoidal supraocular; no preocular; 2\2 small postoculars, upper one slightly larger than lower one; 1\1 narrow, elongate loreal entering orbit, in contact with nasal anteriorly, prefrontal dorsally, second and third supralabials ventrally; 8\8 supralabials; first and second supralabials in contact with nasal; third, fourth, and fifth supralabials in contact with eye; 1\1 anterior temporal; 2\2 posterior temporals; 8\8 infralabials; first pair infralabials contact medially forming a deep, medial groove; first three infralabials in contact with first pair of chinshields; first pair of chinshields elongate, bearing a deep, medial grooves contiguous with groove separating first pair of infralabials.

Body slender; SVL 428 mm; tail incomplete; 191 ventrals; cloacal plate divided; dorsal scales in 17-17-15 rows; vertebral row not enlarged; no apical pits.

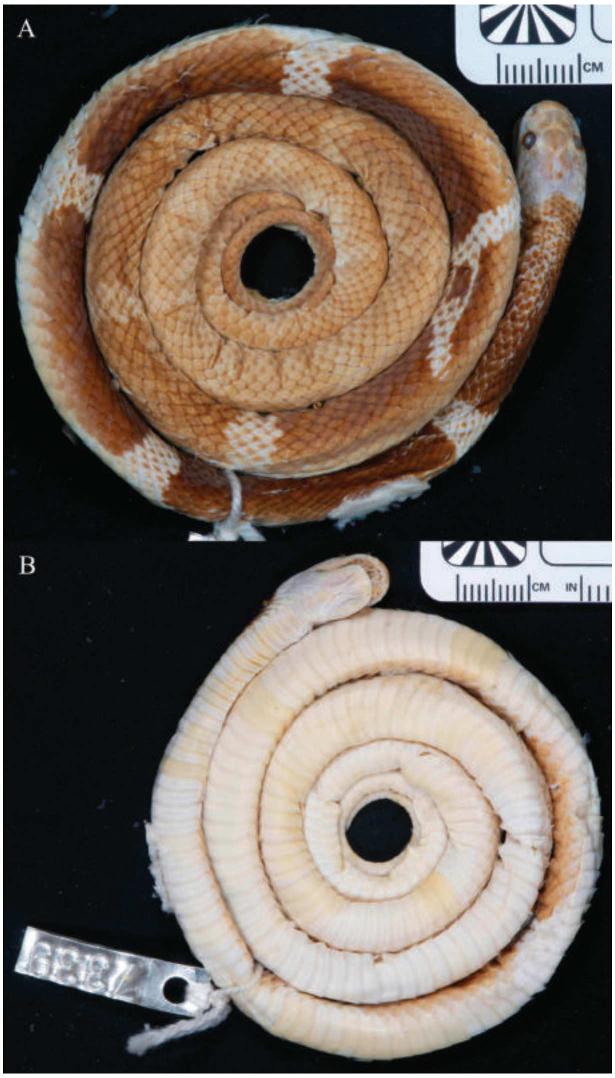

Figure 2. The holotype (USNM 7339) of *Lycodon maculatus* comb. nov. in preservative. **A.** Dorsal view; **B.** Ventral view. Photos are obtained from the website of National Museum of Natural History. Photographer: Teresa Hsu from Division of Amphibians & Reptiles, National Museum of Natural History, Smithsonian Institution.

After long-term immersion in preservative, head almost entirely white with a little light reddish brown on top; dorsal surface of anterior body reddish brown with seven white bands, first six distinct and last one indistinct; dorsal surface of posterior body and tail pale brown with no bands; ventral surface of head, body, and tail white.

Other specimens examined. We examined eight specimens in Kunming Natural History Museum of Zoology, Kunming Institute of Zoology, Chinese Academy of Sciences (KIZ) and two specimens in Museum of Herpetology, Chengdu Institute of Biology, Chinese Academy of Sciences (CIB). KIZ2009061301, adult male, from Tian'e County, Hechi City, Guangxi Autonomous Region, China (Exact locality unknown); KIZ20190902, adult female, from Leyi Village, Chuanshan Town, Huanjiang County, Hechi City, Guangxi Autonomous Region, China (25°5'56"N, 108°0'9"E, at an elevation

Figure 3. *Lycodon maculatus* comb. nov. from China in life. **A.** The adult female (KIZ20190902) from Hechi City, Guangxi Autonomous Region; **B.** The adult female (KIZ2023029) from Guangzhou City, Guangdong Province; **C.** The adult female (KIZ2023030) from Putian City, Fujian Province; **D.** The adult male (KIZ2023031) from Xishuangbanna Prefecture, Yunnan Province; **E.** The adult male (KIZ2023032) from Shenzhen City, Guangdong Province; **F.** An uncollected juvenile from Qingyua City, Guangdong Province.

of 570 m); KIZ2023029, adult female, from Huangpu District, Guangzhou City, Guangdong Province, China (23°5'45"N, 113°16'52"E, at an elevation of 200 m); KIZ2023030, adult female, from Hanjiang District, Putian City, Fujian Province, China (Exact locality unknown); KIZ2023031, adult male, from Menglun Town, Mengla County, Xishuangbanna Prefecture, Yunnan Province, China (21°56'9"N, 101°15'10"E, at an elevation of 550 m); KIZ2023032, adult male, from Luohu District, Shenzhen City, Guangdong Province, China (22°34'7"N, 114°14'9"E, at an elevation of 240 m); KIZ2023034, adult male, from Jinghong City, Xishuangbanna Prefecture, Yunnan Province, China (Exact locality unknown); KIZ2023044, juvenile, from Xiegang Town, Dongguan City, Guangdong Province, China (22°54'20"N, 114°14'19"E, at an elevation of 260 m); and CIB 78124 and CIB 9820, two adult females, both from Sanya City, Hainan Province, China (Exact locality unknown) (Fig. 4).

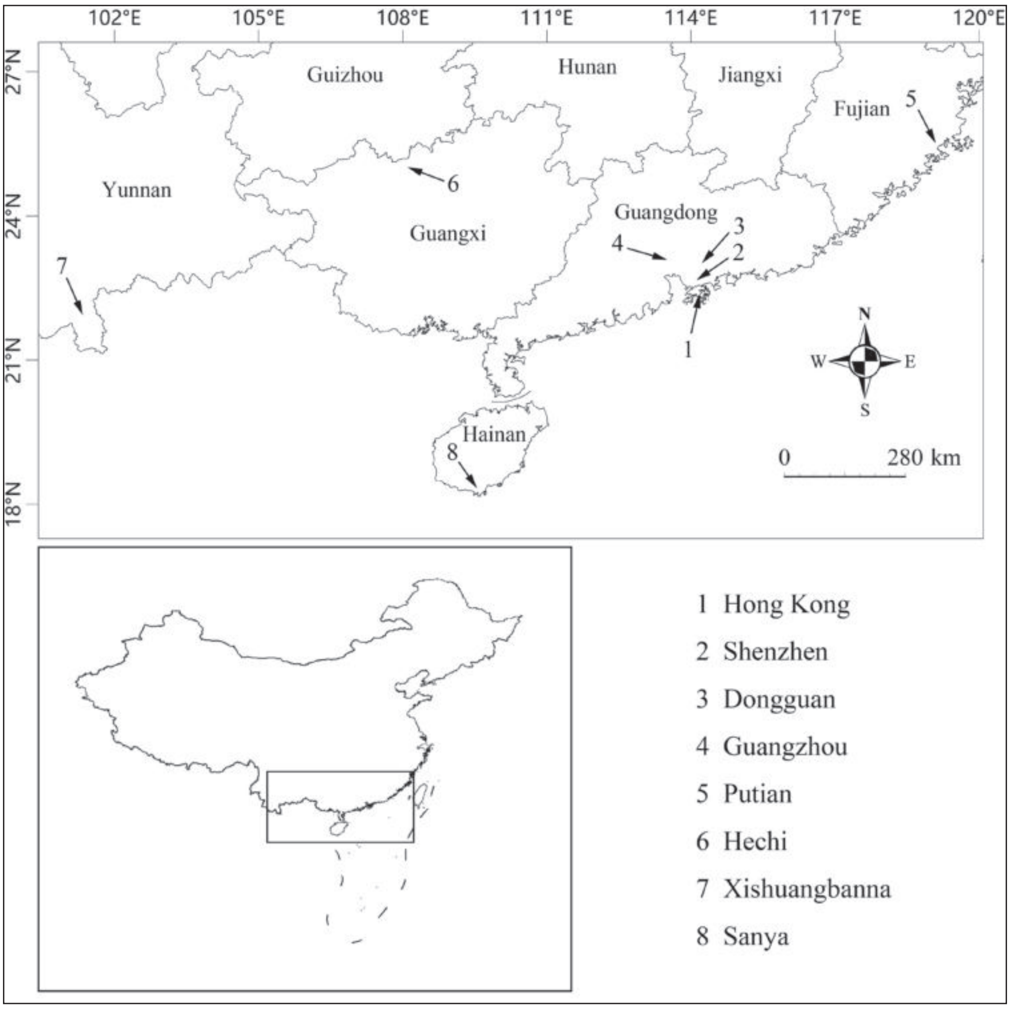

Figure 4. Map showing the type locality of *Lycodon maculatus* comb. nov. in Hong Kong Special Administrative Region, China (1) and the localities of the specimens examined in this study (2–8).

Variation. The morphological data of other specimens are presented in Table 2. All specimens resemble the holotype except that third, fourth, fifth, and sixth supralabials contact eye in some individuals, anterior and posterior temporals vary from one to two, nine infralabials in some individuals, first four infralabials in contact with first pair of chinshields, ventrals vary from 189 to 203, subcaudals vary from 71 to 84, and the bands on dorsal body vary from five to eight in adults and 14 bands on dorsal body and 12 bands on dorsal tail in the juvenile.

Morphological comparison. *Lycodon maculatus* comb. nov. differs from *L. subcinctus* by having fewer ventral scales, namely less than 205 vs. more than 205. *Lycodon maculatus* comb. nov. differs from *L. sealei* by subcaudal scales more than 70 vs. less than 70. In addition, the number of bands on dorsal body and tail is significantly different, although the bands become indistinct in adults, they are usually distinct in juveniles. According to the figures in Siler et al. (2013), Leviton et al. (2018), Francis (2021), and Huang (2021), and Fig. 3F and Table 2 in this paper, the total number of bands on dorsal body and tail of juveniles is less than 15 in *L. sealei* and more than 40 in *L. subcinctus*, whereas the number is 20–27 in *Lycodon maculatus* comb. nov.

Distribution. *Lycodon maculatus* comb. nov. is currently known to be distributed in southern China and Nakhon Ratchasima, Thailand. As Nakhon Ratchasima is located in central southern Thailand and the nearest confirmed distribution site of *Lycodon maculatus* comb. nov. is in Xishuangbanna, Yunnan, China, it can be assumed that the species is likely to occur in the area between Nakhon Ratchasima and Xishuangbanna, specifically in northern Thailand and central and northern Laos. In addition, it is likely that the population in northern Vietnam, previously considered to be *L. subcinctus*, also belongs to *Lycodon maculatus* comb. nov.

Discussion

The name *Anoplophallus maculatus* was synonymized with the name *Lycodon subcinctus* shortly after its proposal (Stejneger 1926) and later this taxon was considered one of the subspecies of *L. subcinctus* (Lanza 1999). At present, “The Reptile Database” (Uetz et al. 2023) regarded this taxon as a synonym of *L. subcinctus*, and Poyarkov et al. (2023) recognized it as a subspecies of *L. subcinctus*. All literature sources on the snakes of China

Table 2. Measurements (in mm) and scalation data of the examined specimens. For abbreviations see Materials and methods. "/" represents injured and incomplete.

	KIZ2009061301	KIZ20190902	KIZ2023029	KIZ2023030	KIZ2023031	KIZ2023032	KIZ2023034	KIZ2023044	CIB 78124	CIB 9820
Sex	Male	Female	Female	Female	Male	Male	Male	Juvenile	Female	Female
SVL	526	464	432	635	425	456	414	237	578	562
TaL	/	108	112	143	114	/	109	56	156	151
HL	18.4	16.6	16.0	20.9	15.9	16.1	15.5	11.5	20.4	17.2
HW	10.3	10.3	9.9	12.1	9.2	9.1	7.4	/	11.7	9.8
HH	7.5	7.8	6.2	8.7	5.3	6.2	5.6	/	7.1	7.0
ED	2.4	2.1	2.2	2.5	2.5	2.0	2.0	1.6	2.6	2.1
SnL	6.5	5.7	5.5	7.0	5.2	5.5	5.2	3.8	6.8	5.9
EN	3.6	3.1	3.3	4.2	2.9	3.1	2.6	1.8	3.6	3.3
InD	4.8	4.1	4.0	5.1	4.0	4.1	4.2	2.0	5.2	4.0
Sl	8\8	8\8	8\8	8\8	8\8	8\8	8\8	8\8	8\8	8\8
IL	9\9	9\9	9\9	9\9	8\9	9\8	9\8	9\9	8\8	9\8
SL-E	345\345	345\345	3456\3456	3456\3456	345\345	3456\3456	345\345	3456\3456	3456\3456	345\345
LoR	1\1	1\1	1\1	1\1	1\1	1\1	1\1	1\1	1\1	1\1
LoR-E	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
PrO	0\0	0\0	0\0	0\0	0\0	0\0	0\0	0\0	0\0	0\0
PtO	2\2	2\2	2\2	2\2	2\2	2\2	2\2	2\2	2\2	2\2
aTMP	1\1	1\1	1\1	1\1	1\1	1\1	2\2	1\1	1\1	1\1
pTMP	2\2	2\1	2\2	2\2	2\2	2\2	2\2	2\2	2\2	2\2
DSR	17-17-15	17-17-15	17-17-15	17-17-15	17-17-15	17-17-15	17-17-15	17-17-15	17-17-15	17-17-15
Ven	199	195	192	199	200	196	202	/	203	189
SC	/	72	76	71	84	/	82	74	74	71
Prec	divided	Divided	divided	divided	divided	divided	divided	divided	divided	divided
BB	8	6	7	5	7	8	7	14	7	7
TB	0	0	0	0	0	0	0	12	0	0

(e.g., Zhao et al. 1998; Zhao 2006; Zhang 2009; Li et al. 2011; Shi et al. 2011; Wang et al. 2020, 2021; Zhu and Rao 2020; Francis 2021; Huang 2021; Wang 2021) report that *L. subcinctus* occurs in China, but do not specify any subspecies. Our phylogenetic analysis revealed three lineages within *L. subcinctus* sensu lato. The first lineage includes sequences from Indonesia (including the Lesser Sundas) and Malaysia and refers to *L. subcinctus* sensu stricto. The second lineage includes sequences from the Philippines that are related to *L. sealei*. The last lineage contains sequences of specimens originating from China and Thailand and refers to *Lycodon maculatus* comb. nov. Currently, *L. subcinctus* sensu stricto is known to be distributed in Indonesia and Malaysia and their adjacent areas, while *L. sealei* is known only in the Philippines, and all populations previously considered being *L. subcinctus* from China belong to *Lycodon maculatus* comb. nov. Therefore, we remove *L. subcinctus* from the herpetofauna of China. Since we have not obtained samples from Laos, Vietnam, Cambodia, region of Thailand except Nakhon Ratchasima, and the Nicobar Islands, we are currently unable to determine the taxonomic status of the populations identified as *L. subcinctus* in these areas. Further studies are needed to clarify what species they belong to.

Many records of *Lycodon subcinctus* (now *Lycodon maculatus* comb. nov.) in China are known from the literature. Pope (1935) described three specimens of this species, two from Hainan and one from Fujian, China. He recorded the ventral scales as 197 and 199 in the Hainan specimens and 221 in the Fujian specimen, and the subcaudal scales as 77 and 78 in the Hainan specimens and 77 in the Fujian specimen, respectively. It is worth noting that two of the three specimens described by Pope (1935) are juveniles, one from Hainan and the other from Fujian. He described the juvenile from Hainan as having 13 light cross-bands on the body, but did not mention any bands on the tail. However, he described the juvenile from Fujian as having 16 distinct light cross-bands throughout. For this species, there are 20–27 light bands throughout in juveniles. Since Pope (1935) only described the bands on the body without mentioning the bands on the tail of the juvenile from Hainan, we cannot know the total number of bands on this specimen. However, the total number of bands on the juvenile from Fujian obviously does not match the characteristics of this species. Therefore, we speculate that the juvenile from Fujian described by Pope (1935) does not belong to this species, and the record of 221 ventral scales is questionable. Zhao et al. (1998) recorded the ventral scales of this species as 193–202 and subcaudal scales as 71–78 based on eight voucher specimens from China. Zhao (2006) recorded the ventral scales of this species as 196–227 and subcaudal scales as 72–105 based on seven specimens from Hainan, China, but did not provide the voucher numbers of the specimens. Li et al. (2011) recorded the ventral scales of this species as 193–202 and subcaudal scales as 71–78, but did not provide voucher specimens details from which this data was derived. Shi et al. (2011) recorded that this species has one preocular and 227 ventral scales, and 105 subcaudal scales based on one specimen from Hainan, China, but also did not provide the voucher number of the specimen. An important diagnosis of *Lycodon maculatus* comb. nov. is the absence of preocular, so it is obvious that Shi et al. (2011) relied on a misidentified specimen, and thus, the numbers of ventral and subcaudal scales they recorded do not belong to this species. Coincidentally, the maximum numbers of ventral and subcaudal scales recorded by Zhao (2006) are the same as those recorded by Shi et al. (2011), and all specimens of this species in Zhao (2006) and Shi et al. (2011) are from Hainan. Thus, the maximum numbers of ventral and subcaudal scales recorded by Zhao (2006) may come from the same specimen as in Shi et al. (2011), which was misidentified as *L. subcinctus* at that time. In this way, the records of 227 ventral scales and 105 subcaudal scales are also not credible. Currently, there are no reliable records of the number of ventral scales exceeding 205.

Conclusion

Based on molecular and morphological data, we resurrect and elevate the junior synonym subspecies, *Lycodon subcinctus maculatus*, as a full, valid species, which we refer to as *Lycodon maculatus* comb. nov. This species is currently confirmed to be distributed in Hong Kong Special Administrative Region, Guangxi Zhuang Autonomous Region, and Guangdong, Fujian, Hainan, Sichuan, and Yunnan provinces, China, and Nakhon Ratchasima, Thailand, based on molecular data. As for whether the populations in the other parts of Thailand and in Laos, Vietnam, Cambodia, and the Nicobar Islands, that were previously considered *L. subcinctus*, also belong to *Lycodon maculatus* comb. nov., further research is needed to verify.

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Range dynamics of *Walterinnesia morgani* (Serpentes, Elapidae) during climatic oscillations in Iran

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Abstract

Reptiles have a crucial part in maintaining global biodiversity and the functioning of dynamic ecosystems, owing to their ecological roles and functions. Nevertheless, these organisms are susceptible to human-induced disruptions and the deterioration of their habitats, leading to their categorization as the third most endangered group of vertebrates on a global scale. Understanding the spatial distribution of reptiles is crucial due to their often specific habitat needs and limited vagility. Morgan's black cobra (*Walterinnesia morgani*) is a secretive venomous snake species that has thus far received little attention in Iranian scientific literature. The aim of the present study was to determine the existing distribution pattern of the cobra and to speculate on how climatic changes might affect it. Maximum entropy modeling was used to examine a dataset consisting of 16 occurrence records gleaned from field observations and the literature. The niche of the species was predicted using current and future climate change forecasts and bioclimatic and topographical characteristics. The models predicted a future reduction in the wide distribution region of *W. morgani* in southern and western Iran. It was discovered that climatic factors like temperature range, precipitation dynamics, and river proximity all played a key role in shaping the pattern of distribution. The predicted suitable areas for *W. morgani* were dependent on water sources; however, future scenarios showed a decline in suitable habitats. This study underscores the importance of conservation efforts in light of the potential implications of climate change on this species. To further understand the range shifts and adaptive strategies of the species, further study of its ecology and dispersal dynamics is required.

