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Phylogenetic position of *Tropidophorus assamensis* Annandale, 1912 with updated morphological data and distributional records

Hmar Tlawmte Lalremsanga[1], Ht Decemson[1], Mathipi Vabeiryureilai[1], Fanai Malsawmdawngliana[1], Van Lalhlimpuia[2], Lal Muansanga & Lal Biakzuala[1]

[1]Developmental Biology and Herpetology Laboratory, Department of Zoology, Mizoram University, Tanhril 796004, India

[2]Department of Environmental Science, Mizoram University, Tanhril 796004, India

The poorly known northeastern water skink *Tropidophorus assamensis* is only known from the type locality (Harigaj Range, Sylhet District) in Bangladesh, and few localities in Assam and Mizoram States, north-eastern India. Little is known about the biology including the systematics of the species. In this study, we present for the first time, genetic data (*16s rRNA*) and inferred its phylogenetic position. In addition to this, we provide updated morphological data along with new distributional records of the species from Mizoram State of north-east India.

Keywords: Distribution, morphology, northeastern water skink, systematics

INTRODUCTION

The genus *Tropidophorus* Dumèril and Bibron, 1839 comprises of 29 extant species of semi-aquatic skinks (Uetz et al., 2021). The genus shows a high level of local endemism (Greer & Biswas, 2004), and can be morphologically diagnosed in having exposed tympanum and a single scale at the corner of the eyelid (Greer, 1970; Hikida et al., 2002; Greer & Biswas, 2004). *Tropidophorus* were known to occur in Indochina including the entire Malay Peninsula, Borneo, Sulawesi, and Philippines (Honda et al., 2006).

The northeastern water skink, *Tropidophorus assamensis* Annandale, 1912 is one of the most poorly known species among the congeners from Indochina (see Pawar & Birand, 2001; Honda et al., 2006; Das et al., 2009). Thomas Nelson Annandale (1876–1924), a Scottish Zoologist, one of the founder, and the first director of the Zoological Survey of India (ZSI), originally described the species from Harigaj range (holotype: ZSI 17029), Sylhet hills, Assam, India (at present Sylhet District, north-eastern Bangladesh) (Annandale, 1912; Das et al., 1998). Almost a century after the original description, the species was recorded from Mizoram State, India by Pawar and Birand (2001) at Nengpui Wildlife Sanctuary. A few years later, Mathew (2006) also collected an individual (VR/ERS/ZSI/241) from a bamboo thicket near a stream from Phairuangkai, Lunglei District, Mizoram State. From Assam, the adjacent State of Mizoram, Das (2008) recorded the species based on three individuals (ZSIC 25813; BNHM 1783; AD/BR 05) from Chambuda area and Adakuchi Basti in Barail Range; which were encountered under bryophyte covered rocks on a dry stream bed and under rocks of slow flowing-stream. It was also noted that unlike other skinks, they remained motionless when uncovered (Das et al., 2009). In the recent phylogenetic study of the genus *Tropidophorus* by Honda et al. (2006), a total of 17 representative species were sampled (11 from Indochina; three from Philippines; two from Borneo; one from Sulawesi), but *T. assamensis* was not included in their analyses. The present study provides the first genetic data for *T. assamensis* by sequencing a fragment of the mitochondrial 16S ribosomal RNA (*16s rRNA*) gene. We reconstruct the phylogenetic relationships of *Tropidophorus*, and provide further data about the morphological data of *T. assamensis* based on the recently collected specimens from Mizoram State, India. This study is based on the museum specimens deposited in the Departmental Museum of Zoology, Mizoram University (MZMU), and Pachhunga University College Zoological Museum (PUCZM). The specimens we collected in this study were anaesthetised using 250 mg/kg of 0.7 % sodium bicarbonate buffered MS-222 (Tricaine Methanesulfonate) solution by intracoelomic injection, followed by euthanasia by a second intracoelomic injection of 0.1 ml unbuffered 50 % (v/v) MS-222 solution following Conroy et al. (2009). Specimens were catalogued and stored in 70 % ethanol.

