

First record of *Rhacophorus verrucopus* Huang, 1983 from Myanmar

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Abstract

We report the first country record of *Rhacophorus verrucopus* Huang, 1983 from Myanmar, based on one specimen collected from Htamanthi Wildlife Sanctuary, Sagaing Division. Morphologically, the specimen shows good agreement with the original description of *R. verrucopus* and phylogenetically, it is clustered with the specimen of *R. verrucopus* from Medog, Tibet, China with strong support. This is also the first record of *R. verrucopus* from outside of China.

Key Words

16S rRNA, Htamanthi Wildlife Sanctuary, new record, tree frog

Introduction

Rhacophorus Kuhl & van Hasselt, 1822 occurs in the tropical and temperate zones of East, South and Southeast Asia and, recently, was partitioned into three genera including *Rhacophorus*, *Leptomantis* Peters, 1867 and *Zhangixalus* Li, Jiang, Ren & Jiang, 2019 (in Jiang et al. 2019). Now the genus *Rhacophorus* includes 44 recognised species with a distribution range from India, Bangladesh, Bhutan, Myanmar, Thailand, Laos, Cambodia, Vietnam, Malaysia, Indonesia and Philippines, as well as extreme southern and south-western China (mainly in Hainan, Guangxi, Yunnan and Tibetan) (Jiang et al. 2019; Frost 2020). *Rhacophorus verrucopus*, a species which was described and named from Medog, Tibet, China, was previously known only from the type locality (Huang 1983; AmphibiaChina 2020; Frost 2020).

Myanmar is an important component of the Indo-Burma biodiversity hot-spot and its northern region lies at a biogeographic crossroads where the faunas of China, Indochina, India and Himalaya converge (Wogan et al. 2008). In recent years, the researchers of Southeast Asia Biodiver-

sity Research Institute, Chinese Academy of Science have found many new species and new records of animals and plants in northern Myanmar (Li and Quan 2017). During our field survey in northern Myanmar in 2019, a specimen of *Rhacophorus* with a small and elongated body and distinct tarsal projections was collected. Molecular comparison indicated this individual to be *R. verrucopus*. Herein, we describe this new record for Myanmar in detail.

Materials and methods

Field surveys were conducted in Htamanthi Wildlife Sanctuary, Sagaing Division, Myanmar. The specimen was collected and euthanised with ethyl acetate and then fixed in 75% ethanol for storage after taking photographs. Liver tissue sample was preserved in 99% ethanol for molecular analysis. The specimen was deposited in Southeast Asia Biodiversity Research Institute, Chinese Academy of Sciences (abbreviation: SEABRI; address: Yezin, Nay Pyi Taw, Myanmar).

Total genomic DNA was extracted from liver tissue. Tissue sample was digested using proteinase K and subsequently purified, following standard phenol/chloroform isolation and ethanol precipitation. A fragment of encoding partial 16S rRNA gene was amplified using primer pairs 16Sar/16Sbr (Palumbi et al. 1991). Polymerase chain reaction (PCR) amplifications were performed in 50 µl reactions using the following cycling conditions: initial denaturing step at 95 °C for 4 min; 35 cycles of denaturing at 94 °C for 60 s, annealing at 51 °C for 60 s and extending at 72 °C for 60 s; and a final extension step of 72 °C for 10 min. Sequencing was conducted directly using the corresponding PCR primers. The new sequence was deposited in GenBank under accession number [MW275978](#). Outgroups were selected according to Jiang et al. (2019) and Nguyen et al. (2020). Homologous and outgroup sequences were obtained from GenBank (Table 1).