Key Words

ecological niche, MaxEnt, Morgan's black cobra, precipitation, topography

Introduction

Reptiles, although they account for one-third of global terrestrial vertebrate diversity, have lagged behind other groups, such as birds and mammals, in terms of ecological studies (Biber et al. 2023). Due to the fact that reptiles are typically characterized by specific habitat needs and limited vagility (Guedes et al. 2018), understanding their spatial distribution is crucial. In this regard, revealing species distribution patterns improves our knowledge of the relationship between landscape and species. Climatic oscillations, like topographic and vegetation cover patterns, have contributed to the establishment of the distribution of species in different biomes (Bobrowski et al. 2018; Şahin et al. 2022a).

Although reptile species are widely distributed throughout the world, they are at risk of extinction due to habitat loss and degradation, pollution, invasive species, diseases, and climate change (Gibbons et al. 2000). Anthropogenic impacts–not only habitat use but also misinformation about their venomosity–have disproportionately threatened snake species (Saha et al. 2018). Therefore, evaluating the conservation status of these quite secretive organisms and determining their distribution patterns are important.

The herpetofauna of Iran comprises 81 species of snakes belonging to 34 genera and 7 Families (Rajabizadeh 2018).

Family Elapidae, including Elapinae (3 genera, 3 species) and Hydrophiinae (1 genus, 10 species), is more diverse than Viperidae, including Crotalinae (1 genus, 1 species) and Viperinae (7 genera, 10 species) (Safaei-Mahroo et al. 2015; Rajabizadeh 2018). Despite increasing efforts to determine herpetofaunal distributions in Iran (Hosseinian Yousefkhani 2019; Vaissi 2021a, b, 2022), snake species remain understudied (Hosseinzadeh et al. 2017; Moradi et al. 2021).

The genus *Walterinnesia* Lataste, 1887 has two species known commonly as desert black snakes or black desert cobras including the type species *Walterinnesia aegyptia* Lataste, 1887 and *Walterinnesia morgani* (Mocquard, 1905) (Fig. 1). Both species occur only in the Middle East. The type locality of *W. morgani* is Khuzestan Province, southwestern Iran (Uetz et al. 2023). Populations of the species in Iran were previously considered as *W. aegyptia* by Farzanpay (1989), Latifi (1985, 1991, 2000), and Leviton et al. (1992). Nilson and Rastegar-Pouyani (2007), based on the lower scale row counts around the neck and a banded juvenile pattern (Fig. 2), recognized the eastern populations of the genus (from Turkey and Saudi Arabia to Iran) as *W. morgani*; however, Rajabizadeh (2018) considered the Iranian populations of the genus as *Walterinnesia aegyptia morgani*.

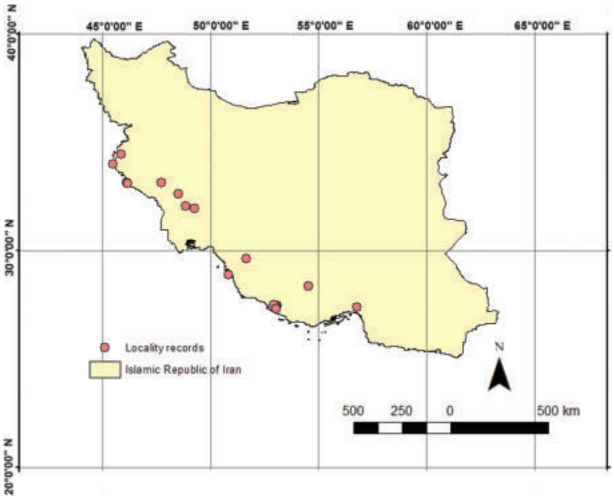

Figure 1. Occurrence records of *W. morgani* in Iran.

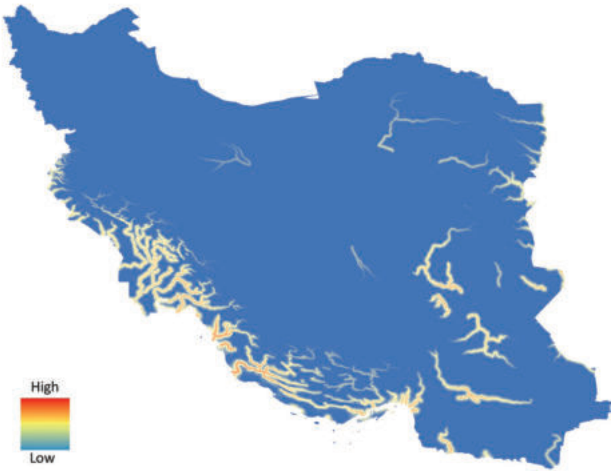

Figure 2. Recent historical (1981–2010) habitat suitability of *W. morgani* in Iran (warmer colors refer to high suitability level).

Walterinnesia morgani, Morgan's black cobra or black desert cobra, is a venomous snake species that inhabits the Arabian Peninsula (Kuwait and Saudi Arabia), the extreme south of Turkey, Syria, and the majority of Iraq, as well as western and southern Iran (Ilam, Kermanshah, Khuzestan, Bushehr, Fars, and Hormozgan Provinces) (Joger 1984; Gasperetti 1988; Latifi 2000; Uğurtaş et al. 2001; Sindaco et al. 2006; Safaei-Mahroo et al. 2015). In a specific concept, the Zagros Mountains display a wide biodiversity pattern, as well as the Central Iranian Plateau and the northern Persian Gulf, in one of the well-known biodiversity hotspots (Irano-Anatolian Biodiversity Hotspot) in the Palearctic Realm (Gholamifard 2011; Mittermeier et al. 2011). *Walterinnesia morgani* is one of the representative charismatic species of this region, where its shiny black color draws attention and, as most of the sightings of this species lead to its death, is very important from the aspect of conservation.

Black desert cobras display nocturnal activity patterns (Uğurtaş et al. 2001). During the hottest parts of the day, they seek refuge in burrows or beneath rocks. The seasonal activity of desert cobras may be influenced by local climate conditions (Baran et al. 2006). In some regions, for instance, they may be less active during the hottest period of summer and more active during the cooler months (Baran et al. 2021). Nonetheless, they are capable of being active even in extreme temperatures and are acclimated to harsh desert environments (Baran et al. 2021).

In spite of the known distribution of Morgan's black cobra, *W. morgani*, (IUCN status is Least Concern (LC)) in the Central and Southern Zagros Mountains, and its medical importance (Abid et al. 2020), the national literature on the species is scarce. The main reason for this is probably the difficulty of studying this mysterious and nocturnally active venomous snake. Therefore, the aim of this study was to reveal the current potential distribution pattern and speculate on the effects of climate oscillations on the potential distribution pattern of the black desert cobra in Iran.

Materials and methods

Study area and input data

This study was conducted within the borders of the Islamic Republic of Iran (25.08–39.77°N, 44.04–63.3°E; Fig. 1). A total of 16 occurrence records of *W. morgani* were obtained from the literature (Farzanpay 1989; Latifi 2000; Nilson and Rastegar-Pouyani 2007; Fathinia et al. 2010; Gholamifard and Rastegar-Pouyani 2012; Gholamifard et al. 2012) and our field observations (Suppl. material 1). In cases in which the locality information was not georeferenced, an online geographic system application (i.e., Google Earth Pro) was used to ascertain the most precise location possible. All records were georeferenced using the WGS84 coordinate system and checked with (QGIS Development Team 2023).

Ecological niche modelling (ENM)

Bioclimatic variables and one topographic layer (elevation) were downloaded from the CHELSA database (https://chelsa-climate.org/) at a spatial resolution of 30 arc-second raster grids (Karger et al. 2017; Brun et al. 2022). Additionally, one other topographic variable (distance to river) was obtained from the study of (Gavashelishvili et al. 2018) (Suppl. material 2). Each layer was clipped for the study area in QGIS. Pearson correlations between variables were calculated in R v4.3 (R Core Team 2023) and highly correlated variables were eliminated (r ≥ |0.8|). Six climatic and two topographic variables were retained and used in model construction based on the Pearson correlation coefficient: mean diurnal air temperature range (Bio_2), annual range of air temperature (Bio_7), daily mean air temperatures of the driest quarter (Bio_9), precipitation amount of the driest month (Bio_14), precipitation seasonality (Bio_15), mean monthly precipitation amount of the driest quarter (Bio_17), distance to river (d_river), elevation (elev). The contributions of the variables are given in Table 1.

All used variables were employed to predict the species niche under recent (1970–2000) and future (2071–2100) climate change projections (GFDL-ESM4, IPSL-CM6A-LR, MPI-ESM1-2-HR, MRI-ESM2-0, and UKESM1-0-LL) with the lowest and the highest limits of the shared socioeconomic pathways (SSPs) from the Coupled Model Intercomparison Project Phase 6 (CMIP6) as these models have the best performance for Eurasia (Eyring et al. 2016; Sun et al. 2022) (Suppl. material 3). Using the R package *spThin*, to reduce the effects of spatial autocorrelation (Boria et al. 2014) we utilized occurrence data that were separated by more than 2 km (Aiello-Lammens et al. 2015). One of the main issues with ENM is that it may lead to model overfitting if a large number of predictor variables are used in conjunction with a small sample size (Fielding and Bell 1997). Therefore, the Akaike Information Criterion corrected for small sample sizes (AICc) was applied to the potential distribution pattern (Hurvich and Tsai 1989).

Maximum entropy modeling was utilized since it is a robust method that can be applied to presence and pseudo-absence data. Based on presence and pseudo-absence data, this algorithm can predict the presence of a species with a probability between 0 and 1 (Phillips et al. 2009). A total of 2000 background points for *W. morgani* were sampled at random throughout the survey area. The potential habitat suitability was modeled by implementing MaxEnt 3.4.1 in R using the ‘kuenm’ package (Phillips et al. 2017; Cobos et al. 2019). To create the models for this cobra species, 80% of the occurrence data was used for generating the candidate models, and the remaining 20% for independent presence as test data.

In order to optimize the model complexity for the cobra species, 31 combinations of MaxEnt’s 5 feature classes [hinge (h), threshold (t), product (p), quadratic (q), and linear (l)] along with 17 regulation multiplier values (0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1, 2, 3, 4, 5, 6, 8, 10) were evaluated. This combination of options enabled us to find the best-fitting model representing our data by generating diverse candidate models (Muscarella et al. 2014; Cobos et al. 2019). Following that, the Akaike Information Criterion corrected for small sample sizes (AICc) (with the lowest values) as well as the AUC values (with the highest values) were both used to determine the best models (Hurvich and Tsai 1989). Significance tests were performed using partial ROC (Peterson et al. 2008) and predictive power with a 5% omission rate (Anderson et al. 2003). Lastly, all model inputs were transformed into binary predictions by using the minimum training presence as the threshold to distinguish suitable areas from unsuitable ones (Pearson et al. 2007; Şahin et al. 2022a).

Results

The regions anticipated to be suitable for *W. morgani* exhibited a significant area under the curve (AUC) value of 0.955±0.014. Furthermore, the recent historical and future bioclimatic predictions (2071–2100) under the SSP126 and SSP585 scenarios had the lowest values for Akaike Information Criterion corrected (AICc) and the delta AICc at 0.169 and 0, respectively. (Figs 2, 3A–J; Table 2).

Table 1. Percentage contribution of the environmental layers used in species distribution modeling of *Walterinnesia morgani*.

Bio 2 mean diurnal air temperature range	Bio 7 annual range of air temperature	Bio 9 daily mean air temperatures of the driest quarter	Bio 14 precipitation amount of the driest month	Bio 15 precipitation seasonality	Bio 17 mean monthly precipitation amount of the driest quarter	d_river distance to river	elev elevation
10	4.3	27.7	0.4	14.7	1.1	31.9	10

Table 2. Summary for selecting the best model for species distribution maps of *W. morgani* via ‘kuenm’ package.

All candidate models	Statistically significant models	MaxEnt features	AICc	wAICc	Delta AICc	AUC	Mean AUC ratio
1054	1054	linear, quadratic, product	418.125	0.169	0.00	0.955±0.014	1.866

AICc: A corrected AIC score, used for a small sample size by increasing the cost for each parameter.
wAICc: The model weight is the relative likelihood for each model, divided by the total relative likelihood for all models that were considered.
Delta AICc: The difference between the model with the lowest score (the “best” model) and the AICc score for each model.
AUC: Area under the curve is a measure of the accuracy of the model.
Mean AUC ratio ≥ 1.00, $p < 0.05$ means predictions are significantly better than a random model.

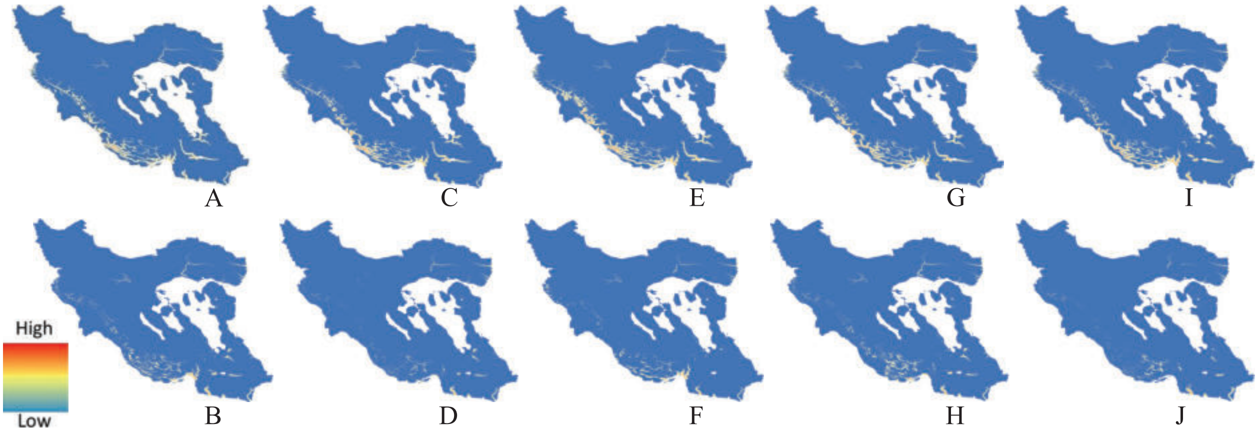

Figure 3. Future habitat suitability (2071–2100) of *W. morgani* in Iran based on **A.** GFDL-ESM4 under optimistic scenario (ssp 126); **B.** GFDL-ESM4 under pessimistic scenario (ssp 585); **C.** IPSL-CM6A-LR under optimistic scenario (ssp 126); **D.** IPSL-CM6A-LR under pessimistic scenario (ssp 585); **E.** MPI-ESM1-2- HR under optimistic scenario (ssp 126); **F.** MPI-ESM1-2- HR under pessimistic scenario (ssp 585); **G.** MRI-ESM2-0 under optimistic scenario (ssp 126); **H.** MRI-ESM2-0 under pessimistic scenario (ssp 585); **I.** UKESM1- 0-LL under optimistic scenario (ssp 126); **J.** UKESM1- 0-LL under pessimistic scenario (ssp 585) (warmer colors refer to high suitability level).

The fact that the AUC data were very close to 1 showed that the potential distribution area revealed by the locality data obtained from the distribution area of the desert cobra displayed a much better performance than a random prediction. For *W. morgani*, 1054 statistically significant models that had different regularization multipliers and feature classes were evaluated. Linear, quadratic and product (LQP) features along with a regularization multiplier of 0.8, and with the lowest delta AICc, was the best model. The most important variables were d_river (31.9%), Bio_9 (27.7%) and Bio_15 (14.7%), respectively.

According to the analysis, the potential distribution area of *W. morgani* covers a water source-dependent pattern in western Iran. Additionally, as can be seen in future climatic conditions, the potential distribution range would be narrower than under recent historical conditions. Habitat loss is expected in each future climatic scenario, but its level varies depending on the ssp level (Table 3). For instance, scenarios in ssp 126 level forecast the species range change percentile to be between -9.814 and -18.028%, however, the ssp 585 scenarios may result in a much greater loss of between 49.337% and 71.262% of the species' suitable habitats in the distribution range (Suppl. material 4: fig. S1A–J; Table 3). As a result of all these scenarios, it is expected that the possible distribution of *W. morgani* will contract in southwestern Iran in the future.

Table 3. Species range change (SRC) of *W. morgani* in recently suitable habitats (gain/loss) by 2081–2100 under optimistic (ssp126) and pessimistic (ssp585) scenarios in different climate-change projections.

	Percent loss	Percent gain	Species range change
GFDL-ESM4 ssp 126	18.859	1.279	-17.58
GFDL-ESM4 ssp 585	49.337	4.739	-44.598
IPSL-CM6A-LR ssp 126	19.613	1.636	-17.977
IPSL-CM6A-LR ssp 585	71.262	0.518	-70.744
MPI-ESM1-2- HR ssp 126	13.15	3.336	-9.814
MPI-ESM1-2- HR ssp 585	65.135	0.274	-64.861
MRI-ESM2-0 ssp 126	19.854	5.286	-14.568
MRI-ESM2-0 ssp 585	58.527	10.632	-47.894
UKESM1- 0-LL ssp 126	30.247	12.219	-18.028
UKESM1- 0-LL ssp 585	77.18	7.599	-69.581

Discussion

Numerous biotic and abiotic factors have significant impacts on the distribution of species (Pearson et al. 2007). To understand the responses of *W. morgani* to climatic oscillations during recent history and under future possible scenarios, we employed ENM as a useful analytical instrument to assess the range of the species. Climate changes affect all aspects of biodiversity, from organisms (organismal diversity) to biomes (ecological diversity) (Gaston and Spicer 2004), and pose a significant threat to the integrity of ecosystems (Bellard et al. 2012). Additionally, it is speculated that vertebrate species will face serious problems of adaptation related to alterations in their climatic niches in the near future (Quintero and Wiens 2013). It should be noted that the distribution of reptiles can be significantly affected by direct and/or indirect anthropogenic activities, such as human population size and human-driven climatic changes (Bickford et al. 2010).

Based on these results, most of the suitable predicted areas were slightly wider than the present potential distribution of *W. morgani*. This might be the possible effect of "d_river" as one of the highest contributors to shape the distribution pattern via the water requirement of the species. Additionally, daily and annual temperature cycles have also contributed to the potential distribution pattern as well as the seasonal factors, especially the driest seasonal precipitation dynamics. This overall bioclimatic and topographic pattern is observed in many herptile species in and nearby the study area (Naumov et al. 2020; Kurnaz and Eroğlu 2021; Şahin et al. 2022a, b; Vaissi 2022; Kurnaz 2023).