Genomic DNA was extracted from the liver tissue of MZMU2080 using QIAamp DNA Mini Kit following the standard protocol provided by the manufacturer. *16s rRNA* was amplified using the polymerase chain

Correspondence: Lal Biakzuala (bzachawngthu123@gmail.com)

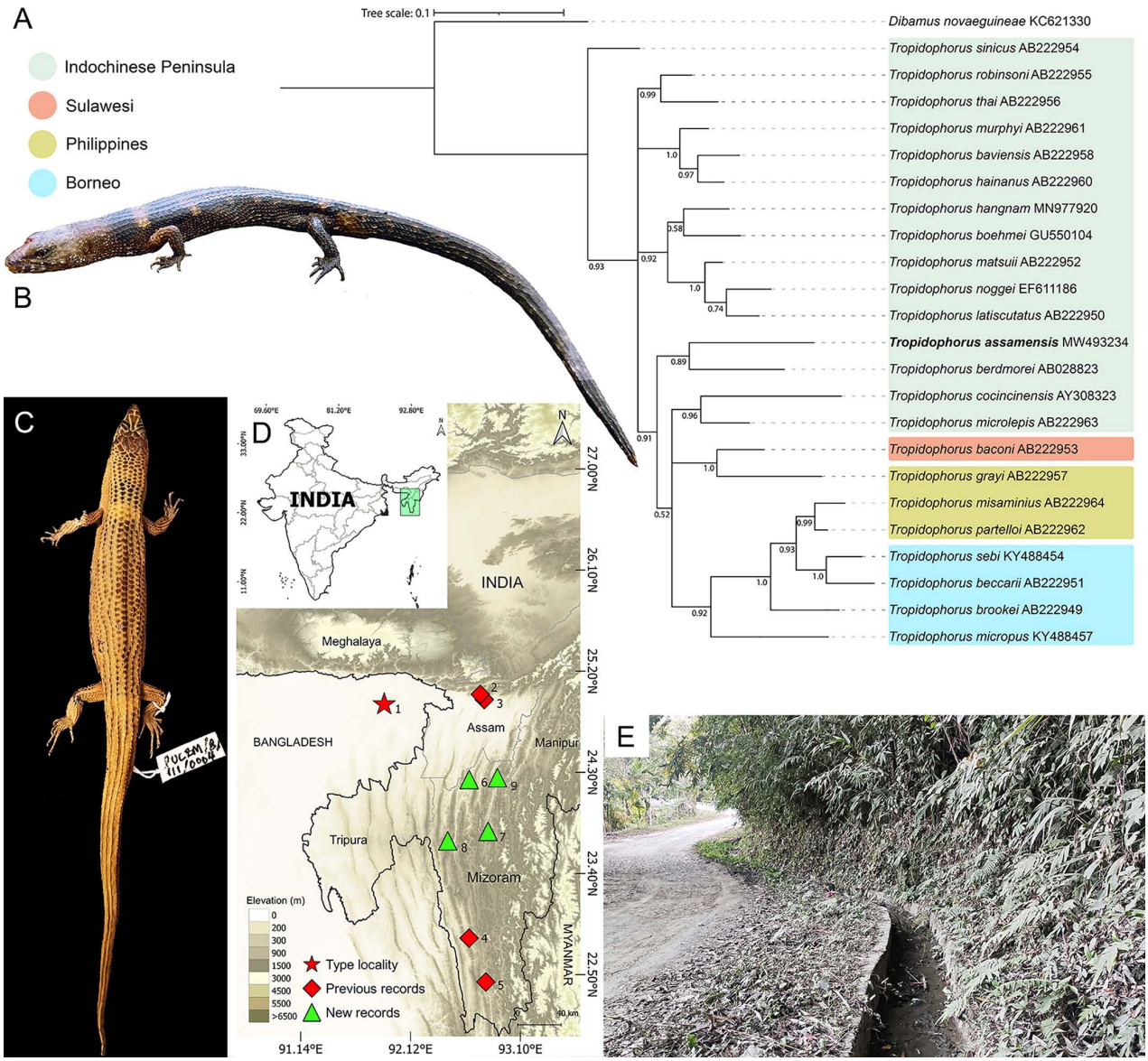

Figure 1. (A) Bayesian Inference phylogeny based on partial *16s rRNA* of *Tropidophorus*. The sample generated in this study is shown in bold. Numbers at each node represent Bayesian posterior probabilities at 20 million generations. **(B)** Adult female *Tropidophorus assamensis* (MZMU2080) in life. **(C)** Preserved specimen of *Tropidophorus assamenis* (PUCZM/B/111/0004). **(D)** Map showing the distribution of *Tropidophorus assamensis*: type locality shown in red star (1. Sylhet District, Bangladesh); previous records from north-east India shown in red diamonds (2–3. Barail Wildlife Sanctuary, Assam State; 4. Phairuangkai, Lunglei District, Mizoram State; 5. Ngengpui Wildlife Sanctuary, Mizoram State); new records from Mizoram State, India shown in green triangles (6. Tuichhuahen river, 7. Tuirial village, 8. Dampa Tiger Reserve, 9. Pualreng Wildlife Sanctuary). **(E)** Microhabitat of *Tropidophorus assamenis* at a roadside drainage in Dampa Tiger Reserve, Mizoram State, India.