Sequences were aligned using ClustalW with default parameters in MEGA 7 (Kumar et al. 2016). Uncorrected pairwise distances between species were calculated in MEGA 7 with the parameters Transitions + Transversions, Uniform rates and Pairwise deletion. The best substitution model GTR+G was the Bayesian Information Criterion (BIC) in jModelTest 2.1.7 (Darriba et al. 2012). Bayesian Inferences were performed in MRBAYES v3.2.6 (Ronquist et al. 2012). Two runs were performed simultaneously with four Markov chains starting from the random tree. The chains were run for 1 000 000 generations and sampled every 100 generations. The first 25% of the sampled trees were discarded as burn-in after the standard deviation

of split frequencies of the two runs was less than 0.01. The remaining trees were then used to create a consensus tree and to estimate Bayesian posterior probabilities (BPPs). Maximum Likelihood analysis was performed in Raxml-GUI 1.5 (Silvestro and Michalak 2012) and nodal support values were estimated by 1,000 rapid bootstrap replicates.

Measurements were taken with a digital caliper to the nearest 0.1 mm. Morphological terminology followed Fei et al. (2009). Measurements included: snout-vent length (SVL, from tip of snout to vent); head length (HL, from tip of snout to rear of jaw); head width (HW, width of head at widest point); snout length (SL, from tip of snout to anterior border of eye); internarial distance (IND, distance between nares); interorbital distance (IOD, minimum distance between upper eyelids); eye diameter (ED, diameter of exposed portion of eyeball); tympanum diameter (TD, greater of tympanum vertical and horizontal diameters); distance from nostril to eye (DNE, from nostril to anterior border of eye); forearm and hand length (FHL, from elbow to tip of third finger); tibia length (TL, distance from knee to heel); and foot length (FL, from proximal end of inner metatarsal tubercle to tip of fourth toe).

Results

The obtained sequence alignment for the 16S gene was 510 bp long. SEABRI2019120056 clustered with *R. verrucopus* from Medog, Tibet, China with strong support (Fig. 1). The genetic distance between

Table 1. Species used for molecular phylogenetic analysis.

| Species | Voucher | Locality | Accession No. |
|----------------------------------|------------------|--------------------------------|---------------|
| Ingroup | | | |
| <i>Rhacophorus annamensis</i> | VNMN 4092 | Gia Lai, Kon Ka Kinh, Vietnam | LC010568 |
| | VNMN 4090 | Dak Nong, Nam Nung, Vietnam | LC010566 |
| <i>Rhacophorus bipunctatus</i> | CAS235303 | Bee Hoe, Chin, Myanmar | JX219444 |
| | CAS229913 | Putao, Kachin, Myanmar | JX219445 |
| <i>Rhacophorus calcaneus</i> | VNMN 4093 | Dak Lac, Chu Yang Sin, Vietnam | LC010573 |
| | KIZ 528 | Bi Doup, Lam Dong, Vietnam | JX219450 |
| <i>Rhacophorus exechopygus</i> | VNMN 4107 | Gia Lai, Kon Ka Kinh, Vietnam | LC010585 |
| | VNMN 4108 | Gia Lai, Kon Ka Kinh, Vietnam | LC010586 |
| <i>Rhacophorus helenae</i> | AMS R 173230 | Binh Thuan, Vietnam | JQ288087 |
| | UNS 00450 | Dong Nai, Vietnam | JQ288088 |
| <i>Rhacophorus hoabinhensis</i> | IEBR A.2016.18 | Hoa Binh, Vietnam | LC331096 |
| | VNMN A.2016.16 | Hoa Binh, Vietnam | LC331097 |
| <i>Rhacophorus kio</i> | VNMN 4110 | Gia Lai, Kon Ka Kinh, Vietnam | LC010589 |
| | VNMN 4111 | Ha Giang, Bac Quang, Vietnam | LC010590 |
| <i>Rhacophorus nigropalmatus</i> | Rao081203 | Malaysia | JX219438 |
| | Rao081204 | Malaysia | JX219437 |
| <i>Rhacophorus orlovi</i> | VNMN 3067 | Ha Tinh, Huong Son, Vietnam | LC010598 |
| | VNMN 4115 | Nghe An, Pu Huong, Vietnam | LC010600 |
| <i>Rhacophorus rhodopus</i> | SCUM 060692L | Mengyang, Yunnan, China | EU215531 |
| | X219440 | Lvchun, Yunnan, China | JX219440 |
| <i>Rhacophorus robertingeri</i> | VNMN 4123 | Gia Lai, Kon Ka Kinh, Vietnam | LC010613 |
| | VNMN 3446 | Kon Tum, Kon Plong, Vietnam | LC010615 |
| <i>Rhacophorus spelaeus</i> | IEBR A.2011.1 | Khammouan, Lao | LC331095 |
| <i>Rhacophorus translineatus</i> | Rao6237 | Medog, Tibet, China | JX219449 |
| <i>Rhacophorus verrucopus</i> | 6254 Rao | Medog, Tibet, China | JX219436 |
| | SEABRI2019120056 | Htamanthi, Sagaing, Myanmar | MW275978 |
| Outgroup | | | |
| <i>Zhangixalus duboisi</i> | VNMN 4102 | Sa Pa, Lao Cai, Vietnam | LC010580 |
| <i>Zhangixalus dugritei</i> | SCUM 051001L | Baoxing, Sichuan, China | EU215541 |
| <i>Leptomantis gauni</i> | FMNH273928 | Sarawak, Bintulu, Malaysia | JX219456 |
| <i>Leptomantis penanorum</i> | ZRC 1.12116 | Sarawak, Bintulu, Malaysia | JN377350 |
| <i>Buergeria buergeri</i> | IABHU 41011 | Hiroshima, Japan | AB127977 |