On the other hand, our results show that there will be a future decrease in suitable habitats for *W. morgani*, but the level of the decreasing trend varies depending on the SSP levels and different future scenario sets (Suppl. material 4: fig. S1A–J; Table 3). This trend is compatible with many ecological niche modeling studies that have been applied to herptiles in the study area and nearby regions (Kurnaz and Şahin 2021; Bozkurt 2022). Additionally, this contraction pattern has also been predicted for future distribution trends in many lizard species (Vaissi 2022). Nonetheless, the genus *Lacerta* and two leopard geckos exhibit the opposite trend in the Anatolian Peninsula and Iran, respectively (Hosseinian Yousefkhani and Nabizadeh 2022; Gül et al. 2023).

When compared to other large vertebrate groups, the ability of reptiles to migrate is quite restricted (Hickling et al. 2006). Moreover, our knowledge about the ecology and dispersal dynamics of *W. morgani* has received little or no attention. Based on Figs 2, 3, the current research assumed an infinite capacity for dispersal across species and made predictions about its range changes through 2100. Despite contractions and expansions, the potential distribution range of the species under each ssp126 scenario remains less changed (Fig. 3A, C, E, G, I; Table 3); however, under ssp585 scenarios, the species will be contract in southwestern Iran (Fig. 3B, D, F, H, J; Table 3). In the pessimistic scenario (ssp585) for the IPSL-CM6A-LR climate projection, in particular, it is expected that there would be 71.26% habitat loss with only 0.51% habitat gain and as a result, there would be a 70.74% negative range change (Suppl. material 4: fig. S1D; Table 3). Additional factors, such as geographic barriers, may influence the migration rate of a species (Morena-Rueda et al. 2012). In our case, distance to the river and elevation parameters, as topographic variables, make significant contributions to the species distribution. Even though the Zagros Mountains can be assessed as a remarkable geographic barrier, the main limitation factor is the “distance to the rivers” for *W. morgani*. Because it seems that the cobra tends not to occur very distant from water resources. Moreover, even though the potential distribution map displays suitable habitats in central or eastern regions of Iran, it should be noted that this could be the consequence of bioclimatic and topographic similarities in distinct areas that the species is biogeographically incapable of inhabiting. A similar pattern to ours was observed in several studies which evaluate narrower regions (Heidari 2021; Bozkurt 2022; Şahin 2022b).

On the other hand, the model algorithm that was used in this study did not take into account some parameters, such as parasitism, disease, habitat loss, and fragmentation that might affect the realistic distribution of the species (Todd et al. 2010). In the meantime, a recent study that focused on snake bite risk in Iran showed that the northern and western parts of Iran would have greater risk than the rest of the country (Yousefi et al. 2023). However, our results pointed out that southwestern Iran could be a potentially suitable region for Morgan’s black cobra. No common potential distribution model was reported for *W. morgani*, neither country-based nor across-range, by including up-to-date topographic variables. Thus, our results suggest that *W. morgani* should be monitored in the near future, with more occurrence records collected in order to better understand its distribution and to increase awareness of human-cobra conflict as a result of anthropogenic activities. To conclude, it is desirable to predict the potential distribution of this species in Iran from the aspect of medical treatment of possible bites of the species and to prepare its antivenom.

Author contributions

A. Gholamifard and M.K. Şahin conceived the study. A. Gholamifard collected the field data and documentation. M.K. Şahin carried out the sampling design, statistical analysis and performed modeling. A. Gholamifard and M.K. Şahin contributed equally to writing the manuscript.

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Supplementary material 1

Species occurrence records of *W. morgani* in Iran from literature and our field trips

Authors: Ali Gholamifard, Mehmet Kürşat Şahin

Data type: docx

Link: https://doi.org/10.3897/herpetozoa.36.e107947.suppl1

Supplementary material 2

Variables used for modeling the distribution of black desert cobra (*Walterinnesia morgani*)

Authors: Ali Gholamifard, Mehmet Kürşat Şahin

Data type: docx

Link: https://doi.org/10.3897/herpetozoa.36.e107947.suppl2

Supplementary material 3

Climate forcing data and source for the CHELSA CMIP6 ISIMIP3 data

Authors: Ali Gholamifard, Mehmet Kürşat Şahin

Data type: docx

Link: https://doi.org/10.3897/herpetozoa.36.e107947.suppl3

Supplementary material 4

Species range change (SRC) of *W. morgani* in recently suitable habitats (gain/loss) by 2081–2100 under optimistic (ssp126) and pessimistic (ssp585) scenarios

Authors: Ali Gholamifard, Mehmet Kürşat Şahin

Data type: docx

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Redescription of *Rhacophorus tuberculatus* (Anderson, 1871) and the validity of *Rhacophorus verrucopus* Huang, 1983

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Abstract

Rhacophorus tuberculatus and *Rhacophorus verrucopus* are two morphologically similar species described in 1871 and 1983 respectively. Their taxonomic distinctiveness has been questioned in the past. In the current study, we encountered frogs that we confer to *R. tuberculatus* based on morphological similarity to a syntype of this species. We redescribe the species based on a re-examination of a syntype, which is designated as a lectotype here, and additional specimens from Garo hills of Meghalaya. We also present molecular data, natural history notes, and report a range extension of this species. Molecular phylogenetic analysis based on the 16S rRNA fragment revealed minimal genetic divergences (0.20–1.74% uncorrected p-distance) between specimens identified as either *R. tuberculatus* or *R. verrucopus* from different locations. On the basis of molecular data and morphological characteristics, we conclude that *R. verrucopus* is a junior synonym of *R. tuberculatus*.

Key Words

Darwinian shortfall, Indo-Burma hotspot, range extension, Rhacophoridae, synonymy, systematics

Introduction

Frogs of the genus *Rhacophorus* Kuhl & Van Hasselt, 1822 occur across South and Southeast Asia and are represented by 44 extant species (Frost 2023). Despite many species of *Rhacophorus* being widespread, most of them are poorly studied, and information on their distribution is sparse (Ohler and Delorme 2005). *Rhacophorus tuberculatus* (Anderson, 1871) is one such poorly known species. It was described in 1871 from "Seebsaugor, Assam" (now Sivasagar 26.98515°N, 94.63878°E) and was subsequently reported from Arunachal Pradesh, Meghalaya, and West Bengal, India (Annandale 1912; Chanda 2002; Sen 2004; Das and Dutta 2007; Ahmed et al. 2009; Mathew and Sen 2010; Roy et al. 2018). Huang (1983) described *Rhacophorus verrucopus* from "Beibeng, Medo Xian" (Beibeng Township, Motuo County, Xizang Autonomous Region, China 29.23942°N, 95.17644°E), which closely resembles *R. tuberculatus* and this species was known only from its type locality and northern Myanmar (Fei 1999; Fei et al. 2009, 2010; Li et al. 2010; Fei et al. 2012; Liu et al. 2020; Fei 2020; Zug 2022). By studying the type and near-topotypical specimens of *R. verrucopus* and comparing them with the descriptions of *R. tuberculatus*, Che et al. (2020) considered that *R. verrucopus* and *R. tuberculatus* could be the same species.

However, since Che et al. (2020) did not obtain any molecular data or examined the types of *R. tuberculatus* from India and only made comparisons of external morphology, Frost (2023) does not currently adopt their view and still treats *R. verrucopus* as a valid separate species.

We encountered a *Rhacophorus* species, the identity of which was difficult to determine, during our recent surveys between 2021 and 2022 in the West Garo hills of Meghalaya, India. Herein, we ascertain the identity of that *Rhacophorus* species as *R. tuberculatus* based on comparison with the syntypes of *R. tuberculatus* and provide a redescription of the species after more than 150 years since its original description, provide photographs of live individuals, morphological measurements and genetic divergence of *R. tuberculatus* with other congeners using 16S rRNA gene and discuss its relationship with *R. verrucopus*.

Materials and methods

Study area

We conducted surveys in Sasatgre village (25.5250°N, 90.3350°E, ca. 940 m) and Baladingre village (25.514213°N, 90.398204°E, ca. 835 m) of West Garo Hills district of Meghalaya between 2020 and 2022.

Voucher collection

Frogs were caught by hand, photographed first and euthanized using 20% Benzocaine following Torreilles et al. (2009). A small portion of the liver tissue was extracted by making a narrow slit on the ventral aspect of the specimens and stored in Molecular Biology Grade Ethanol (BP2818). Specimens were later fixed in 90% Ethanol for two hours and then transferred to 70% Ethanol for long-term storage as museum specimens. In total, seven individuals were collected for this study: six adult males collected from near Sasatgre village (25.5250°N, 90.3350°E, 940 m asl.) and an adult female from near Baladingre village (25.514213°N, 90.398204°E, 835 m asl.). The specimens used for morphological and molecular analyses were deposited at the herpetological collection facility at Sálim Ali Centre for Ornithology and Natural history (SACON).

DNA extraction and molecular analysis

Total genomic DNA was extracted from tissue samples of two specimens of *R. tuberculatus* (SACON VA 148 and VA 800) with a DNA extraction and purification kit, following the manufacturer's protocols. 16S rRNA gene was amplified using the primers 16sAR-L (5′-CGCCTGTTTATCAAAAACAT-3′) and 16sBR-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 5× Phusion HF buffer, 0.4 µl of 10mM 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 (National Centre for Biological Sciences, Bengaluru). Sequences were edited and manually adjusted using SeqMan in Lasergene 7.1 (DNASTAR Inc., Madison, WI, USA) and MEGA 11 (Tamura et al. 2021). Species of the genus *Zhangixalus* were selected as outgroups following Liu et al. (2022). Homologous and outgroup sequences were obtained from GenBank (Table 1). The technical computation methods for sequence alignment, genetic distance calculation, the best substitution model selection, Bayesian Inference (BI) and Maximum Likelihood (ML) phylogenetic analyses were the same as those in Liu et al. (2021).

Morphometric measurements

The following measurements were recorded to the nearest 0.02 mm from the specimens using 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), upper eyelid width (UEW, the maximum width of the upper eyelid), interorbital distance (IO, distance between the margins 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 top of the 4th toe). Webbing formulae follows Savage and Heyer (1997).

Geographic range estimation

Geographic range of the target species was calculated by plotting the known occurrences of the species on a

Table 1. List of specimens and GenBank accession numbers for all 16S rRNA sequences included in this study.

Taxon	Voucher No.	Locality	GenBank No.
Rhacophorus annamensis	VNMN 4090	Dak Nong, Nam Nung, Vietnam	LC010566
Rhacophorus baluensis	FM235958	Sabah, Malaysia	KC961089
Rhacophorus bengkuluensis	UTA A-62770	Lampung, Sumatra, Indonesia	KM212948
Rhacophorus bipunctatus	PUCZM/IX/SL360	Mizoram, India	MH087073
Rhacophorus borneensis	BORN:22410	Maliau Basin, Sabah, Malaysia	AB781693
Rhacophorus calcaneus	VNMN 4093	Dak Lac, Chu Yang Sin, Vietnam	LC010573
Rhacophorus catamitus	ENS 14726	Sumatra, Indonesia	KX398877
Rhacophorus exechopygus	VNMN 4107	Gia Lai, Kon Ka Kinh, Vietnam	LC010585
Rhacophorus helenae	AMS R 173230	Binh Thuan, Vietnam	JQ288087
Rhacophorus hoabinhensis	VNMN A.2016.16	Hoa Binh, Vietnam	LC331097
Rhacophorus indonesiensis	MZB: Amp:23619	Indonesia	AB983367
Rhacophorus kio	VNMN 4110	Gia Lai, Kon Ka Kinh, Vietnam	LC010589
Rhacophorus lateralis	SDB.2010.330	Karnataka, Bygoor, India	KC571277
Rhacophorus malabaricus	Rmal-In	Madikeri, India	AB530549
Rhacophorus margaritifer	ENS 16162	Java, Indonesia	KX398889
Rhacophorus modestus	ENS 16853	Sumatra, Indonesia	KX398904
Rhacophorus napoensis	GXNU YU000171	Napo, Guangxi, China	ON217796
Rhacophorus nigropalmatus	Rao081203	Malaysia	JX219438
Rhacophrus norhayatiae	NNRn	Endau Rompin, Johor, Malaysia	AB728191
Rhacophorus orlovi	VNMN 3067	Huong Son, Ha Tinh, Vietnam	LC010598
Rhacophorus pardalis	FMNH273243	Sarawak, Bintulu, Malaysia	JX219454
Rhacophorus poecilonotus	ENS 16480	Sumatra, Indonesia	KX398920
Rhacophorus pseudomalabaricus	SDB.2011.1010	Kerala, Kadalar, India	KC593855
Rhacophorus reinwardtii	Rao081205	Malaysia	JX219443
Rhacophorus rhodopus	SCUM 060692L	Mengyang, Yunnan, China	EU215531
Rhacophorus robertingeri	VNMN 4123	Gia Lai, Kon Ka Kinh, Vietnam	LC010613
Rhacophorus spelaeus	IEBR A.2011.1	Khammouan, Lao	LC331095
Rhacophorus translineatus	Rao6237	Motuo, Xizang, China	JX219449
Rhacophorus tuberculatus	KIZ014154	Motuo, Xizang, China	MW111522
Rhacophorus "*verrucopus*"	Rao6254	Motuo, Xizang, China	JX219436
	SEABRI2019120056	Htamanthi, Sagaing, Myanmar	MW275978
Rhacophorus tuberculatus	SACON VA – 148	Meghalaya, India	OR836578
	SACON VA – 800	Meghalaya, India	OR836579
Rhacophorus vampyrus	VNMN 4125	Hon Ba, Khanh Hoa, Vietnam	LC010616
Zhangixalus dennysi	SCUM 060401L	Shaoguan, Guangdong, China	EU215545
Zhangixalus dugritei	SCUM 051001L	Baoxing, Sichuan, China	EU215541

distribution map generated using ARCGIS 10.5. The area within the minimum convex hull was computed by connecting the outermost occurrence points to calculate the extent of occurrence as defined by the IUCN (2001).

Results

The suggested best substitution model for BI was GTR+F+I+G4 and for ML was TIM2+F+I+G4, both analyses showed an essentially consistent topology (Fig. 1). The sequences of the newly collected specimens from Meghalaya, India clustered with the sequences of *R. verrucopus* from Myanmar with strong supports by both BI and ML (0.97/99), and they together clustered with the sequences of *R. verrucopus* from China with strong supports by both BI and ML (1/100). The genetic divergence (uncorrected p-distance) between the sequences of the newly collected specimens and the sequences of *R. verrucopus* from China ranged from 1.50% to 1.74%, the genetic divergence (uncorrected p-distance) between the sequences of the newly collected specimens and the sequence of *R. verrucopus* from Myanmar ranged from 0.20% to 0.44% (Table 2).

Morphologically, the newly collected specimens (*R. tuberculatus*) from Meghalaya, India agree with the Syntype (ZSI 10154) and subsequent descriptions and figures by Annandale (1912) and Mathew and Sen (2010) of *Rhacophorus tuberculatus* in most aspects, especially in having a distinct tympanum, almost half as large as the eye; absence of vomerine teeth; presence of partial, sheath-like webbing on fingers and fully developed webbing on toes; pointed projection at tibio-tarsal articulation; well-developed and expanded discs on toe. Hence, we consider these newly collect-

Table 2. Genetic divergences (uncorrected p-distance in %) (%) between specimens identified as either *Rhacophorus tuberculatus* or *R. verrucopus* from different locations.

Species	Voucher	1	2	3	4
Rhacophorus tuberculatus	SACON VA – 148 India				
	SACON VA – 800 India	0.22			
Rhacophorus "*verrucopus*"	SEABRI2019120056 Myanmar	0.20	0.44		
	Rao6254 China	1.50	1.74	1.24	
	KIZ014154 China	1.54	1.74	1.28	0.00

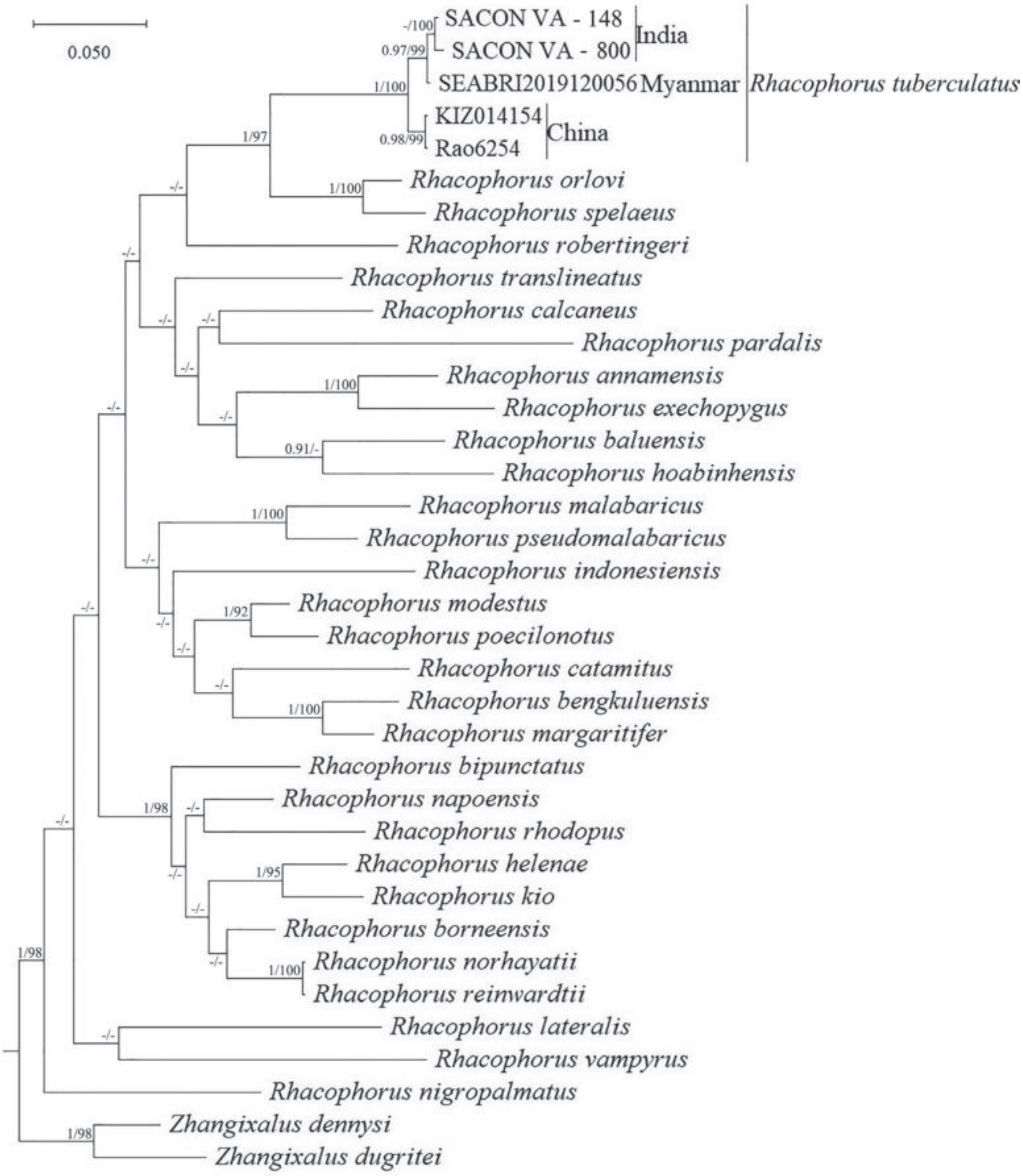

Figure 1. Bayesian inference tree of the genus *Rhacophorus* based on partial 16S rRNA fragments. Numbers before slashes indicate Bayesian posterior probabilities (≥ 0.90 remain) and numbers after slashes indicate ultrafast bootstrap support for ML analyses (≥ 90 remain).

ed specimens to belong to *R. tuberculatus*. In addition, there were also no obvious morphological differences between the newly collected specimens of *R. tuberculatus* and *R. verrucopus* from China and Myanmar (see Table 3).