reaction (PCR) with the forward L02510 (Palumbi, 1996) and reverse H03063 (Rassmann, 1997) primers in 20 µL reaction volumes, containing 1X amplification buffer, 2.5 mM $MgCl_2$, 0.25 mM dNTPs, 0.2 pM each forward and reverse primer, 1µL genomic DNA, and 1U Taq DNA polymerase. The PCR thermal cycling was performed as 5 min at 95 °C for initial denaturation, followed by 35 cycles of [1 min at 95 °C for denaturation, 30 s for annealing at 50.3 °C, elongation for 1 min at 72 °C], and a final elongation for 5 min at 72 °C. PCR products were checked by gel electrophoresis on a 1.5 % agarose gel containing ethidium bromide. Sample was sequenced using Sanger's dideoxy method, and sequencing reactions were carried using the ABI 3730xl DNA Analyzer at Barcode BioSciences, Bangalore, India. The generated partial *16s rRNA* gene sequence is deposited on the GenBank repository (523 base pairs; accession number MW493234). We included 22 sequences of *Tropidophorus* species available from the NCBI database, and one sequence of *Dibamus novaeguineae* (KC621330) as an outgroup. The nucleotide sequences were aligned using the MUSCLE algorithm (Edgar, 2004) with default parameters, and uncorrected p-distance was calculated in MEGAX (Kumar et al., 2018). The best-fitting model for the nucleotide substitution was selected under the Bayesian Information Criterion (BIC) in ModelTest-

NG (Darriba et al., 2020). The Bayesian inference (BI) phylogeny was constructed in MrBayes 3.2.5 by selecting nst=6, rates=gamma and statefreqpr=dirichlet (1,1,1,1) for the GTR + G model. The MCMC (one cold and three hot chains) was run for 20,00,000 generations sampling one tree each 1,000 generations, and the analysis was terminated when the average standard deviation of split frequencies become less than 0.001 (Ronquist & Huelsenbeck, 2003). Burn-in was set to 25 %, and the remaining trees were used to assess Bayesian posterior probabilities (BPP) for nodal support (Fig. 1A). Maximum Likelihood (ML) analysis was performed with 1,000 bootstrap replicates in MEGA X software (Kumar et al., 2018) using the selected model (GTR + G) based on the lowest BIC score (Nei & Kumar, 2000). The ML phylogenetic tree is presented in Supplementary Material.

The first specimen of *T. assamensis* (MZMU613) was collected by H.T. Lalremsanga on 24 August 2011 from the banks of Tuichhuahen river, Kolasib District, Mizoram, India (24°14'10.42"N, 92°38'34.25"E, 62 m/asl.). On 27 November 2020, at ca. 2300, a female individual of *T. assamensis* (MZMU2080) was collected by H.T. Lalremsanga and a team from a roadside water canal located close by the Teirei Forest Guest House, Dampa Tiger Reserve (DTR), Mizoram, India (23°41'26.00" N, 92°27'6.12" E, 260 m/asl., Fig. 1B). In this study, we also examined an individual (PUCZM/B/111/0004) collected by Van Lalhlimpuia on 10 July 2014 from a streamlet at Tuirial village, Aizawl District, Mizoram, India (23°44'13.72"N, 92°47'57.41"E, 370 m/asl., Fig. 1C). Another male individual (MZMU2534) with snout-vent length 61.28 mm was collected on 15 August 2021 by Lal Muansanga from a water-filled muddy crevice of dried pond inside the buffer zone of Pualreng Wildlife Sanctuary, Kolasib District, Mizoram (24˚25'48.51"N, 92˚81'29.78"E, 538 m/asl.). The taxon type locality (Sylhet, Bangladesh), previous records (Mizoram and Assam States, India), and the collection sites of the new specimens (Mizoram State, India) are shown in Fig. 1D. Notably, the partially submerged skink (MZMU2080) within a muddy microhabitat of drainage at Dampa Tiger Reserve (Fig. 1E) not even react or try to flee when encountered and while capturing, instead remained still. Such behaviour is rather similar to the observations of Das et al. (2009).

