



DNA Barcoding Reveals the First Record of the Burmese Whipping Frog, *Polypedates mutus* (Anura: Rhacophoridae), from India

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Introduction

The Burmese Whipping Frog (*Polypedates mutus*) was originally described by Smith (1940) from N' Chang Yang, Kachin, northern Myanmar (25.833313 N, 94.799994 E; elev. 789 m asl). The species belongs to the family Rhacophoridae, consisting of 434 species in 22 genera. The genus *Polypedates* Tschudi (1838) currently includes 25 nominal species and has a distribution ranging from eastern India throughout much of southeastern Asia (Frost 2022). According to Dinesh et al. (2020) and Frost (2022), the genus includes 13 species from India, two of which (*P. teraiensis* and *P. braueri*) occur in Mizoram.

Polypedates mutus is a cryptic species that was removed from the synonymy of the *P. leucomystax* complex by Liu and Hu (1961). These treefrogs inhabit broadly disturbed habitats with shrub or tree borders (Zug 2022). Their distribution ranges from mainland (Bago, Kachin, Shan) and peninsular (Tanitharyi) Myanmar through adjacent southern China, northern Laos, northern Thailand, and Vietnam (Frost 2022; Kuraishi et al. 2012). Liu et al. (2018) described call characteristics that differ from its congener, *P. megacephalus*. The species is listed by the International Union for Conservation of Nature (IUCN) Red List of Threatened Species as being of Least Concern (LC) (IUCN SSC Amphibian Specialist Group 2022). The species is diagnosable based on the combination of characters provided by Smith (1940), Pham et al. (2017), and Ziegler et al. (2006).

Methods

At 1315 h on 18 September 2021, we collected a subadult *Polypedates* sp. (Fig. 1) that was not readily identifiable to species on damp ground surrounded by vegetation near the human settlement of Sialhawk, Khawzawl District, Mizoram (23.291645 N, 93.095818 E; elev. 1,300 m asl) (Fig. 2). It was euthanized following methods of Conroy et

al. (2009), preserved in 70% ethanol, and catalogued in the Departmental Museum of Zoology, Mizoram University (MZMU2612). Measurements to the nearest 0.1 mm were taken with Mitutoyo dial Vernier Callipers (Model 505–671).

We extracted genomic DNA from liver tissue using DNeasy Blood and Tissue Kit (Qiagen™, Valencia, California, USA) following the standard protocol provided by the manufacturer. We amplified and sequenced the partial 16S rRNA gene using a pair of primers: forward L02510-CGCCTGTTTATCAAAAACAT (Palumbi 1996) and reverse H03063-CTCCGGTTTGAAGCTCAGATC (Rassmann 1997) before performing a PCR reaction for a 20-µL reaction mixture containing 1X amplification buffer, 2.5 mM MgCl₂, 0.25 mM dNTPs, 0.2 pM each forward and reverse primers, 1 µL genomic DNA, and 1 U Taq DNA polymerase with a pair of partial 16S rRNA primers (above). The PCR thermal regime for amplification was 5 min at 95 °C for initial denaturation, followed by 35 cycles of 1 min at



Fig. 1. Dorsal (left) and ventral views of a subadult Burmese Whipping Frog (*Polypedates mutus*) collected in Sialhawk, Mizoram, India. Photographs by Vanlal Siammawii.

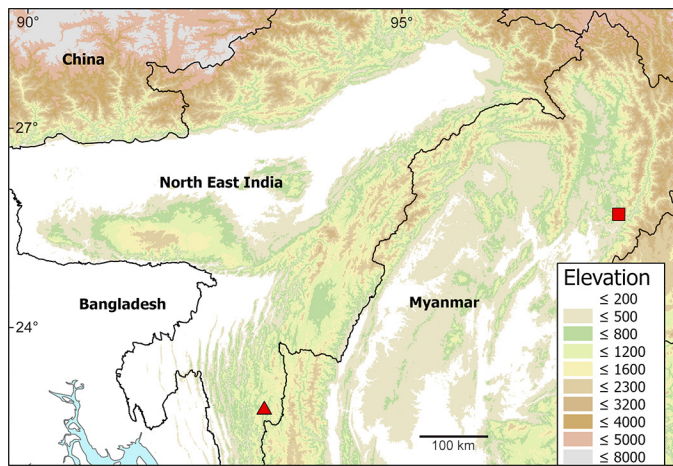


Fig. 2. Map showing the locality records of the Burmese Whipping Frog (*Polypedates mutus*) from the Indo-Bangladesh Region. The type locality N'Chang Yang, Kachin, in northern Myanmar is indicated by the red square and new record in Sialhawk, Mizoram, India, is marked by the red triangle.