Integrating the results of morphological data and also considering the shallow genetic divergence that is usually considered as intraspecific variation in the genus *Rhacophorus*, we second Che et al. (2020) in stating that *R. tuberculatus* and *R. verrucopus* are conspecific, and formally place *R. verrucopus* under the subjective synonymy of *R. tuberculatus*. Below, we give a formal synonymy list and provide the description of the specimen ZSI 10154 and designate it as the lectotype of *Rhacophorus tuberculatus*. Additionally, we also provide measurements of the newly collected material for comparison.

Systematics

Rhacophorus tuberculatus (Anderson, 1871)

Polypedates tuberculatus Anderson, 1871.
Rhacophorus tuberculata – Boulenger, 1882.
Rhacophorus (*Rhacophorus*) *tuberculatus* – Ahl, 1931.
Rhacophorus schlegelii tuberculatus – Wolf, 1936.
Rhacophorus verrucopus Huang, 1983, syn. nov.
Rhacophorus tuberculatus – Inger, 1985.
Rhacophorus (*Rhacophorus*) *verrucopus* – Dubois, 1987 «1986».

Redescription of *Rhacophorus tuberculatus* (Anderson, 1871). Specimens examined: ZSI 10154, lectotype by present designation, adult female, collected by Anderson from “Seebsaugor, Assam”; SACON VA – 143, 144, 145, 146, 147 and 148, adult males collected

Table 3. Morphometric measurements (in mm) of *R. tuberculatus* and *R. turpes* from the current study (*Designated as lectotype).

Species	***Rhacophorus tuberculatus* (Voucher no., sex and values)**								***Rhacophorous turpes* (Voucher No., sex and values)**	
Voucher no: Morphometric variables	***ZSI 10154**	**SACON VA 143**	**SACON VA 144**	**SACON VA 145**	**SACON VA 146**	**SACON VA 147**	**SACON VA 148**	**SACON VA 800**	**BMNH 1940.6.1.30**	**BMNH 1974.828-832**
	Female	**Male**	**Male**	**Male**	**Male**	**Male**	**Male**	**Female**	**Female**	**Female**
SVL (mm)	39.10	30.32	29.38	30.88	28.16	27.02	30.76	34.02	37.42	35.80
AG	17.44	12.80	12.20	14.06	12.84	12.48	13.74	16.82	21.28	18.02
BW	16.92	7.52	5.82	7.28	6.76	6.10	8.44	7.44	14.22	11.46
HL	11.40	8.74	9.18	10.00	8.40	7.92	10.02	11.62	12.22	10.76
HW	11.10	9.38	9.00	9.34	8.92	8.86	9.40	12.00	9.74	9.78
HD	6.04	4.32	4.08	4.60	3.92	3.62	3.54	6.32	4.68	4.28
ED	4.42	4.28	4.10	3.70	3.70	3.84	4.18	4.90	3.54	2.98
EN	2.80	2.98	2.00	2.32	2.84	2.84	2.56	3.08	2.74	3.30
ES	4.42	3.78	3.64	4.32	4.52	4.22	4.32	5.62	4.90	5.43
TYD	1.82	1.68	1.80	2.08	1.20	1.72	1.70	2.38	2.44	2.76
ET	1.24	0.48	0.80	0.52	0.60	0.40	0.72	0.66	1.12	1.18
UEW	2.86	3.70	4.20	3.70	3.66	3.68	3.70	5.54	2.44	2.02
IO	4.64	3.12	3.70	2.82	2.98	2.72	3.02	4.14	4.28	4.12
IN	3.50	2.40	2.36	1.74	2.50	2.80	2.98	3.22	2.80	3.08
UAL	6.46	5.72	5.62	4.72	4.92	4.04	4.50	5.42	5.24	6.98
LAL	7.62	5.64	5.42	5.66	5.24	5.94	5.34	8.00	7.00	7.68
PAL	9.42	6.84	7.32	7.20	6.90	6.70	7.40	9.58	9.18	8.64
FEL	16.92	13.56	13.62	11.72	11.74	11.72	13.96	15.06	14.88	16.22
TBL	18.80	15.48	14.82	13.8	14.4	13.74	14.32	17.28	16.90	18.28
TAL	12.44	8.48	9.34	7.34	8.44	7.20	9.14	9.90	8.94	9.32
FOL	19.76	11.12	10.88	9.38	9.84	9.28	11.88	12.96	10.82	10.36

from near Sasatgre (25.5250°N, 90.3350°E, ca. 940 m asl.) by RSN between 13th – 26th May 2020 and SACON VA – 800 Adult female collected from near Baladingre (25.514213°N, 90.398204°E, ca. 835 m asl.) by RSN on 26th February 2022.

Diagnosis. *Rhacophorus tuberculatus* can be differentiated from all known congeners by the following suite of external morphological characters: small to medium adult size (mean SVL 29.4 mm; range 27.0–30.9 mm); distinct tympanum, almost half as large as the eye; absence of vomerine teeth; presence of a prominent calcar at tibio-tarsal articulation; presence of partial, sheath-like webbing on fingers and fully developed webbing on toes; well-developed and expanded discs on toes; and a dorsal colouration of uniform pale brown with mild traces of an irregular patch on the head and mossy greenish patches near the shoulders in some individuals.

Description of Lectotype (Fig. 2). Head flat, almost as long as wide (HL:HW 1.03); snout slightly pointed in dorsal view, rounded in lateral aspect, projecting slightly beyond margin of the lower jaw; canthus rostralis distinct, bluntly angular; nostrils much closer to tip of snout than eye; eyes large (ED:HL 0.39); tympanum distinct almost half as large as eye (TYD:ED 0.41); Supra-tympanic fold distinct, originating from the posterior of eye to the axilla; Upper eyelids wide, (UEW 2.86), narrower than the interorbital space (UEW:IO 0.62). Inter-orbital space broader than the inter-narial space (IO:IN 1.33). Upper arms short (UAL:SVL 0.17), shorter than the lower arms (UAL:LAL 0.85); palm length longer than the upper arms (UAL:PAL 0.69); pointed projection (calcar) at tibio-tarsal articulation; relative length of fingers I < II < IV < III, tips of all fingers with well-developed discs with distinct circum-marginal grooves. Fingers partially webbed. Relative length of toes I < II < III < V < IV; tips of toes with well-developed disks with distinct circum-marginal grooves; disks smaller than those of toes. Dorsal skin smooth; flanks wrinkled; underside of chin and chest smooth, abdomen and thigh coarsely granular; the granulation much denser around the cloacal region; outer margin of both limbs with low dermal ridges.

Colouration in preservative. Dorsal colouration uniformly pale brown with mild traces of an irregular bluish black patch on the head. Ventral aspect of body pale cream coloured (Fig. 2).

Colouration in life of frogs recorded from Meghalaya. Dorsum, overall pale to medium brown in colour with small scattered black dots and three or four dark blackish brown transverse bands across the thigh and tibial region, tarsus feet and webbing between toes orangeish-red in colour. Webbing in fingers translucently yellow. Ventral sides mild brownish white and groins, thighs and the rest of the legs brownish yellow to dark red in colour. Some individuals with irregular florescent green patches on the head and mid body region (Fig. 4).

Natural history. The specimens of *R. tuberculatus* from Meghalaya examined during this study were collected from two different locations within West Garo Hills. A small shallow stream running parallel to the eastern boundary of the Sasatgre community reserve, the stream was bounded on both side by cardamom and banana plantations. The frogs were encountered at 1900 – 2300 hrs in the month of May, found perched on leaves of yam and cardamom plants, one to two meters above ground level. The other location was a similar habitat from another cardamom plantation near a forest patch near Baladingre village.

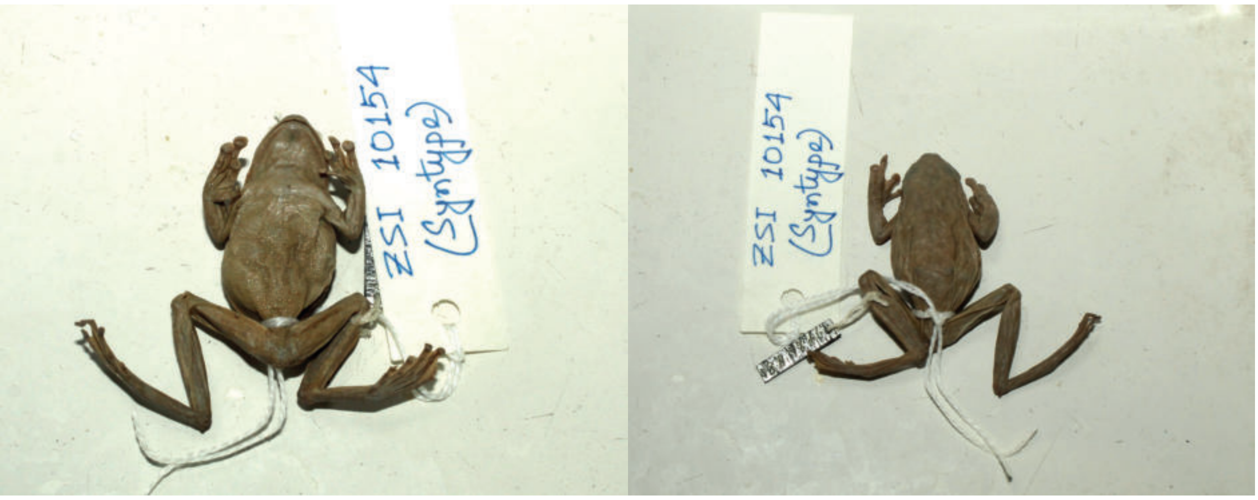

Figure 2. The ventral and dorsal view of the lectotype of *Rhacophorus tuberculatus* ZSI 10154.

Figure 3. The ventral and dorsal view of a syntype of *Rhacophorus turpes* BMNH 1940.6.1.30.

Figure 4. *Rhacophorous tuberculatus* in life from Garo hills, Meghalaya.

Table 4. Morphological (in mm) comparisons between our newly collected specimens of *Rhacophorus tuberculatus* from India and *R. verrucopus* from China and Myanmar. Data for *R. verrucopus* from China were obtained from Huang (1983) and Che et al. (2020), and data for *R. verrucopus* from Myanmar were from Liu et al. (2020) (“ – “ data unavailable).

Morphological variables	*R. tuberculatus*		*R. verrucopus*		*R. verrucopus*
	India		China		Myanmar
	Mean (Range) Male, n=6	Female, n=1	Mean (Range) Male, n=7	Female, n=1	Female, n=1
SVL	29.4 (27.0–30.9)	34.00	37.9 (36.0–40.6)	41.60	52.00
HL	9.0 (7.9–10.0)	11.60	12.5 (12.0–13.0)	12.20	17.60
HW	9.2 (8.9–9.4)	12.00	11.4 (10.9–12.3)	11.60	15.70
ED	4.0 (3.7–4.3)	4.90	4.3 (3.9–4.9)	5.20	5.40
ES	4.1 (3.6–4.5)	5.60	5.7 (5.1–6.7)	5.90	7.70
TYD	1.7 (1.2–2.1)	2.40	2.2 (2.0–2.4)	2.70	3.10
UEW	3.8 (3.7–4.2)	5.50	2.9 (2.5–3.6)	3.40	–
IO	3.1 (2.7–3.7)	4.10	4.3 (3.8–5.0)	3.60	–
IN	2.5 (1.7–3.0)	3.20	3.5 (3.2–4.1)	3.10	4.30
LAL+ PAL	12.6 (12.1–12.9)	17.60	16.5 (16.0–17.0)	18.80	17.80
PAL	7.1 (6.7–7.4)	9.60	10.6 (10.1–11.1)	11.70	–
FEL	12.7 (11.7–14.0)	15.10	17.3 (16.3–17.9)	20.50	22.90
TBL	14.4 (13.7–15.5)	17.30	17.9 (17.3–18.3)	21.20	24.20
FOL	10.4 (9.3–11.9)	13.00	15.3 (14.6–16.2)	19.00	20.40
HL/SVL	0.31 (0.29–0.33)	0.34	0.33	0.29	0.34
HL/HW	0.99 (0.89–1.07)	0.97	1.10	1.05	1.12
ED/HL	0.44 (0.37–0.49)	0.42	0.35	0.43	0.31
TYD/HL	0.19 (0.14–0.22)	0.21	0.17	0.22	0.18
FEL/SVL	0.43 (0.38–0.46)	0.44	0.46	0.49	0.44
TBL/SVL	0.49 (0.45–0.51)	0.51	0.47	0.51	0.47
TBL/FEL	1.14 (1.03–1.23)	1.15	1.04	1.03	1.06

Discussion

“Darwinian shortfall” is a major challenge faced by conservationists today, the lack of availability of molecular data for several extant species is a common phenomenon across taxa, leading to a situation where phylogenetic information is absent for most organisms, thus inhibiting a robust understanding of phylogenetic relationships within a particular group (Diniz-Filho et al. 2013). *Rhacophorus tuberculatus* is one such species which was described by Anderson (1871), based on three adult specimens. The original description was brief and lacked photographs or diagrams since it was from more than 150 years ago. Although several subsequent studies such as Ahmed et al. (2009) and Roy et al. (2018) reported photographic records of this species, there had been no studies involving a detailed taxonomic assessment of the species to date. As a result, the species had been overlooked in several studies, including the description of *Rhacophorus verrucopus* by Huang (1983). The validity of this species has been doubted by Che et al. (2020) who suggested that *R. verrucopus* could be a junior synonym of *R. tuberculatus* but due to the lack of molecular evidence this was not accepted. In the current study, we provide genetic divergence between *R. tuberculatus* and other congeners, including ‘*R. verrucopus*’ using 16s rRNA gene, thus filling an important knowledge gap. Further examination of *R. verrucopus* from China and Myanmar and *R. tuberculatus* from India based on re-examination of types and also freshly collected specimens used in this study revealed that there is an extensive overlap in morphology, and shallow molecular divergence (of a level that usually qualifies to be considered an intraspecific variation in the genus) between *R. verrucopus* and *R. tuberculatus*. Based on these lines of evidence we endorse the conclusions of Che et al. (2020) and formally place *R. verrucopus* under the junior synonymy of *R. tuberculatus*. Examination of the types (BMNH 1940.6.1.30 and BMNH 1974.828-832) of *Rhacophorus turpes* described from Kachin region of Northern Myanmar (26.24972°N, 97.23878°E) by Smith (1940) revealed that these specimens also closely match the morphological characters of *R. tuberculatus* except for a slightly truncated snout, a relatively less prominent tibio-tarsal projection, and fewer granulations on the ventral surface (Fig. 3). However, further studies utilizing molecular approaches based on fresh material are necessary to assess the taxonomic status of this species.

The current study also addresses the re-assessment of the threat status of *R. tuberculatus* as per IUCN Red List criteria of this Data Deficient species. *Rhacophorus tuberculatus* was known with certainty only from its type locality until now. However, in this study, we were able to resolve the taxonomic confusion with this species thus mapping its actual distribution range for the first time. The new records of this species from West Garo hills of Meghalaya mark the westernmost limit of the distribution of the species (Fig. 5). The up-listing or down-listing of species from one threat category to another of the IUCN Red List requires an assessment against all the five criteria (A–E, with 11 sub-criteria) but only one criterion needs to be fulfilled for designation of threat categories (IUCN 2001). In the case of *R. tuberculatus*, the information on geographic distribution seems the most accurate and reliable among all other criteria and hence, used for a conservative estimate of the extent of occurrence (Criteria B, B1). The current Extent Of Occurrence for this species estimated based on all known localities is about 1,07,600 km^2 and hence,

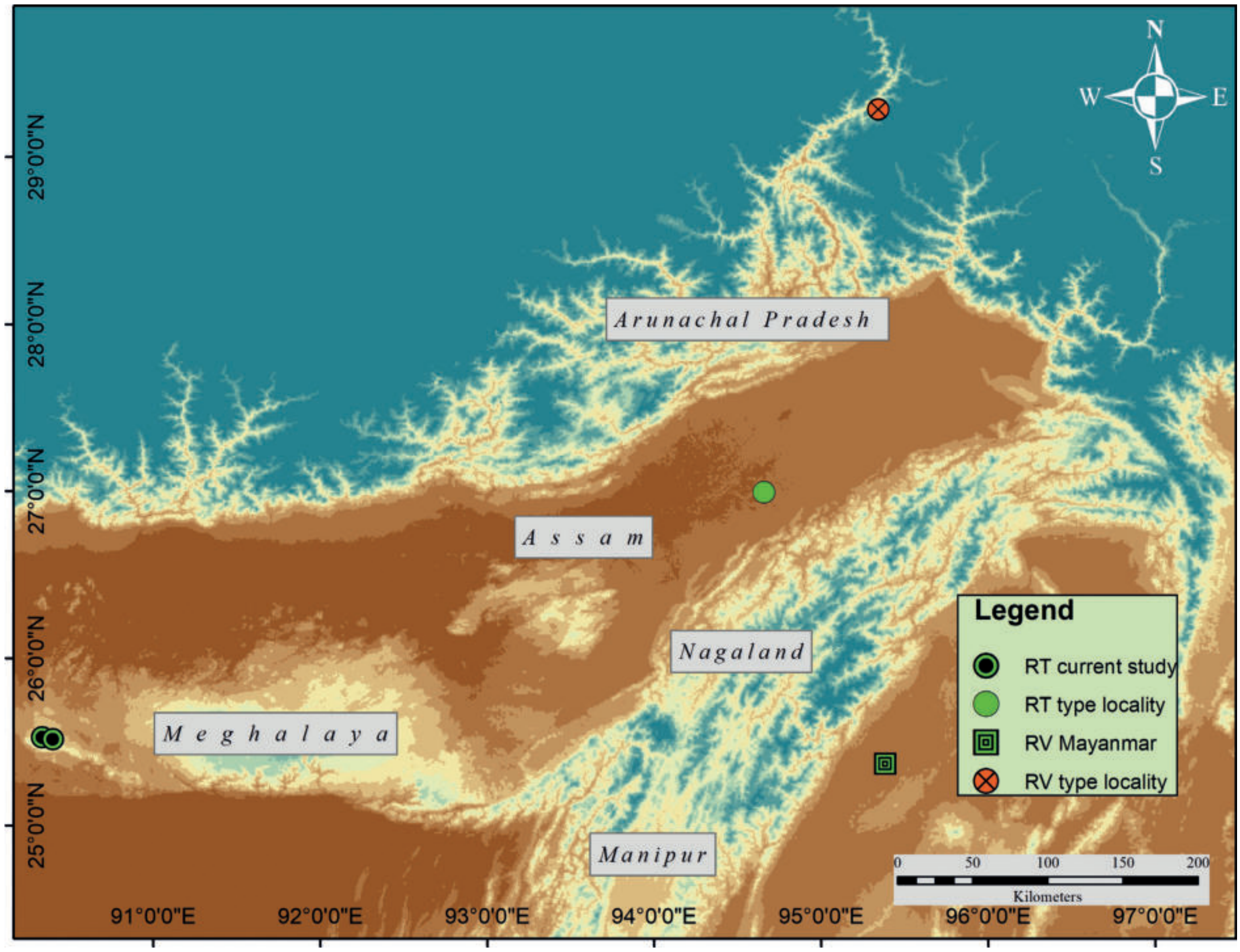

Figure 5. Updated distribution map of *R. tuberculatus* including records from Meghalaya (Current study) in green and black circle, from Myanmar in green and black square, type locality of "*R. verrucopus*" in red circle and type locality of *R. tuberculatus* in green circle.

with all the updated information presented here, we recommend transferring the species from Data Deficient to Least Concern.