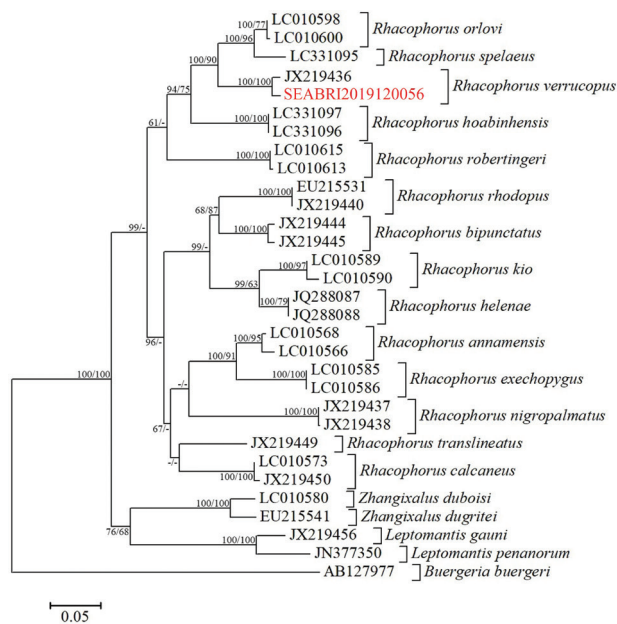


Figure 1. Maximum Likelihood tree of partial *Rhacophorus* species inferred from 16S rRNA gene sequences (Numbers before slashes indicate Bayesian posterior probabilities and numbers after slashes indicate bootstrap support for Maximum Likelihood analyses. The symbol “–” represents values below 60). The specimen collected from Myanmar is indicated by red.

SEABRI2019120056 and *R. verrucopus* from Medog, Tibet, China was 1.2% (Table 2). Therefore, we considered SEABRI2019120056 belongs to *R. verrucopus*.

Taxonomic account

Rhacophorus verrucopus Huang, 1983

Fig. 2

Specimen examined. Adult female (SEABRI2019120056) collected on 21 December 2019 by the local guides from Htamanthi Wildlife Sanctuary, Sagaing Division, Myanmar (25°21'59"N, 95°22'59"E, 90 m elevation).