The specimens agree with the original description (Anandale, 1912) and other literature (Smith, 1935; Mathew, 2006, 2007). Unfortunately, our first collected specimen MZMU613 has possibly been lost during shifting specimens from the old Museum building to the current Departmental Museum of Zoology, Mizoram University. So, the following morphological attributes are solely from the other two specimens, and provided the values as PUCZM/B/111/0004 & MZMU2080, respectively: snout-vent length 70.62 mm & 74.50 mm, exceeding the size of the largest known individual for the species (68.65 mm) previously recorded by Das (2008); tail length 92.42 mm & 90.20 mm; trunk length 41.38 mm & 37.04 mm; snout length 5.60 mm & 5.56 mm; snout width (at nostril) 2.80 mm & 2.85 mm; head length at angle of jaw 11.40 mm & 14.38 mm; head width at angle of jaw 8.61 mm & 9.20 mm; head depth 6.73 mm & 6.84 mm; eye diameter 3.40 mm & 3.90 mm; tympanum exposed and large with diameter 1.86 mm & 2.10 mm; inter-narial distance 1.71 mm & 2.90 mm; eye to nostril distance 3.37 mm & 3.70 mm; tympanum to eye distance 4.73 mm & 5.80 mm; forelimb length 16.58 mm & 18.54 mm; hindlimb length 24.70 mm & 25.92 mm; leg when stretched almost reaches to the wrist; 91 & 80 caudal plates; scales around mid-body 34 & 31; longitudinal mid-dorsal scale from nuchal to level of hindlimb insertion 43 & 46; two large preanal scales; single large frontal; two prefrontal contact each other; supralabials 8 on either sides & 6 on left and 8 on right; infralabials 8 on left and 7 on right & 6 on left and 7 on right; lower eyelids scaly; supra oculars 4 & 4; lamellae on fourth finger 11 & 12, and 18 & 14 on fourth toe; relative finger length F4>F3>F2>F5>F1; and toe length T4>T3>T5>T2>T1; ventrals smooth; dorsal and lateral scales strongly keeled and mucronate; snout sharply pointed; rostrum convex; single nasal; mental large. In life, dorsum dark brownish with obscured yellow patches; prominent yellowish band across hindlimbs, and another across the forelimbs; three or four broken yellowish bands between the forelimbs and hindlimbs; head region including the nape paler than dorsum colour; tail abstrusely banded.

The *16s rRNA* dataset of *Tropidophorus* species consisted of a final alignment of 446 characters after removal of ambiguous aligned sites, of which 141 sites were diagnosed as variable. Our BI and ML analyses showed that *T. assamensis* is forming a sister species with *T. berdmorei* by a considerable nodal support (BPP=0.89; bootstrap value=78), and the clade (*T. assamensis* + *T. berdmorei*) is inferred as sister to the clade consisting of certain Indochinese species (*T. cocincinensis* + *T. microlepis*), Sulawesi (*T. baconi*), Philippines (*T. grayi* + *T. misaminius* + *T. partelloi*), and Borneo species group (*T. sebi* + *T. beccarii* + *T. brookei* + *T. micropus*) (BPP=0.91; bootstrap value=55). The branching patterns from our BI topology largely accorded with the cladogram in Honda et al. (2006), except on the position of *T. berdmorei* and *T. sinicus*. In the studied dataset, the interspecies mean uncorrected p-distance was 10.3 %, ranging from 9.7 % to 13.9 %. The studied sequence of *T. assamensis* showed 9.7 % genetic distance with the sister species *T. berdmorei*, while the maximal genetic distance 13.9 % was diagnosed with *T. beccarii* of the Borneo species group.

This study inferred the phylogenetic position of *T. assamensis* with its congeners, and presents additional distributional localities adjoining previous records with updated morphological and first genetic data (GenBank accession no. MW493234). We assume these new information will help improving the limited knowledge of this rare and poorly known water skink species. As of now, the species is documented from five Districts of Mizoram State and we speculated that it is widespread in the region despite its rarity. Regarding some discordances between our present BI topology and Honda et al.

(2006) in the phylogenetic reconstructions of the genus, we encourage further studies to assess the monophyly of *Tropidophorus* species, and also to determine the definite phylogenetic status of *T. assamensis* with better accuracy.

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Authors' contribution:
Hmar Tlawmte Lalremsanga and Lal Biakzuala collected specimens, conceived the research questions, designed the works, examining specimens, and wrote manuscript. Ht Decemson assists in specimen collection and manuscript writing. Mathipi Vabeiruyreilai assists in specimen collection and molecular works. Fanai Malsawmdawngliana accompanied the field survey and help in examining specimens. Van Lalhlimpuia and Lal Muansanga collected the specimen and help in manuscript revision.

Ethical statement:
The specimens in this study are collected after obtaining permission from Chief Wildlife Warden, Environment, Forest and Climate Change Department, Government of Mizoram, India (Permit No. A. 33011/2/99-CWLW/225).

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