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Geographic distribution of the rare and endangered *Telmatobufo venustus* (Philippi, 1899) (Anura, Calyptocephalellidae), with the description of a new locality and comments on the type locality

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Abstract

Telmatobufo venustus was one of the rarest endemic amphibians of Chile until 2020. Prior to that year, this species had been known in four localities, three of them with uncertain location, including the type locality. However, three new precise localities have been reported successively since 2020, all based on a few individuals. In this study, we review the geographic information on the species and, based on literature and other documents, tentatively locate the three localities with uncertain location published before 2020. Furthermore, we describe a new locality near the uncertain southern end of its distribution. Although the number of localities has considerably increased since 2020, the species still has a highly fragmented known distribution, its type locality and southern limit cannot be located with certainty, and it is found in only two state-protected areas. Due to these reasons and because threats to the habitat have been identified in some localities, we suggest keeping the species as Endangered.

Key Words

altitudinal range, Altos de Lircay National Reserve, *Nannophryne variegata*, tadpoles, southern limit, type series

Introduction

Calyptocephalella gayi (the Chilean giant frog) and *Telmatobufo* (four species, Mountain false toads) comprise the family Calyptocephalellidae, one of the most ancient anuran lineages from South America (Feng et al. 2017). This family, endemic to south-central Chile (western side of the Andes), is more related to the Australasian family Myobatrachidae than to any South American lineage, which is consistent with a Gondwanan origin (Correa et al. 2008; Mörs et al. 2020). The family has a rich fossil record, mainly in Argentinean Patagonia, where several extinct species of the genus *Calyptocephalella* have been described (reviewed by Nicoli et al. 2022), but no fossils of the genus *Telmatobufo* are known. Due to its old evolutionary age, wider geographical distribution in the past, low number of current species, and high morphological and ecological disparity of the genera that compose it, Calyptocephalellidae can be considered as a relict lineage.

The two genera exhibit different distribution patterns. *Calyptocephalella gayi* (Duméril & Bibron, 1841) inhabits lentic environments, from the semi-arid zone (28°S) to the temperate forests of southern Chile (41°30'S), covering the entire intermediate Mediterranean zone (Mora et al. 2021). In contrast, the four species of *Telmatobufo* are distributed exclusively in the temperate forests of south-central Chile (35°30'S–41°S). *Telmatobufo ignotus* Cuevas, 2010, whose presence has only been confirmed at the type locality and surroundings (~ 36°S), and *T. bullocki* Schmidt, 1952 (37–38°S) have allopatric distributions in the Coastal Range, while *T. venustus* (Philippi, 1899) (35°30'S to ~ 38°S) inhabits the foothills of the Andes, and *T. australis* Formas, 1972 (39°25'S to ~ 41°S) is found in both mountain ranges (Formas et al. 2001).

Telmatobufo venustus is the species of the genus with the largest latitudinal distribution, but with the second fewest records. Since its description as *Bufo venustus* (Philippi 1899), there were no published records of the species until the year 1982. Formas and Veloso (1982) clarified the taxonomic status and generic assignment of *T. venustus* with material from a new locality, Alto de Vilches (Altos de Lircay National Reserve). In 1983, a tadpole was found in the locality of Ralco (Díaz et al. 1983), but the species has not been observed there again. Only recently, since 2020, three new localities have been reported (Caro-Lagos and Charrier 2020; Díaz-Páez and Alveal 2021; González-Véliz et al. 2022), one of which slightly extended the range of the species to the north (Caro-Lagos and Charrier 2020) (Table 1).

The description of *Telmatobufo venustus* (Philippi 1899) was based on specimens from two localities: Hacienda San Ignacio de Pemehue and Cordillera de Chillán, whose exact locations are currently unknown. Formas et al. (2001) reviewed the geographic distribution of the species and indicated that the type locality is Cordillera de Chillán, but they do not mention Hacienda San Ignacio de Pemehue. This locality potentially corresponds to the southern limit of its distribution and has been omitted from almost all sources of geographic information of the species, except for IUCN (2022). The two locations reported later, Alto de Vilches (Formas and Veloso 1982) and Ralco (Díaz et al. 1983), were described with low precision (sexagesimal coordinates up to minutes). In both cases, the coordinates do not agree with the named sites where the specimens were supposedly collected. Later, Araya and Cisternas (2008) provided a satellite map with points within and around the Altos de Lircay National Reserve, but no coordinates. Regarding Ralco, Fenolio et al. (2011) indicated that the place was flooded due to the construction of a dam. Only the localities reported since 2020 have been published with precise coordinates.

Telmatobufo venustus has always been considered rare. Philippi (1899, 1902) had already mentioned its rareness in Hacienda San Ignacio de Pemehue. Until 1998 (i.e., 99 years after its description), adults had not been collected again (Fenolio et al. 2011). They were observed that year in the Altos de Lircay National Reserve (Formas and Cuevas 2000). The most recent reports have been based on a few individuals, while only the Altos de Lircay National Reserve seems to host a stable population (Fenolio et al. 2011). In addition to its low abundance, the species appears to have a highly fragmented distribution. It is known from fewer than 10 areas, spaced over 300 km. The low number of known localities and the fragmentation of its distribution justify its classification as Endangered by the IUCN (2022) and the Chilean state categorization system, the Reglamento de Clasificación de Especies Silvestres (RCE).

Here we review the geographic information of *T. venustus* in the literature and online databases (Global Biodiversity Information Facility, GBIF, https://www.gbif.org; iNaturalist, https://www.inaturalist.org) to map all the points of presence described to date. We address the problem of locating the type locality, which is linked to the fact that no holotype was designed by Philippi (1899). We also propose possible locations for three historical localities reported until 1983, including the type locality and the point that would define the southern limit of the species, and clarify the altitudinal limits of the species. Finally, we describe a new population near the southern limit and the population of Ralco, where the species has not been seen for more than 40 years.

Materials and methods

Compilation of localities and related information

This review is based on all publications with information on *T. venustus*, including journal articles, books, book chapters, guides and online sources. Some of these sources contain dot maps (Formas et al. 2001; Caro-Lagos and Charrier 2020; Díaz-Páez and Alveal 2021; González-Véliz et al. 2022), surface maps (Charrier 2019; IUCN 2022) or less precise or incomplete information on the geographic range and/or altitudinal limits (e.g. Formas 1995; Veloso 2006; Rabanal and Nuñez 2008; Stuart et al. 2008; Lobos et al. 2013). Presence points were downloaded from GBIF and iNaturalist; both sites were accessed on March 30, 2023. In addition to the coordinates and altitude, for each point some clarifications on its location were added (Table 1, column Remarks).

Map

A map with all the localities collected was made in ArcMap v10.8, using satellite imagery as a base. We used the exact coordinates provided in each publication, except in the case of the localities of the Altos de Lircay National Reserve and surroundings (Araya and Cisternas 2008), which were extracted directly from their satellite map. The two original localities, Hacienda San Ignacio de Pemehue and Cordillera de Chillán (Philippi 1899, 1902), where the species has not been recorded again,

Table 1. Localities and areas where *Telmatobufo venustus* has been recorded. For each locality/area, the number with which it appears on the map in Fig. 1 is indicated in parentheses after the name. Only the source(s) where each locality/area was mentioned for the first time and where relevant information was added later are indicated. For each locality/area, published coordinates, atitude, and some clarifications about their location (Remarks column) are provided.

Locality/area	Source(s)	Published coordinates	Altitude (m)	Remarks
Hacienda San Ignacio de Pemehue (the four red segments of Germain's reconstructed routes, Fig. 2B)	Philippi (1899, 1902)	Not provided	914–1219 (originally, 3000–4000 feet above sea level, Philippi 1899)	One of the two localities, together with Cordillera de Chillán, from where the specimens used to describe the species came from
Cordillera de Chillán (9)	Philippi (1899)	Not provided	914–1219 (originally, 3000–4000 feet above sea level); ~ 1200 m (Formas and Veloso 1982; Formas et al. 2001)	One of the two localities, together with Hacienda San Ignacio de Pemehue, from where the specimens used to describe the species came from; omitted by Philippi (1902); type locality according to Formas and Veloso (1982) and Formas et al. (2001); it could correspond to the vicinity of Recinto (this study, see Results)
Alto de Vilches (= Altos de Vilches, Vilches Alto, Río Lircay, Altos de Lircay National Reserve)	Formas and Veloso (1982)	Not provided in Formas and Veloso (1982); 35°28'S, 71°11'W (Formas and Cuevas 2000; Nuñez and Formas 2000; Cuevas and Formas 2001); 35°32'S, 70°50'W (Araya and Cisternas 2008); 35°35'41"S, 71°04'27"W (Núñez and Gálvez 2015)	1280 (Formas and Veloso 1982); 900 (Cuevas and Formas 2001)	Coordinates of Formas and Cuevas (2000), Nuñez and Formas (2000) and Cuevas and Formas (2001) fall outside of the Altos de Lircay National Reserve; coordinates of Araya and Cisternas (2008) do not match their map, in which six exact points of presence within or around the Río Lircay National Reserve are shown (detailed below); coordinates of Núñez and Gálvez (2015) are only referential
Arroyo Puente del Tronco (inside the Altos de Lircay National Reserve) (2)	Araya and Cisternas (2008)	Not provided, but the site is shown on a map	1638	Located here approximately at 35°35'33"S, 71°00'04"W
Chorro de Checo (inside the Altos de Lircay National Reserve) (3)	Araya and Cisternas (2008)	Not provided, but the site is shown on a map	1554	Located here approximately at 35°35'40"S, 71°00'49"W
Arroyo Nido de Carpinteros (inside the Altos de Lircay National Reserve) (4)	Araya and Cisternas (2008)	Not provided, but the site is shown on a map	1543	Located here approximately at 35°35'53"S, 71°01'35"W
Río Lircay Camino Viejo (inside the Altos de Lircay National Reserve) (5)	Araya and Cisternas (2008)	Not provided, but the site is shown on a map	1346	Located here approximately at 35°35'44"S, 71°02'13"W
Estero Piedras Tacitas (near the Altos de Lircay National Reserve) (6)	Araya and Cisternas (2008)	Not provided, but the site is shown on a map	1164	Located here approximately at 35°36'33"S, 71°04'17"W
Pantano (near the Altos de Lircay National Reserve) (7)	Araya and Cisternas (2008)	Not provided, but the site is shown on a map	1435	Located here approximately at 35°37'06"S, 71°04'21"W
Ralco (12, two points)	Díaz et al. (1983)	37°59'S, 71°24'W; 37°53'28"S, 71°38'03"W (Núñez and Gálvez 2015)	Not provided	The tadpole was collected "in the Bío-Bío River", but the coordinates of Díaz et al. (1983) fall around 8 km east of this river; Fenolio et al. (2011) affirm that the locality was flooded by a dam; the referential coordinates of Núñez and Gálvez (2015) fall in the Biobío River, but ~ 22 km northwest of the point of Díaz et al. (1983); in the vicinity of the "Ralco River" according to González-Véliz et al. (2022)
Radal Siete Tazas National Park (1)	Caro-Lagos and Charrier (2020)	35.498174°S, 70.929807°W	1524	On a tributary of the Claro River (Caro-Lagos and Charrier 2020)
Altos de Malalcura, Cajón de las Pulgas (10)	Díaz-Páez and Alveal (2021)	37°23'06"S, 71°29'45"W	1062	On a tributary of the Malalcura River
ca. San Fabián de Alico (8)	González-Véliz et al. (2022)	36.740589°S, 71.376011°W	1228	Coordinates fall in Quebrada Los Baños, 25 km southeast of the town San Fabián de Alico
Estero Pichipangue (Fig. 2A), Pitrilon sector (11)	This study	37.8983°S, 71.5085°W	1150	Tributary of the Pangue River, which flows into the right bank of the Biobío River

were published without coordinates. Therefore, we resorted to literature and other documents from the late 19th and early 20th centuries to pin down their location more precisely. One of these sources (Germain 1894) was used to trace the possible route of the trip where that author supposedly collected one of the type specimens. Areas within the altitudinal limits specified in the description of the species, between 914 and 1219 meters (3000–4000 feet), were mapped using ArcMap to restrict the location of possible collection places for this specimen. Moreover, a layer was added with the areas protected by the Sistema Nacional de Áreas Silvestres Protegidas del Estado de Chile (SNASPE) to determine which localities are state protected. Finally, a similar map was produced for the GBIF and iNaturalist points.

New locality

The new locality of *T. venustus* was discovered by chance by one of the authors (J.I. Osses). The coordinates and altitude were recorded in situ with a cell phone and verified on Google Earth. The size of the adult individuals (snout-vent length, SVL) was estimated with the lid of a camera (diameter 52 mm) placed next to them. None of the observed individuals was captured.

Results

Compilation of localities

We compiled 14 localities of *Telmatobufo venustus*, including the new one described by us (Table 1). We did not include the locality of Niblinto (Ibarra et al. 1999) in Table 1, cited as an imprecise record by González-Véliz et al. (2022). The original source does not specify if it is in the National Reserve or in the Nature Sanctuary called Los Huemules del Niblinto, it was mentioned without coordinates, and it is not supported by collected or photographed material. Therefore, we consider that the presence of the species in that area requires confirmation. We mapped only 13 localities (Fig. 1), since Alto de Vilches (Formas and Veloso 1982) was replaced with more precise sites subsequently reported within and around the reserve (Araya and Cisternas 2008). Five of the mapped locations are within state-protected areas (SNASPE): one in the Radal Siete Tazas National Park, which constitutes the northern end of the distribution of the species, and four in the Altos de Lircay National Reserve (Fig. 1). Eighteen points were downloaded from iNaturalist and nine from GBIF (Suppl. material 1), but six of the latter (the only ones with coordinates) come from iNaturalist and have the same associated coordinates. Most of the iNaturalist points fall in low-lying anthropized areas, along roads or in difficult-to-access mountainous areas, but all around the Altos de Lircay National Reserve (Suppl. material 2). Therefore, we assume that the observations were made in that reserve, but the coordinates have been obscured by default (with an accuracy of ~ 28.7 km) because it is a threatened species (taxon geoprivacy). Thus, we do not include these points in the collection of localities (Table 1, Fig. 1) because we consider them redundant with the published geographic information of the species.

Type locality and type series

Formas and Veloso (1982) were the first to indicate that the type locality of *T. venustus* is Cordillera de Chillán (Andean foothills, east of the city of Chillán), which has been included in some maps despite being an indeterminate place (Formas et al. 2001; Díaz-Páez and Alveal 2021). The mention of Cordillera de Chillán as the type locality is based on a specimen from the collection of the Museo de Zoología de Concepción (MUZUC or MZUC 205051) labeled as the holotype (as it appears in the photograph of the same source), and recognized as such in subsequent publications (e.g. Formas 1995; Formas et al. 2001). The problem with this type locality is that Philippi (1899) described the species from four specimens from Hacienda San Ignacio de Pemehue and Cordillera de Chillán, without defining a holotype, and the only remaining specimen of the type series (MZUC 205051) apparently has a wrong collection number and is currently missing. The only specimen from the Hacienda San Ignacio de Pemehue had already been lost at the time of the description (Philippi 1899). In the 1970s, only one of the three specimens from Cordillera de Chillán could be in the collection of the Museo Nacional de Historia Natural of Santiago (MNHN). Apparently, this specimen is one of the two that existed in the MNHN collection at the beginning of the 20th century, whose localities of origin were Chillán and El Recinto (Quijada 1914). This specimen appears photographed in Donoso-Barros (1972) and is the same one identified as MZUC 205051 by Formas and Veloso (1982). There is no published information on the specimen MZUC 205051 before 1982, so we speculate that it was entered into the collection and identified as the holotype by Roberto Donoso Barros in the early 70's, but without a nomenclatural act formally published in the peer-reviewed literature. Furthermore, since Philippi (1899) did not select a specimen from the type series as the holotype, this only remaining syntype should have been designated as the lectotype (International Code of Zoological Nomenclature, ICZN 1999), thereby also automatically defining the type locality (Cordillera de Chillán). Unfortunately, we could neither find any specimen accurately identifiable as *T. venustus* in the MZUC collection nor a record with the number 205051, since the number of the collection does not reach 50,000 specimens yet. The apparent loss of the only remaining specimen of the type series and confusion about its collection number prevent us from amending its typification (designating it the lectotype), and from recognizing the currently accepted type locality of the species. Consequently, following the rules of the ICZN (1999), in the absence of a holotype or lectotype, the type locality of *T. venustus* corresponds to the two localities of origin of the type series: Hacienda San Ignacio de Pemehue and Cordillera de Chillán.