Morphological description. Morphological characters of the specimen from Myanmar agreed well with the

original description of Huang (1983) and subsequent descriptions of Fei et al. (2009, 2012). Body small and elongate, SVL 52.0 mm; head length (HL 17.6 mm) larger than width (HW 15.7 mm); snout slightly sharp, canthus rostralis distinct, loreal region slightly oblique; nostril at tip of snout, internarial distance (IND 4.3 mm) narrower than interorbital distance (IOD 9.3 mm); tympanum (TD 3.1 mm) rounded, slightly larger than half eye diameter (ED 5.4 mm), separated from eye by 1.6 mm; pupil transverse, eye diameter larger than half snout length (SL 7.7 mm). Vomerine teeth present; tongue attached anteriorly, deeply notched posteriorly.

Forearm and hand length (FHL 17.8 mm) no more than half SVL; relative length of fingers I < II < IV < III; tips of all fingers expanded into discs with transverse grooves, disc of first finger small, disc of third finger largest, its width nearly equal to tympanum; webbing formula I 1-1 II 0-1 III 1-1/2 IV; subarticular tubercles distinct, formula 1, 1, 2, 2; rows of tubercles present on palms; inner metacarpal tubercle large and flat, outer metacarpal tubercle absent.

Hind limbs long, tibia (TL 24.2 mm) about half SVL and longer than thigh (22.9 mm) and foot (FL 20.4 mm); relative length of toes I < II < III < V < IV; tips of toes expanded into discs, smaller than those of fingers; webbing formula I 0-0 II 0-1 III 0-1 IV 1-0 V, subarticular tubercles distinct, formula 1, 1, 2, 3, 2; rows of tubercles present on pes; inner metatarsal tubercle flat, outer metatarsal tubercles absent.

Skin of dorsum smooth; supratympanic fold thin and distinct; outer edge of forearm with light coloured granules arranged in serrated shape; dermal calcars present on heels forming tarsal projections; granules above vent forming transverse skin fold; ventral skin covered with small flat granules.

Colour in life. Colour pattern similar to the holotype of *R. verrucopus*. Dorsal surface greyish-yellow with scattered small brownish-black spots; limbs with very indistinct transverse stripes; each side of thigh and inner sides of shank, tarsus and foot orangish-red; webbing between fingers orangish-yellow, webbing between toes orangish-red; ventral surface greyish-white, belly sides light yellow; pupil black, iris greyish-yellow.

Table 2. Divergence (P-distance; %) between and within homologous species and *Rhacophorus verrucopus* estimated from 16S gene sequences.

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 |
|--|------|------|------|------|------|------|------|------|------|------|------|------|------|-----|
| 1 <i>Rhacophorus annamensis</i> | | | | | | | | | | | | | | |
| 2 <i>Rhacophorus bipunctatus</i> | 9.6 | | | | | | | | | | | | | |
| 3 <i>Rhacophorus calcaneus</i> | 10.4 | 10.8 | | | | | | | | | | | | |
| 4 <i>Rhacophorus exechopygus</i> | 6.9 | 9.5 | 10.1 | | | | | | | | | | | |
| 5 <i>Rhacophorus helenae</i> | 10.5 | 7.2 | 10.7 | 12.3 | | | | | | | | | | |
| 6 <i>Rhacophorus hoabinhensis</i> | 9.1 | 13.4 | 10.4 | 13.5 | 9.6 | | | | | | | | | |
| 7 <i>Rhacophorus kio</i> | 10.8 | 9.0 | 12.2 | 11.9 | 5.8 | 12.3 | | | | | | | | |
| 8 <i>Rhacophorus nigropalmatus</i> | 9.9 | 13.4 | 12.0 | 10.5 | 10.5 | 14.1 | 13.3 | | | | | | | |
| 9 <i>Rhacophorus orlovi</i> | 10.9 | 11.5 | 10.8 | 13.2 | 12.2 | 8.7 | 12.0 | 11.8 | | | | | | |
| 10 <i>Rhacophorus rhodopus</i> | 10.9 | 9.1 | 11.6 | 11.3 | 9.5 | 14.4 | 11.1 | 13.5 | 12.4 | | | | | |
| 11 <i>Rhacophorus robertingeri</i> | 11.0 | 11.0 | 9.9 | 13.3 | 11.7 | 10.3 | 11.3 | 12.2 | 10.6 | 11.9 | | | | |
| 12 <i>Rhacophorus spelaus</i> | 11.8 | 11.5 | 10.7 | 13.5 | 12.0 | 9.2 | 12.4 | 12.6 | 3.6 | 12.4 | 9.9 | | | |
| 13 <i>Rhacophorus translineatus</i> | 9.0 | 10.8 | 9.2 | 9.5 | 10.6 | 12.2 | 12.1 | 12.1 | 10.8 | 11.5 | 10.5 | 11.0 | | |
| 14 <i>Rhacophorus verrucopus</i> (China) | 12.2 | 13.5 | 11.3 | 13.7 | 9.9 | 10.6 | 10.7 | 14.1 | 7.5 | 13.9 | 10.5 | 9.0 | 12.7 | |
| 15 <i>Rhacophorus verrucopus</i> (Myanmar) | 12.3 | 9.8 | 10.5 | 13.7 | 9.5 | 10.7 | 11.2 | 11.1 | 7.8 | 12.4 | 11.0 | 9.2 | 11.0 | 1.2 |