Proposed locations for historical localities

The only specimen of *T. venustus* from Hacienda San Ignacio de Pemehue known to date (Philippi 1899) was collected by the entomologist Philibert Germain. At the time, that estate ("hacienda") covered more than 240,000 hectares (Flores 2013), from near the city of Mulchén to the town of Lonquimay in the south, and eastward to the border with Argentina. We reconstructed the possible route that Germain followed during his exploration of the estate in December 1893 (Germain 1911), which is described in Germain (1894). The trip can be divided into two parts. In the first stage, Germain started from a place called El Cisne, following the right bank of the Renaico River, then, crossed a small mountain range, Pichinitrun (also known as Pichinitro, Pichinitron or Pichi-Nitron), to reach the Vilucura River basin (also known as Vilicura or Villucura), and followed the right bank of this river to reach a place called Lolco. We used the map of the Comisión Chilena de Límites (1908) to plot this part of the journey as there is a road that exactly matches the description of Germain

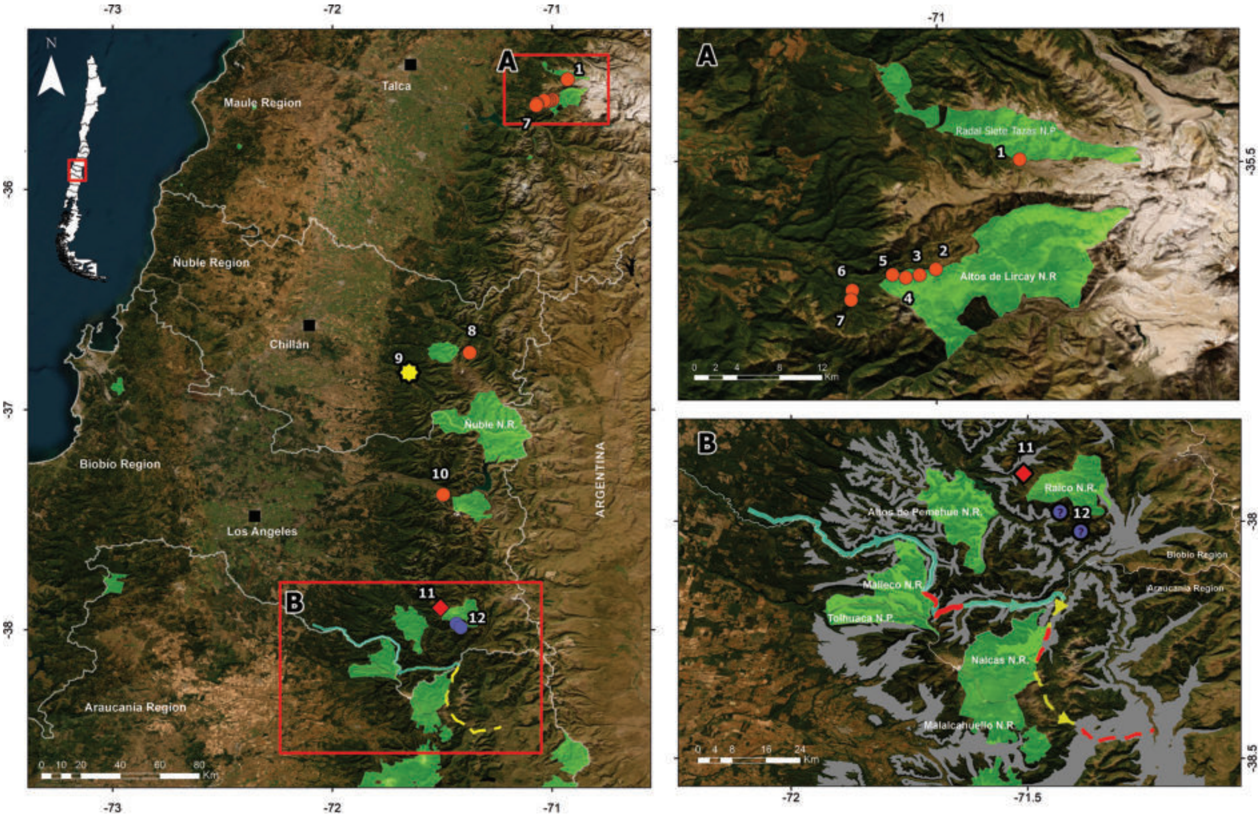

Figure 1. Geographic distribution of *Telmatobufo venustus*. Orange circles: literature localities; yellow octagram: possible location of Cordillera de Chillán according to this study; red diamond: new locality described here; blue circles: possible locations of Ralco. The insets to the right show enlargements of the northern (**A**) and southern (**B**) ends of its distribution. Inset B shows the possible routes (greenish solid and yellow dashed lines) that Philibert Germain followed, from west to east, on his journey inside the Hacienda San Ignacio de Pemehue in 1893 (see details in Results). The red segments of the solid and dashed lines represent the sectors of Germain's routes that lie between 914 and 1219 meters (gray areas of inset B). The semi-transparent green areas represent the areas protected by the state of Chile (SNASPE). The thin white lines within Chile correspond to the boundaries of the administrative regions (named with white letters).

(1894) (Fig. 1B, greenish solid line). The second part of the trip was not described in detail by Germain (1894). He apparently set out from Lolco up the right bank of the river almost to its source (the Headwaters of Lolco), crossed a bare alpine area of the Cordillera de Toluaca (Tolhuaca Range) to reach the basin of the Lonquimay River, and finally followed a tributary of this river (not named) until reaching the Lonquimay "pampa", where the administrator's house of this sector of the estate was located. This part of the trip (Fig. 1B, dashed yellow line) was traced partly following current paths (Google Earth); the location of the administrator's house was located according to the map by Soza (1891). We identified four areas along the reconstructed tracks, one of which could correspond to the southern limit of the range of the species (red segments of the paths in Fig. 1B), considering the elevation range specified (3000–4000 ft) in the species description (Philippi 1899). Regarding the accepted type locality until now, Cordillera de Chillán (Formas and Veloso 1982), González-Véliz et al. (2022) highlighted that the new locality near San Fabián de Alico described by them would be close to it. In fact, the new locality is in the Andean foothills, approximately 66 km east of Chillán, but there is no direct way to get there from this city. Instead, we propose a more precise place for the locality of Cordillera de Chillán, in the vicinity of the town of Recinto, located about 47 km southeast of the city of Chillán, on the road that ends at Termas de Chillán. This is based on a specimen of *Bufo venustus* that appears in the catalog of amphibians housed in the MNHN at the beginning of the last century published by Quijada (1914), labeled "El Recinto, 1897". According to Philippi (1899), Germain brought to the Museum the specimens from the Cordillera de Chillán that were used to describe the species in January 1897. Germain described numerous insects in Chile, including some beetles from Termas de Chillán (Germain 1911). Thus, it is possible that he collected the individuals of *T. venustus* from Cordillera de Chillán at an intermediate point of the way such as Recinto. Finally, we also propose possible locations for the locality of Ralco (Díaz et al. 1983). The only specimen observed there, until now, was collected supposedly in the Biobío River, but this type of river is different from the typical environment where the species is known up to now (Fenolio et al. 2011). In addition, the imprecise coordinates that appear in Díaz et al. (1983) fall about 8 km east of the Biobío River, on the side of a mountain at ~ 1885 m elevation and far from currently recognizable roads. Therefore, we tentatively locate “Ralco” in the upper part of the two tributaries (Malla and Quepuca rivers; points 12 of Fig. 1B) of the Biobío River closest to the coordinates given by Díaz et al. (1983). We also emphasize that there is no additional data or information published about the locality reported by Díaz et al. (1983) to affirm that it was flooded after 1983 as it appears in the literature (Fenolio et al. 2011; Díaz-Páez and Alveal 2021).

Altitudinal range

There is no current consensus in the literature about the altitudinal limits of *T. venustus*. For example, Formas et al. (2001) defined the range between 600 and 1280 m, while an earlier source (Formas 1995) specified higher limits (1500–1700 m), which appear in most of the subsequent publications and sources of information about the species (e.g. Díaz-Páez and Ortiz 2003; Rabanal and Nuñez 2008; Stuart et al. 2008; Fenolio et al. 2011; Díaz-Páez and Alveal 2021), and even in the species summary information file of the Ministry of the Environment of Chile (https://clasificacionespecies.mma.gob.cl/wp-content/uploads/2019/10/Telmatobufo_venustus.pdf). Considering the records with precise altitudes collected here (Table 1), the highest point is Arroyo puente del tronco, at 1638 m, inside the Altos de Lircay National Reserve (Araya and Cisternas 2008). Regarding the lower limit, we discard that of Formas et al. (2001) (600 m) because there is no reliable precedent that allows to locate any population at that altitude. On the other hand, Cuevas and Formas (2001) reported the presence of *T. venustus* at the type locality of *Alsodes hugoi* Cuevas & Formas, 2001, Altos de Lircay National Reserve, at 900 m, specifically on the banks of the Lircay River. In the extreme northwest of the Reserve, there are sectors on the south bank of the Lircay River at around 900 m, which could be the area where the type series of *A. hugoi* was collected. Furthermore, this limit would be close to the originally minimum altitude (3000 ft = 914 m) reported by Philippi (1899). Therefore, we define the altitudinal limits of *T. venustus* as between ~ 900 and 1638 m.

New locality

The new locality, Estero Pichipangue (Fig. 2A), corresponds to a melt stream with crystalline waters located on the northwest slope of the Callaqui volcano and that flows into the Pangue river, which in turn flows into the Biobío River, in a sector called Pitrilon (southeast of the Biobío Region). On February 16, 2022, eight *T. venustus* adults (Fig. 2B) were observed at night on the

Figure 2. Stream and anurans observed at the new locality of *Telmatobufo venustus*, Estero Pichipangue, Biobío Region, Chile. **A.** Stream near the site where the adults and larvae of *T. venustus* were found; **B.** Adult male of *T. venustus* from Estero Pichipangue; SVL ~ 6.5 cm; **C.** Tadpole of *T. venustus*; **D.** Adult of *Nannophryne variegata*.

banks of Estero Pichipangue, at an approximate height of 1150 m. The individuals were easily recognized as members of the genus *Telmatobufo* by the presence of parotid glands, the numerous and prominent glands on the back, highly developed interdigital webbing on the hindlegs and the vertical pupil, and at the species level by their orange-reddish spots on a dark background. An average SVL of 65 mm was estimated for the eight observed adults (range, ~ 60–70 mm). The stream where the adults were observed presents a gentle slope, with a maximum depth of 30 cm, and a rocky and sandy bottom. Inside the stream, three *T. venustus* larvae were observed clinging to the bottom rocks (Fig. 2C). The tadpoles were identified as *Telmatobufo* by their dorsoventrally flattened bodies, the presence of a suctorial oral disk, and by its thick tail, particularly at the base (Díaz et al. 1983; Fenolio et al. 2011). The habitat where the adults and larvae of *T. venustus* were found corresponds to mountain mixed forest dominated by *Nothofagus dombeyi*, and, to a lesser extent, by *N. alpina*; the understory is dominated by *Chusquea quila*, *Fuchsia magellanica* and abundant *Gunnera tinctoria*. About 3.2 km upstream from the site where the *T. venustus* individuals were observed, close to the tree line, an adult of *Nannophryne variegata* Günther, 1870 (SVL = ~ 50 mm) was observed on the bank of the stream, among the riparian vegetation (Fig. 2D). This new record of *N. variegata* is located about 50 km south from the northernmost known point of the species (Cisternas-Medina et al. 2019). The Estero Pichipangue is far from human settlements and shows very few signs of human intervention. No salmonids were observed in the stream, a potential threat to *T. venustus* larvae and other amphibians (Fenolio et al. 2011), but the area around the stream could be threatened by livestock, since it is used for “veranadas” (summer pastures).

Discussion

After 124 years since its description, *T. venustus* is still only known from a few precise localities and its altitudinal and southern distribution limits cannot be clearly inferred from the literature. In fact, almost half of the known points are within or around the Altos de Lircay National Reserve, the only area where the species can currently be observed with relative ease (Fenolio et al. 2011; C. Correa and J. Morales, personal observations, February 2022). This is partly because it is a protected area with several trails to walk that receives many visitors, mainly in summer. Regarding online databases, GBIF and iNaturalist do not contain additional points that contribute to better define the distribution of the species, since they are all concentrated around the Altos de Lircay National Reserve, and do not have precise coordinates or names. The latter adds more uncertainty to the scarce precise geographic information on the species, which is why we recommend not using the *T. venustus* records of those databases.

The present review allowed us to propose locations for some historical localities and better clarify the altitudinal limits of the species. Specifically, we identified possible places for the two localities that we consider here as the type locality, Hacienda San Ignacio de Pemehue and Cordillera de Chillán. Furthermore, we specified the altitudinal limits, although we emphasize that, with the available information, the lower limit (900 m) cannot be determined with certainty. According to the literature, *T. venustus* is a rare and secretive species (Stuart et al. 2008; Fenolio et al. 2011). The scarcity of records and the small number of individuals observed in most localities over 120 years support this perception. The low number of known localities is probably due to a lack of exploration, since the four locations described as of 2020, including the one in this study, are found in hard-to-reach areas and generally far from public roads. In fact, there are still relatively large gaps throughout the distribution; for example, more than 120 km between Altos de Lircay National Reserve and the vicinity of San Fabián de Alico; and more than 50 km between Cordillera de Chillán and Altos de Malalcura, and between this last locality and Estero Pichipangue (Fig. 1).

Telmatobufo venustus is listed currently as Endangered by the IUCN (2022; assessment from 2015) and the RCE (Decree of 2011), based on criteria related to geographic distribution (B1 and B2, IUCN; only B2, RCE). The IUCN considers that the species only occurs in two threat-defined locations, Altos de Lircay National Reserve, where the only stable subpopulation would be, and Ralco, and has an area of occupancy (AOO) of 499 km^2 and an extent of occurrence (EOO) of 996 km^2. Using the geographic information updated here, a precise estimate of the EOO cannot be obtained, as long as the southern distribution limit of *T. venustus* is not clear, but following the IUCN recommendations (4 km^2 per location) and defining nine threat-defined locations, the AOO could be 36 km^2. This estimate may vary depending on how many locations are defined for the six points of the Altos de Lircay National Reserve and surroundings (here they were grouped into two, considering their distances, water connectivity and their protection status; see Fig. 1A) and if the populations of historical records are considered extinct or not. However, the value falls below the limit of subcriterion B2 (< 500 km^2) to preliminarily ratify the species as Endangered.

To assign a category of threat under criterion B, the species also must meet two or more conditions related to some characteristics of its geographic distribution and the quality of its habitat. The first condition (a), severely fragmented distribution, is maintained despite the sustained increase in known localities since 2020. Fenolio et al. (2011) provided arguments to apply the second condition (b), based on observations in the Altos de Lircay National Reserve: a decrease in the quality of the habitat where the larvae develop due to the presence of rainbow trout and the potential presence of chytrid fungus (not detected for them). After more than a decade, one more threat may be added. Caro-Lagos and Charrier (2020) describe how several streams within the Radal Siete Tazas National Park have been progressively drying up due to the megadrought that has been affecting central Chile for more than a decade (Garreaud et al. 2020), phenomenon that could also be occurring in the Altos de Lircay National Reserve, located only 12 km further south (Fig. 1A). In addition, the megadrought is closely associated with the increase in the frequency and intensity of forest fires (González et al. 2018), another threat identified for the species by Veloso (2006) and for *T. ignotus* (Ortiz & Briones, 2022) in the Coastal Range. Thus, the detected and potential threats to the habitat justify maintaining the Endangered category for the species (under subcriterion B2ab(iii)), despite the recent increase in known populations.

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Supplementary material 1

Some data associated with iNaturalist and GBIF geographic records

Authors: Claudio Correa, José Ignacio Osses, Jesús A. Morales, Juan Carlos Ortiz

Data type: csv

Link: https://doi.org/10.3897/herpetozoa.36.e107328.suppl1

Supplementary material 2

Geographic records of *T. venustus* obtained from iNaturalist and GBIF

Authors: Claudio Correa, José Ignacio Osses, Jesús A. Morales, Juan Carlos Ortiz

Data type: tif

Explanation note: The yellow circles represent the points from iNaturalist, while the blue squares are the ones that appear on both platforms. Orange circles are the nearby points described in the literature (1–7, Fig. 1A and Table 1)

Link: https://doi.org/10.3897/herpetozoa.36.e107328.suppl2

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Migration strategy of the Great crested newt (*Triturus cristatus*) in an artificial pond

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Abstract

In animals, migration is an evolutionary adaptation to manage seasonally varying habitats. Often driven by climatic changes or resource availability, amphibians then migrate from their hibernation sites to their breeding grounds. This research focused on the migratory habits of the Great crested newt (*Triturus cristatus*). The study explored factors like gender, body size, and environmental determinants, noting that immigration and emigration events proved distinct during the year. Results unveiled that males typically reached ponds first, with temperature being pivotal: males preferred up to 5 °C, females around 10 °C, while juveniles moved as temperatures increase. Wind velocity affected larger newts, around 120 mm, prompting them to migrate with stronger winds. Notably, heavy rainfall favored migration of newts of roughly 60 mm size. Humidity displayed gender-based trends: males associated positively with average levels, females showed aversion above 50%, and juveniles leaned towards drier conditions. Emigration patterns mirrored these findings, emphasizing roles of temperature, wind, and humidity. The effect of moonlight is not statistically significant. These findings provide valuable insights into the environmental factors influencing the migration of *T. cristatus*, which may guide future conservation efforts.

Kurzfassung

Bei Tieren ist die Migration eine evolutionäre Anpassung an saisonal wechselnde Lebensräume. Oft sind es klimatische Veränderungen oder die Verfügbarkeit von Ressourcen, die dazu führen, dass Amphibien von ihren Überwinterungsplätzen zu ihren Brutgebieten wandern. Diese Studie befasste sich mit den Wanderungsgewohnheiten des Kammmolchs (*Triturus cristatus*). Die Studie untersuchte Faktoren wie Geschlecht, Körpergröße und Umweltfaktoren und stellte fest, dass sich Ein- und Auswanderungsereignisse im Laufe des Jahres unterscheiden. Die Ergebnisse zeigten, dass die Männchen in der Regel zuerst die Teiche erreichten, wobei die Temperatur ausschlaggebend war: Die Männchen bevorzugten Temperaturen bis zu 5 °C, die Weibchen etwa 10 °C, während die Jungtiere mit steigenden Temperaturen abwanderten. Die Windgeschwindigkeit wirkte sich auf größere Molche (ca. 120 mm) aus und veranlasste sie, bei stärkerem Wind zu wandern. Vor allem Molche mit einer Größe von etwa 60 mm bevorzugten bei ihrer Wanderung starke Regenfälle. Die Luftfeuchtigkeit zeigte geschlechtsspezifische Tendenzen: Männchen assoziierten sich positiv mit durchschnittlichen Werten, Weibchen zeigten eine Abneigung gegen Werte über 50%, und Jungtiere neigten zu trockeneren Bedingungen. Die Auswanderungsmuster spiegeln diese Ergebnisse wider und unterstreichen die Rolle von Temperatur, Wind und Feuchtigkeit. Der Einfluss des Mondlichts ist statistisch nicht signifikant. Diese Ergebnisse bieten wertvolle Einblicke in die Umweltfaktoren, die die Migration von *T. cristatus* beeinflussen, und können als Grundlage für künftige Schutzbemühungen dienen.

PENSOFT.

Key Words

emigration, immigration, moon phase, rainfall, temperature, *Triturus cristatus*, wind

Schlüsselwörter

Auswanderung, Einwanderung, Mondphase, Niederschlag, Temperatur, *Triturus cristatus*, Wind

Introduction

Migration is an adaptive behavior seen in animals that enables them to deal with environments that change seasonally (Alerstam and Lindström 1990; Duellman and Trueb 1994). This phenomenon also includes breeding migrations, like amphibians in temperate regions that annually transition from terrestrial habitats to wetlands for offspring development (Russell et al. 2005; Dingle 2014). Migrations serves multiple purposes, such as congregating individuals for mating, facilitating the use of alternate resources, or helping avoid physiological limitations that might impact survival (Grayson et al. 2011). Species' migratory responses can differ due to variations in physiological tolerance and desiccation resistance, especially in the context of changing climates (Todd and Winne 2006). Changes in rainfall, for instance, can impact pond-breeding amphibian populations by affecting their habitats and increasing larval mortality (Semlitsch and Wilbur 1988; Carey and Alexander 2003; Daszak et al. 2005). Understanding how climatic factors influence over-land migration patterns in diverse amphibians is crucial to assessing future impacts of climate change and possible strategies for mitigation. The great crested newt (*Triturus cristatus*) is a notable amphibian species which has suffered significant population declines (Beebee and Griffiths 2000; Arntzen et al. 2009). Movement behavior is of paramount importance in conservation biology as it governs the spatial interactions between individuals, other organisms, and the environmental elements around them (Vojar 2007; Nathan et al. 2008). The seasonal activities and migratory patterns of *T. cristatus* have been recorded for more than a century, with historical records dating back to study in 1897 (Dürigen 1897). *T. cristatus* move to ponds for breeding (Kupfer and Kneitz 2000). The migration period usually takes place between February and April (Langton et al. 2001). The early migrations of *T. cristatus* often occur at night when the air temperature rises above 4–5 °C. Migration activity peaks during and immediately following successive humid nights (Jehle et al. 2011). Ralph (1957) found that the lunar cycle significantly influenced salamander activity patterns. Deeming (2008) similarly revealed that *T. cristatus* showed heightened activity just prior to the new moon.

It's worth mentioning that the migration of newts, including crested newts, is not a synchronized process, and individuals can be seen migrating several months after their initial arrival at the pond (Langton et al. 2001). On average, male newts tend to reach the pond a few days before the females, a behaviour believed to enhance their chances for successful mating. This early arrival tactic might give males a competitive edge in securing partners during the mating season. Newts use their breeding ponds for more than just reproduction; they also use them for feeding, an essential method of resource location (Jehle et al. 2011).

Adults of various species frequently display non-random migration patterns when departing from breeding sites, illustrating a propensity to both enter and exit the same locations and expressing a preference for specific habitats as transit routes over others (Marty et al. 2005; Sztatecsny and Schabetsberger 2005; Rittenhouse and Semlitsch 2006). After the adults leave the breeding pond, the larvae undergo metamorphosis and transition to a terrestrial environment distant from the pond. A portion of the juveniles will remain near the breeding site, eventually reaching sexual maturity and returning to reproduce in their birth pond, thus becoming part of the local breeding adult population. The remaining surviving juveniles will disperse into the adjacent terrestrial habitat, presumably to colonize ponds beyond their birth site (Rittenhouse and Semlitsch 2006). Despite the information provided, it remains unclear which climatic factors significantly impact migration.

Our study was centred on immigration and emigration activity of *T. cristatus*. The research was conducted at a location known for its suitability to *T. cristatus* and where these species have been documented in the past (Weber et al. 2019). Our objective was to understand whether there exist any differences in immigration and emigration based on gender. Furthermore, we investigated migration patterns including direction of individuals varying in length (as per snout to vent length). Lastly, we delved into the possible impacts of environmental factors on migration, paying specific attention to aspects such as temperature, rainfall, humidity, wind, moon phase, and the week of the year. A thorough understanding of migratory behavior can prove instrumental in shaping and directing conservation strategies (Marsh and Trenham 2001).

Materials and methods

The research was conducted in the artificial pond located in Czech Republic in the village of Tovéř (49°38.433'N, 17°19.691'E), which is situated northeast of the town of Olomouc at an elevation of 227 meters above sea level. This pond is a small retention reservoir with a water surface area of approximately 500 m^2. Since it lacks a permanent water inflow, its water levels are dependent on current rainfall, the usual depth in spring is 1.8 m, sometimes in warm summers it completely dries up. The pond was eutrophic with algal growth on the surface. From the south and northeast sides, the shore of the pond has a gentle slope. The littoral zone of the pond is mainly dominated by pondweed (*Lemna minor*), and submerged grasses are also present in the area surrounding the pond. Based on the Habitat Suitability Index (HSI) assessment, which evaluates the suitability of habitats for the occurrence of *T. cristatus*, the Tovéř locality is classified as “good”. The water body does not contain any fish. However, waterfowl, particularly mallards (*Anas platyrhynchos*), can be found in the area. One of the potential amphibian predators present at the site is the grass snake (*Natrix natrix*). In addition to the great crested newt, other syntopic amphibian species found here include the common newt (*Lissotriton vulgaris*) and the alpine newt (*Ichthyosaura alpestris*). The fire salamander (*Salamandra salamandra*) has also been observed in the vicinity. Among the frogs, individuals of the European fire-bellied toad (*Bombina bombina*), the European tree frog (*Hyla arborea*), the common toad (*Bufo bufo*), the European green toad (*Bufotes viridis*), and the agile frog (*Rana dalmatina*) have been captured in this area.

The monitoring took place from 4 March to 18 November 2017, for a total of 259 days. During this study, amphibians were captured using drift fencing lined with pitfall traps (n=47) around the whole pond. The 75 cm high PE (polyethylene) UV-resistant half-sheet was used as a guidance drift fence during the study. Approximately 10 cm of the drift fence was embedded in the ground to prevent individuals from burrowing under the barrier. As part of the trapping method, white plastic buckets measuring 30 cm in height and 25 cm in diameter were buried around the perimeter of the pond as traps. These trapping buckets were spaced approximately 3 meters apart and were sunk into the ground so that the top of the bucket was level with the ground (Crosswhite 1999). Plastic buckets were positioned on both sides (inside n=24, outside n=23) of the drift fence to capture individuals during both emigration and immigration. Each bucket was equipped with a small, moistened sponge and a small number of leaves to retain moisture and provide shelter for the animals. The drop traps were numbered sequentially from 1 to 47, creating a directional rosette of migrants. The traps were divided into two sections based on the different environments representing potential wintering sites. The section with the forest was designated for traps numbered 1 to 25, while the section near the village was assigned to traps numbered 26 to 47 (Fig. 1). Every morning the sex and length of each of the *T. cristatus* were recorded, along with the corresponding trap number in which it was found. The length of the individual was measured from snout to vent (SVL). Individuals with indistinct adult sex characteristics were considered as juveniles in size between 10–80 mm. For each captured individual, an identification photograph of the belly was taken for capture-mark-recapture purposes (used for another study), then individuals were released immediately in the direction of their migratory path. It was assumed that individuals would move in a relatively straight line from the pond towards the fence and fall into one of the two nearest traps, which is a common assumption in drift fence studies. Additionally, all traps were considered equally effective at capturing newts, thus providing a statistically representative sample

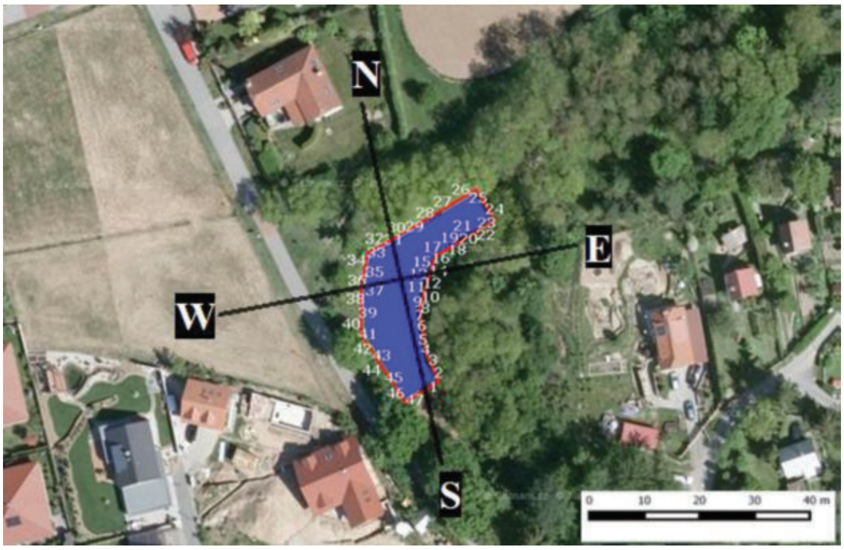

Figure 1. Map of the site with marked water area (blue), drift fence (red), individual trapping containers (white numbers) and distribution of cardinal directions.

of migrating newts in all directions (Malmgren 2002). Meteorological data used for this study were measured at location on DAVIS Vantage PRO meteorological station. Air temperature (°C) and humidity (%) were measured at 2 m above ground level, wind speed (m/s) at 10 m above ground level, and precipitation (mm) at 1 m above ground level with length of sunshine (h). Daily averages were used for temperature, humidity, and wind speed, while the total daily rainfall was considered for precipitation.

The effect of meteorological data on migration activity of different body size and both sexes were evaluated using Canonical Correspondence Analysis (CCA) using Canoco for Windows 5.0. Models for both immigration as well as emigration activity of newts were done. Body size and sexes were used as species data, whereas environmental data were factors: week of the year (week), average temperature (T_avg), minimum temperature (T_min), maximum temperature (T_max), average wind strength (F_avg), maximum wind strength (F_max), precipitation (SRA), average humidity (H_avg), direction from forest (forest) and length of sunshine (light). Environmental variables that significantly explained variation of activity of newts were uses to calculate predictive Generalized Additive Models (GAM). The direction of migration (both immigration and emigration) was visualized in program Oriana for Windows with applied Rayleigh-Test.

Results

Migration based on sex

During the immigration process, the highest number of captures was observed for males, with 543 individuals recorded. Females followed closely with 532 captures, while only 21 juveniles were observed arriving in the pond. In terms of emigration, 530 males, 386 females, and 191 juveniles were recorded as captures. The sex ratio of *T. cristatus* individuals found at the Tověř site was 1.21:1, with a slight majority of males compared to females. The primary immigration of *T. cristatus* to the study site, accounting for 75.36% of the total number of immigrants, occurred between March 4th and March 31st, spanning a period of 27 days. During this period, a total of 390 females (accounting for 73.31% of the total arrivals), 433 males (79.74%), and 3 juveniles (14.29%) arrived at the study site. A subsequent small increase in immigration was observed from around April 28th to May 8th. On the other hand, the primary emigration period for individuals began on June 5th and lasted until July 12th, totalling 37 days. During this time, approximately 40.43% of the overall outmigration was attributed to the leaving individuals. During this period, 336 females (representing 87.05% of the total number of females leaving) and 421 males (79.14%) departed. However, no juveniles were observed to emigrate during this period. The juveniles experienced an emigration wave from August 7th to September 6th (n=130; i.e., 68%), followed by a shorter period from September 15th to September 27th. Autumn migrations of tens of individuals have also been recorded. *T. cristatus* individuals were observed immigrating to the pond predominantly from the southwest, which includes village area (Rayleigh test; mean direction ± IC95 is μ=215.111±11.27°; length of vector r=0.211; p<0.0001). Conversely, during emigration, individuals predominantly departed to the southeast, where the forest is situated (Rayleigh test; μ=148.598±8.84°; r=0.265; p<0.0001) (Fig. 2).

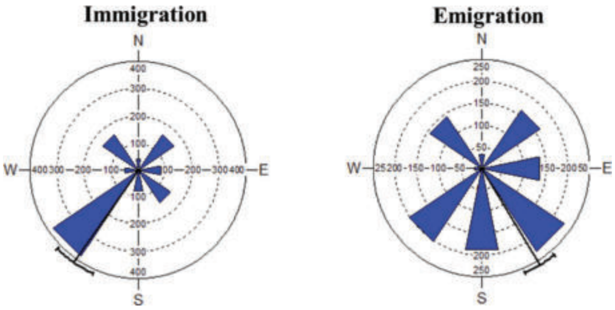

Figure 2. Directional rosettes of immigration and emigration for all individuals of *T. cristatus*. The black section shows the mean angle of migration, and the arcs extending to either side represent the 95% confidence intervals of the mean.

When examining the direction of immigration separately for each sex, we find that for females (Rayleigh test; μ=209.735±17.43°; r=0.196; p=0.510) and juveniles (Rayleigh test; μ=39.639±67.54°; r=0.153; p=0.616), the results are non-significant, indicating no clear preference. However, for males, there is a significant preference (Rayleigh test; μ=219.5±13.94°; r=0.241; p<0.0001) that aligns with the overall direction of immigration, indicating a preference for coming from the southeast, which corresponds to the direction from the village (Fig. 3).

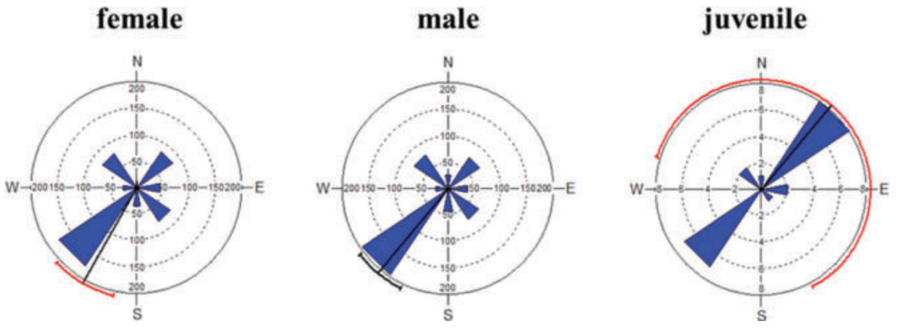

Figure 3. Directional rosettes in immigration, by gender. The black section shows the mean angle of migration, and the arcs extending to either side represent the 95% confidence intervals of the mean. Red colour sings nonsignificant results.

Migration based on body length

The average length (SVL) of the male individuals captured during the study was 102.3 mm, while the average size of the female individuals was 108.4 mm. The average length for juveniles was 61.1 mm (Table 1). Significant difference in size were found between all sex groups (Tukey's range test, $p<0.05$). The average increase in body length between immigration and emigration period observations was identical at 7 mm for both males and females.

Table 1. Size structure (SVL) in mm for immigration and emigration (Q1 = lower quartile, Q3 = upper quartile, med = median).

sex	Immigration					Emigration				
	max	min	med	Q1	Q3	max	min	med	Q1	Q3
female	141	60	105	98	115	142	78	112	105	117
male	124	54	99	92	105	126	10	106	102	110
juvenile	70	48	62	59	68	76	39	62	56	66

The highest number of females (n=149) immigrated in the length of around 100 mm; the highest number of males (n=152) also immigrated in this length. As for juveniles, the highest number (n=11) was observed with the SVL around 60 mm. For emigration, both males and females shifted up by one average length. Most females (n=152) emigrated in body size around 110 mm, followed by 242 males in the same size. Among juveniles, SVL around 60 mm was the most preferred size, with 84 captures.

Migration based on environmental factors

The Canonical Correspondence Analysis (CCA) model, when applied to single environmental factors during immigration, displays explanatory variables accounting for 8.8%. It highlights several influential factors under simple term effects, including week of the year, average, minimum and maximum temperature, average humidity and length of sunshine. No notable preference for these factors is observed among males and females. However, the immigration of juveniles is seen to be dependent on the week of the year and males immigrate to the pond at lower temperatures (Fig. 4).

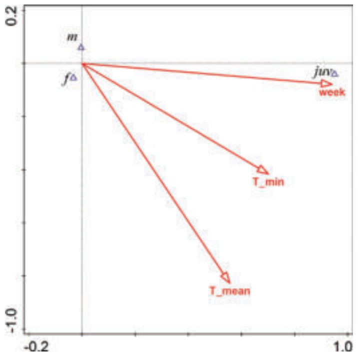

Figure 4. CCA model for immigration for sex with the environmental factors (the week in year (week), average temperature (T_mean), minimum temperature (T_min). Only significant variables are shown.

In the Generalized Additive Model (GAM) for the sequence of the week of the year, it's observed that males arrive first at the pond, followed by females. Juveniles started immigration around mid-year. One of the key factors here is the average temperature, as the GAM indicates that females predominantly arrive at around 10 °C, males prefer cooler temperatures up to 5 °C, and the number of juveniles was observed to increase as the temperature rises from 10 °C. The GAM for minimum and maximum temperature were similar as GAM for average temperature. Males show a positive correlation with average humidity, whereas females display a negative correlation when humidity levels are 50% or higher. Juveniles seem to prefer lower humidity (Fig. 5). A GAM analysis between factors and body length shows that smaller males immigrate to the pond first followed by larger females. The largest individuals immigrate at temperatures between 10 and 15 °C, corresponding to females. It is also apparent that immigration of larger individuals above 100 mm decreases with increasing humidity up to 80%, but then increases again with increasing humidity (Fig. 6).

The Canonical Correspondence Analysis (CCA) model applied to individual environmental factors during emigration accounts for 26.15% of the variation. The significant factors within simple term effects include the week of the year, maximum and average wind strength, precipitation, average humidity, length of sunshine and minimum, maximum, and average temperature. In this model, males emigrated from the pond during lower rainfall, stronger winds on warmer days. Females emigrated on sunny days with warmer temperatures. For juveniles, the week of the year was important and they emigrated at higher humidity and precipitation (Fig. 7).

The Generalized Additive Model (GAM) for the order of week in the year reveals that males typically emigrated by the 25th week, followed by a decrease in emigration and a new surge starting around the 35th week. Females peak in their emigration around 20th week and then start their emigration again in the 35th week. Juveniles have an emigration peak during the 35th week. Male emigration decreases with increasing mean temperature, showing a slightly increase at 20 °C, while females prefer temperatures between 15–20 °C for their emigration. Juvenile emigration firstly decreases up to 20 °C, then increases with rising temperatures. The GAM for minimum temperature mirrors that of the mean temperature, with males preferring cooler temperatures for emigration, females around 15 °C, and juveniles leaving as the minimum temperature rises. In the GAM for maximum and mean wind strength, males tend to favour stronger winds for emigration, whereas females cease their migration at higher wind strengths. In terms of precipitation, juveniles tend to emigrate during heavier rainfall, while females and males demonstrate a decrease in emigration during such conditions. Regarding humidity, males find it optimal to emigrate around 60% average humidity, while females cease emigration as humidity increases. Contrarily, juvenile emigration elevates with increasing humidity (Fig. 8). The GAM further show that larger in-

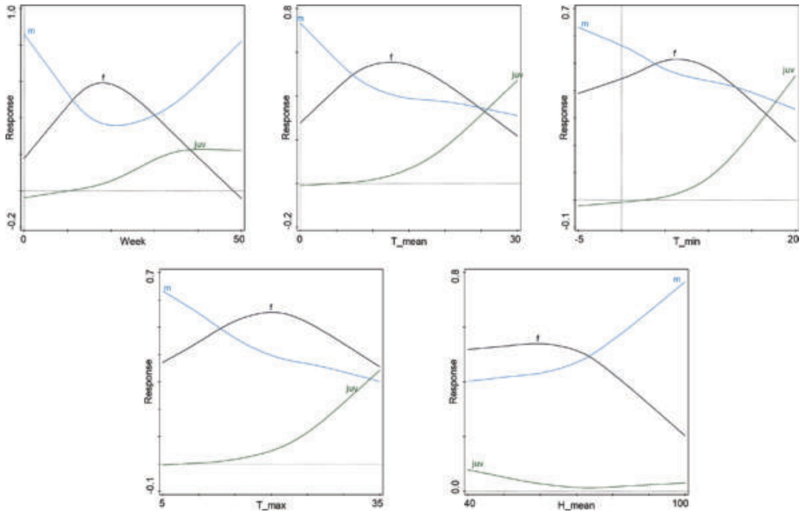

Figure 5. GAM of immigration acitivity for the week (Week), average temperature (T_mean), minimum temperature (T_min), maximum temperature (T_max), average humidity (H_mean)). Only significant variables are shown.

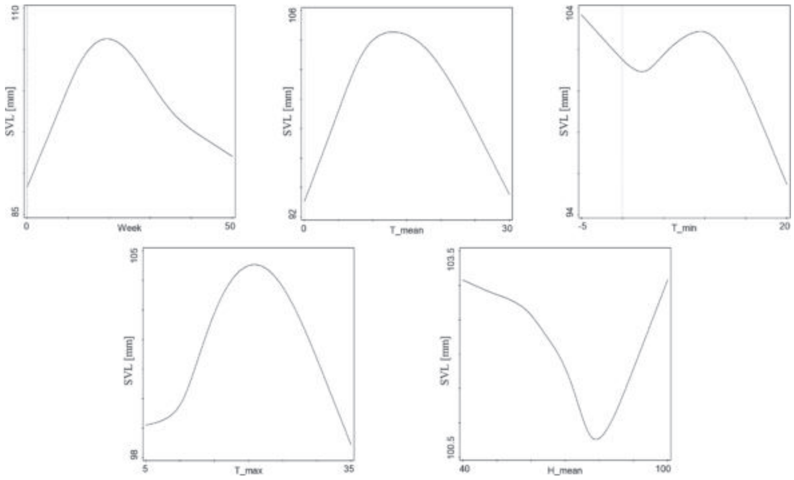

Figure 6. GAM of size of the newts of immigration activity for the week (Week), average temperature (T_mean), minimum temperature (T_min), maximum temperature (T_max), average humidity (H_mean). Only significant variables are shown.