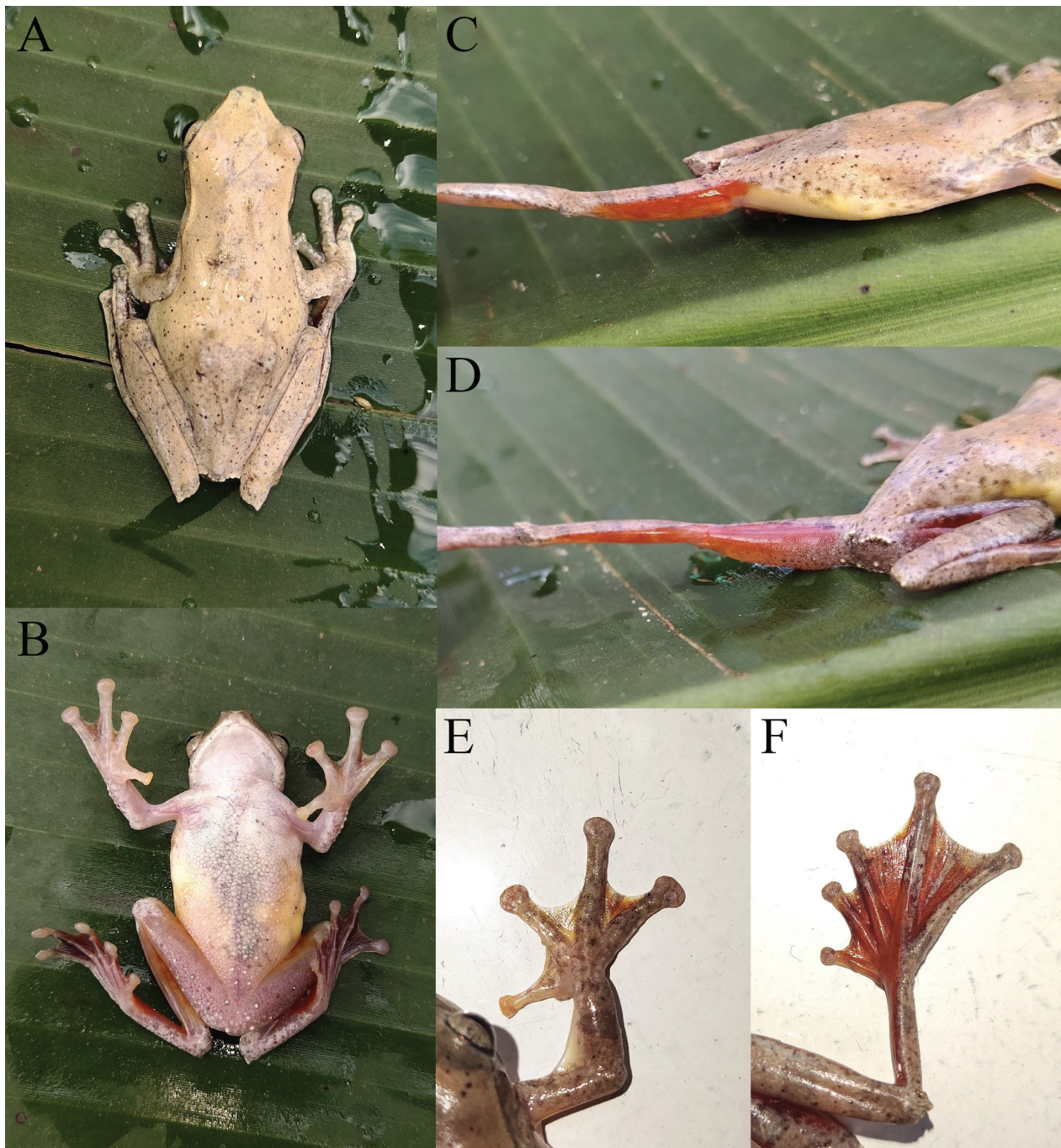


Figure 2. The specimen (SEABRI2019120056) collected from Myanmar in life. **A.** Dorsal view; **B.** ventral view; **C.** lose-up view of anterior part of thigh; **D.** close-up view of posterior part of thigh; **E.** close-up view of hand; **F** close-up view of foot.

Ecological notes. The specimen was found at night on a bush approximately 0.8 m above the ground near a large river. Several eggs were visible through the skin of the belly. The eggs were yellow and large. The breeding period of this species remains unknown.

Discussion

Morphologically, the specimen of *R. verrucopus* from Myanmar shows good agreement with the original description, except for minor differences in colouration. In

the original description, the colour of the upper eyelids was greyish-brown and there were greyish-brown transverse bands present on