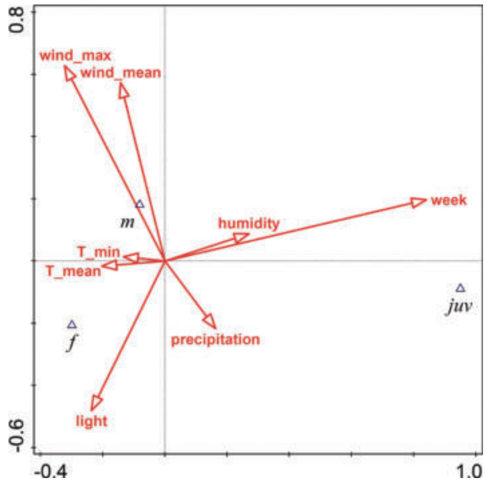

Figure 7. CCA model for emigration for sex with the environmental factors (the week in year (week), average temperature (T_mean), minimum temperature (T_min), maximum wind strength (wind_max), average wind strength (wind_mean), average humidity (H_mean), precipitation (precipitation), light (light). Only significant variables are shown.

dividuals emigrate during week 20, with a resurgence of emigration during week 35. Individuals above 100 mm peak emigration at 15 °C and continue to emigrate at higher wind strengths. Conversely, emigration of larger individuals decreases with increasing mean humidity, which can also be seen in the GAM for precipitation, where larger individuals stop migrating with increasing precipitation (Fig. 9).

The last factor we investigated was the impact of the lunar phase (illumination). We noted that as the moon's phase or brightness increased, there was a rise in the number of both immigrants and emigrants, encompassing all genders. Nevertheless, the correlation discovered between moonlight and both immigration and emigration proved to be statistically non-significant. For immigration, we found a correlation coefficient r = 0.42, but with a p-value of 0.5, being not statistically significant. Similarly, the correlation for emigration was also statistically non-significant, with a correlation coefficient r = 0.049 and a p-value of 0.53 (Fig. 10).

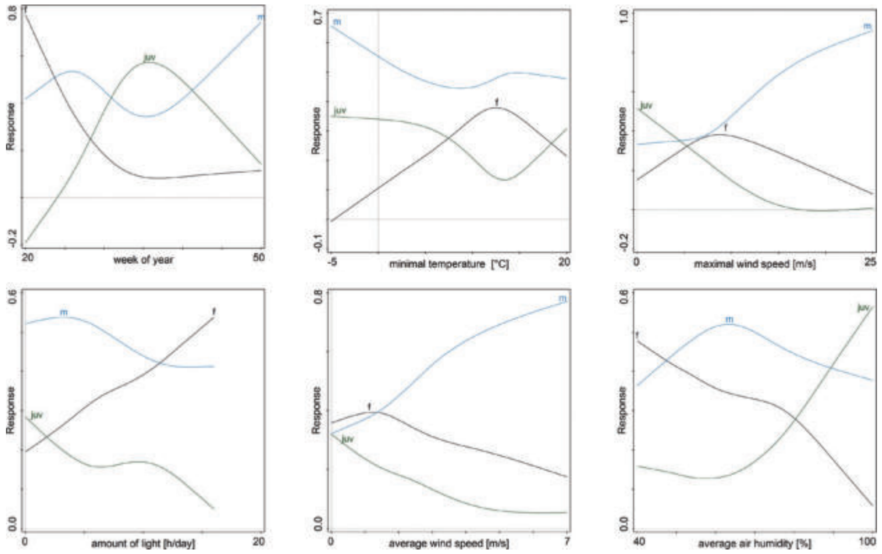

Figure 8. GAM of emigration acitivity for the week of year, minimum temperature, maximum wind speed, amount of light, average wind speed, average humidity. Only significant variables are shown.

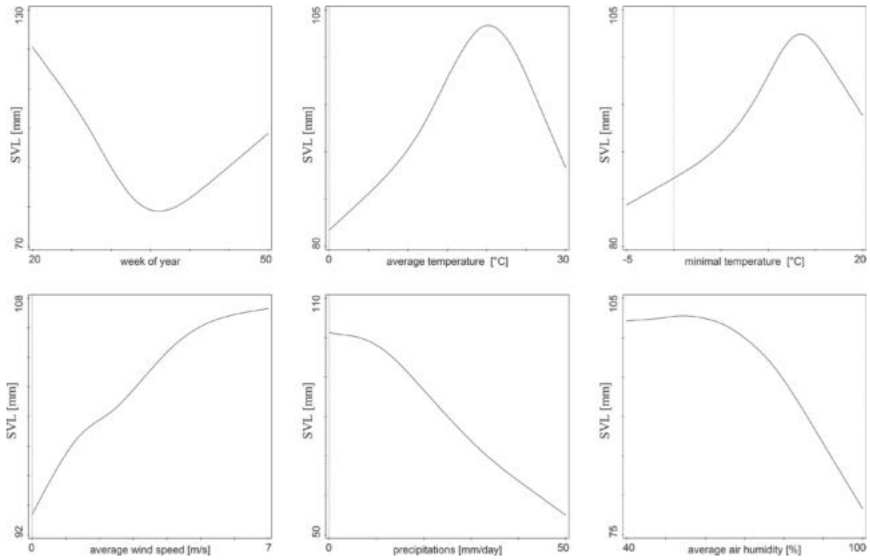

Figure 9. GAM of size of the newts of emigration activity for the week of year, average temperature, minimum temperature, average wind speed, precipitations, average humidity. Only significant variables are shown.

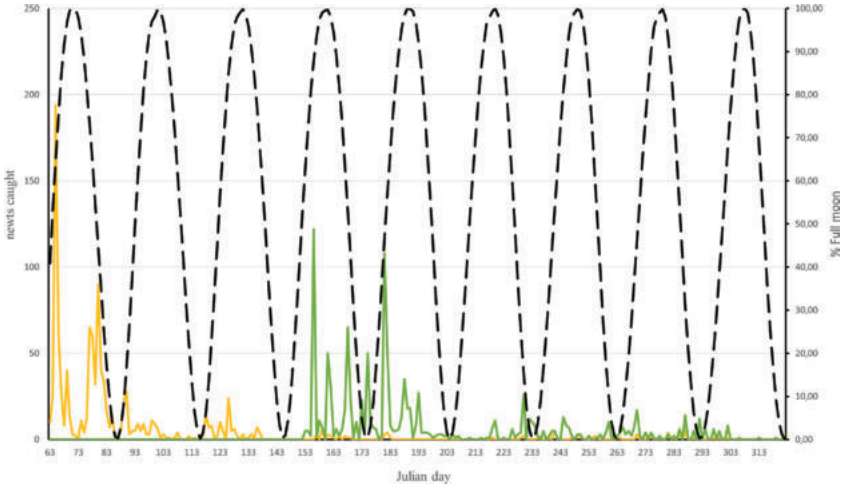

Figure 10. The number of newts (orange line for immigration, green line for emigration) caught plotted against the phase of the moon (dashed line).

Discussion

Our study noted that the major immigration period occurred between March 4th and March 31st. This accounted for a significant 75.36% of the total immigration events, over a period of 27 days. This immigration period is somewhat earlier compared to study (Verrell and Halliday 1985), where the majority of adult newts had entered the pond by May 5th. Our results are, however, broadly in line with the patterns reported by Blab and Blab (1981), who documented spring immigration lasting from February to May. Blab and Blab (1981) also observed an emigration period running from mid-July to mid-October, followed by a second immigration wave during October and early November. These findings appear to generally align with the data obtained in our study, offering similar migration patterns for this species. Our results indicated that male *T. cristatus* arrived at the pond in advance of the females, which is similar in other species of newts (Diego-Rasilla 2003; Diego-Rasilla and Luengo 2007) and salamanders (Douglas 1979; Sexton et al. 1990). This could suggest a selective advantage for males to reach the breeding grounds earlier, potentially increasing their opportunities to mate with various females. Alternatively, females may be selectively inclined to arrive later to broaden their selection group of potential mates (Douglas 1979; Russell et al. 2005).

In our study, we observed that the main emigration period began on June 5th and ended on July 12th, spanning a total of 37 days. This period accounted for roughly 40.43% of the overall outmigration, with individuals predominantly leaving during this time. Interestingly, no juveniles were noted to emigrate during this primary emigration phase. Instead, we recorded a significant juvenile emigration wave from August 7th to September 6th, during which approximately 68% (n=130) of the juveniles left. This was followed by a secondary, shorter wave from September 15th to September 27th. Autumn immigration suggests that certain individuals remain in the habitat over the winter period, indicating potential overwintering within the water. This finding may help to explain the phenomenon noted by Verrell and Halliday (1985), when fewer amphibians are captured upon leaving a pond than entering it, particularly when using a drift fence to intercept migrating individuals. While the exact reason for this discrepancy is not entirely clear, factors like predation could explain it. Our findings align with study research in Western France (Arntzen 2002), which suggested that males tend to leave the ponds before females despite a slightly longer overall aquatic phase. Our results are, however, shorter than the seven-month aquatic phase reported by Griffiths and Mylotte (1987) in an upland area. The observed emigration pattern is also consistent with study Verrell and Halliday (1985), which recorded emigration from late April to early November, with metamorphosed larvae departing the ponds starting in early August. In agreement with our study, they also mentioned an autumnal migration period, with several males and females captured outside of the drift fence in November.

Our findings suggest that migration directions of *T. cristatus* individuals can be influenced by the presence of specific environmental factors such as developed land, forests, and possibly the shelters that gardens provide during the terrestrial period. The use of gardens in the village's area as overwintering sites is intriguing and shows that these amphibians can adapt to utilize human-modified habitats for their survival needs. This adaptability can provide crucial survival strategies in a rapidly changing world where natural habitats are increasingly being modified or lost. However, there is also evidence to suggest that *T. cristatus* may not necessarily exhibit directional preference when moving to and from a pond (Verrell and Halliday 1985). This disparity might be explained by differences in local environmental conditions across different study sites. Some studies have suggested that newts, when leaving a breeding pond, tend to move in straight lines towards favorable habitat patches, indicating their efforts to optimize the use of available space (Verrell 1987; Sinsch 1991; Macgregor 1995; Jehle and Arntzen 2000; Jehle et al. 2011). It's interesting to note that while metamorphs of *T. cristatus* might have the ability to follow cues left by adults (Hayward et al. 2000; Oldham et al. 2000), this behavior was not observed in this study. Instead, juveniles exhibited a non-preferred direction of migration, it could be part of dispersion, suggesting different factors influence their movements, potentially including the surrounding habitat's structure and resources. The finding that *T. cristatus* individuals of all sizes exhibit non-random migration directions when emigrating from the pond (Malmgren 2002) aligns well with our results. This study, like ours, noted that these newts prefer to leave the pond in areas where forests adjoin rather than open fields, indicating the importance of these specific habitat types. Furthermore, despite having no prior experience in the terrestrial environment, juveniles showed a preference for areas near the forest during dispersal, again supporting the notion that specific environmental cues or conditions may guide their migration directions.

Our results confirm that females are generally larger than males. This concurs with the findings in study Verrell and Halliday (1985), where adult females entering the water were significantly longer than males. Moreover, our study notes that females emigrating from ponds also tend to be slightly larger than males. The growth observed between immigration and emigration periods was identical for both sexes, registering an increase of 7 mm. This indicates that the environmental conditions during these periods were conducive to growth for both genders equally. When it comes to juveniles, their average length in our study was significantly larger than previous research, with a recorded SVL of 61.1 mm. This contrasts with the results of Verrell and Halliday (1985), where juvenile length ranged from 40 to 51 mm. This stage of *T. cristatus* can span two to five years, characterized by rapid growth (Dolmen 1983; Francillon-Viellot et al. 1990). By Arntzen and Teunis (1993) the growth of juveniles over the summer was swift, with an average increase in SVL of 8.3 mm over a span of 10 weeks.

Our study found that female *T. cristatus* tend to start their migration at around 10 °C, while males seem to prefer cooler temperatures of up to 5 °C, and juveniles start their migration as temperatures increase. Initial migrations occur post-sunset at temperatures above 4–5 °C, with most activity during consecutive humid nights (Jehle et al. 2011). It's also interesting to mention that the temperature threshold for migration in crested newts is higher compared to smaller newt species (Griffiths and Raper 1994). Migration in *T. cristatus* has been found to be influenced by the daily minimum temperature, with significant newt movement observed when temperatures reach or exceed 5 °C. On the other hand, limited movement was reported during cooler periods from late March to late April (Verrell and Halliday 1985). During March, when most incoming individuals were captured, the number of animals caught each day showed a positive correlation with the minimum temperature. The correlation coefficient (Pearson r) was 0.6, indicating a moderate positive relationship, and the correlation was statistically significant ($p < 0.001$) (Verrell and Halliday 1985). In colder springs, the migration period was short, lasting 23 days compared to early and warm springs where it lasted 40 days (Dervo et al. 2016).

Our research reveals complex connections between weather conditions and the migratory behaviours of *T. cristatus*. We discovered that rainfall and humidity play substantial roles in influencing behaviours, but their effects vary across different body size and genders. For example, newts between 50 and 70 mm prefer heavier rainfall, while larger newts decreases their arrival frequency with increasing rainfall. Our data also imply that precipitation affects emigration patterns, with juveniles tending to emigrate during heavier rainfall, while both genders show decreased emigration under such conditions. Juvenile amphibians, due to their smaller size and resultant greater surface area to volume ratios, are theoretically more prone to desiccation risks during day-time migrations compared to their adult counterparts (Spight 1968). Consequently, it might have been anticipated that these young amphibians would be less inclined to migrate during the day. Regarding humidity, optimal emigration conditions for males occur around 60% average humidity, while females halt emigration as humidity increases. Conversely, juvenile emigration rises with increasing humidity. Smaller individuals (to 80 mm) require higher humidity for emigration, whereas larger individuals (over 110 mm) stop emigration when humidity surpasses 60%. These findings challenge some previous research. While many studies correlate rainfall with amphibian breeding timing (Byrne 2002; Vaira 2005; Scott et al. 2008) others report no such link (Gittins et al. 1980; Reading 1998). Verrell and Halliday (1985) concluded rainfall wasn't necessary for *T. cristatus* migration. Often, nocturnal rainfall triggers migrations as it lowers desiccation risks for migrating amphibians (Semlitsch and Pechmann 1985). Amphibians' water balance heavily relies on sufficient moisture, thereby making them vulnerable to desiccation, particularly during periods without rain (Jørgensen 1997; Hillyard 1999). A multitude of studies indicate that amphibian migrations often correlate with heavy rainfall (Todd et al. 2011). However, not all species respond similarly, with recently metamorphosed *B. terrestris* showing a weak correlation between migration and rainfall due to their higher desiccation tolerance (Thorson and Svihla 1943). Interestingly, even periods of no rain can trigger migration in certain amphibians, like recently metamorphosed *Bufo quercicus*, particularly after prolonged dry spells (Greenberg and Tanner 2005). Our findings reveal that these relationships are more nuanced, pointing to the importance of considering both humidity and size classes in understanding amphibian migrations. In conclusion, we underscore that rainfall is typically the most critical determinant of amphibian movements. Notwithstanding, responses to climatic factors, such as rainfall, vary considerably among amphibian species and age classes, with some demonstrating a greater dependence than others.

While our findings did not display a significant correlation between the moon's phase and immigration/emigration numbers, it's worth noting that we haven't considered possible interference from cloud cover. Clouds can obscure the moon's illumination and thus might impact the activity patterns of the animals, or human activity in this case. This could be a relevant factor that may affect the visibility of the moon and hence potentially influence our observed results. Future research could explore this aspect to gain a more comprehensive understanding of the influence of lunar phases on migration patterns. There have been previous studies showing that the moon's phase can affect the behavior of certain species. By Ralph (1957) salamanders' activity patterns were determined by the lunar cycle. Deeming (2008) observed that *T. cristatus* were most active right before the new moon. The pattern of heightened activity during the darkest nights could potentially be a survival strategy, reducing the risk of predation when individuals move away from breeding ponds to forage or migrate between ponds (Deeming 2008). Another possible explanation could be the influence of secondary phenomena like insect activity. Certain insects are known to have activity patterns that align with the lunar cycle, often peaking during full moon phases. If the organisms under study – salamanders, newts, or another species – rely on these insects as a primary food source, then their migration and foraging patterns might align more closely with the activity patterns of their prey rather than directly with the moon's phase or brightness. This indirect influence could create a seeming correlation between lunar phases and migration patterns. To properly assess this, future research could delve into a multivariable analysis considering these potential ecological interactions (Deeming 2008).

Finally, our study underscores that climatic conditions may play a crucial role in the migratory behaviours of *T. cristatus* as notable amphibian species. The documented decline in amphibian populations worldwide might be directly or indirectly associated with climate change, considering that key climatic elements like precipitation and temperature significantly influence essential processes in amphibian population dynamics (Blaustein et al. 2001). By Dervo et al. (2016) the migration phenology of *T. cristatus* was noticeably affected by the combined impact of temperature and precipitation. They observed an overall trend towards an earlier commencement of breeding migration with increasing temperatures. As climate patterns continue to alter, it's plausible that amphibian phenology could also evolve in response to these changing selection pressures, as has been witnessed in various other organisms (Bradshaw and Holzapfel 2001). However, despite the robust phenological reactions of amphibians to climate change, there's a lack of detailed information on whether these responses stem from genetic adaptations or are entirely due to phenotypic plasticity (Urban et al. 2014). The study Todd et al. (2011) alerts to the potential consequences of rapid shifts in regional weather patterns on amphibian migrations. Such changes could differentially impact species, potentially leading to altered arrival times of reproductive adults or varying success rates of migrations. Thus, the study reinforces the need for conservation efforts to consider these climatic impacts and the differing responses of amphibian species to environmental changes.

Conclusions

Our study provides a comprehensive examination of the migratory patterns of *T. cristatus*, noting that a significant portion of immigration events occur over a period of 27 days, starting from March 4th. Our data align with previous studies, revealing similar migration patterns. Protandry, with males reaching breeding grounds before females, was observed, indicating potential mate selection advantages. Emigration was prevalent during a 37-day period from June 5th, while juveniles mainly emigrated from August to September. Autumn migration with overwintering within water habitats was also confirmed. In addition, the influence of environmental factors such as land development, forests, and human-made shelters was noted on migration directions. *T. cristatus* showed the ability to adapt to human-modified habitats. However, juveniles demonstrated non-preferred migration directions, indicating the influence of local habitat structure and resources. Temperature played a significant role in migration, with gender and size-specific preferences. Furthermore, rainfall and humidity considerably influenced migratory behaviours with variable effects across different sizes and genders. Our data suggested no significant correlation between moonlight and immigration/emigration, although an upward trend was observed. More research, considering factors like cloud cover, is suggested to understand this aspect better. Finally, the study emphasized the crucial influence of climatic conditions on amphibian migration, underscoring the need for further research and effective conservation strategies amid global climate change.

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