limbs. However, the upper eyelids of the specimen from Myanmar were the same colour as the dorsal surface and the transverse bands on the limbs were nearly invisible.

Rhacophorus verrucopus was known previously only from Tibet, China. This is the first record of *R. verrucopus* from Myanmar and from outside of China (Fig. 3). According to the original description, this species inhabited the area between 850 m and 1500 m elevation in the type locality (Huang 1983). The new location in Myan-

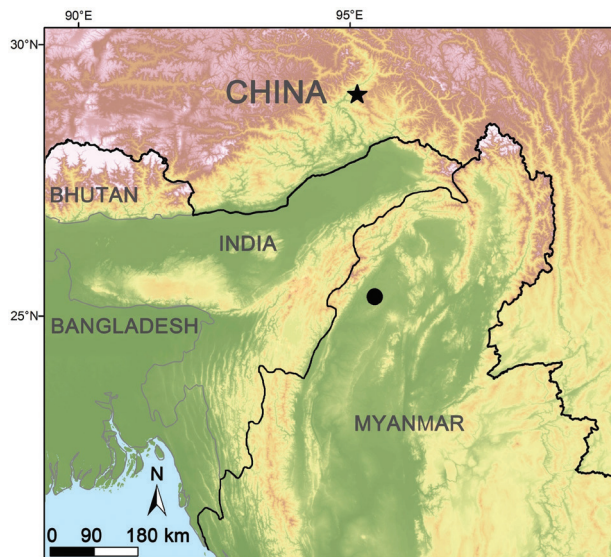


Figure 3. Collection site of *Rhacophorus verrucopus* (black dot) in Myanmar and the type locality (black star) in Tibet, China.

mar is approximately 440 km away from the type locality in China and the altitude (90 m elevation) of the new location is much lower than the type locality.

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