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 amplicons were confirmed by gel electrophoresis on a 1.5% agarose gel containing ethidium bromide. Samples were sequenced using Sanger's dideoxy method and sequencing was carried out for both forward and reverse directions (Barcode Bioscience, Bangalore, India). The newly generated partial 16S rRNA sequences were deposited in NCBI's GenBank repository to obtain an accession number.

The phylogenetic analyses included 15 nucleotide sequences consisting of 496 bp for 14 congeners as ingroup

taxa with *Duttaphrynus melanostictus* as the outgroup (Table 1). All alignments were performed using MUSCLE (Edgar 2004) in MEGA X (Kumar et al. 2016) with default parameter settings and uncorrected pairwise distances were calculated using a Maximum Likelihood (ML) approach by RAxML 2.0 at default setting (ML + rapid bootstrap) (Edler et al. 2021). We selected the best-fitting nucleotide substitution model (GTR+G) for Bayesian Inference (BI) analysis based on the lowest AIC (Akaike Information Criterion) score in MrModeltest v2.3 (Nylander 2004). We then estimated Bayesian phylogenies using MrBayes 3.2.6 (Ronquist et al. 2012) and employing the best-fit substitution model, we ran 2 parallel chains in the Markov Chain Monte Carlo (MCMC) for 10,000,000 generations each. The posterior was sampled every 1,000 generations. We discarded the initial 25% of posterior samples as burn-in. The MCMC chain was run until the average standard deviation of split frequency was lower than 0.002, indicating that the chain had converged. Both phylogenetic trees were visualized and edited using FigTree v1.4.4.

Results

The phylogenetic analysis revealed similar topologies. Our specimen was placed in the same clade with *Polypedates megacephalus* (MW204592), *P. impresus* (MK481034), and *P. leucomystax* (HM770388) with BPP/BS support values of 0.98/93. Our specimen was conspecific with *P. mutus* from Myanmar with significant BP/bootstrap value (0.99/58) (Fig. 3). The genetic sequence of *P. mutus* and its congeners were compared and uncorrected pairwise divergence based on the 16S rRNA gene fragment was calculated (Table 2). The

Table 1. Samples used for phylogenetic estimation using 16s rRNA sequence data.

Species	GenBank Accession Number	Source
<i>Polypedates mutus</i>	ON072235	This study
<i>Polypedates mutus</i>	MG935985	Mulcahy et al. 2018
<i>Polypedates megacephalus</i>	MW204592	Koehler and Thammachoti, unpublished
<i>Polypedates leucomystax</i>	HM770388	Brown et al. 2010
<i>Polypedates impresus</i>	MK481034	Luo and Zhou, unpublished
<i>Polypedates teraiensis</i>	MW165469	Lalremsanga et al., unpublished
<i>Polypedates braueri</i>	MW165467	Lalremsanga et al., unpublished
<i>Polypedates discantus</i>	KF303483	Rujirawan et al. 2013
<i>Polypedates himalayensis</i>	MT983150	Khatiwada et al. 2021
<i>Polypedates maculatus</i>	KY111850	Senevirathne et al., unpublished
<i>Polypedates cruciger</i>	GQ204692	Meegaskumbura et al. 2011
<i>Polypedates macrotis</i>	JN377345	Haas et al., unpublished
<i>Polypedates colletti</i>	EF624066	Haas and Das 2008
<i>Polypedates otolophus</i>	JN377346	Haas et al., unpublished
<i>Duttaphrynus melanostictus</i>	MW165455	Lalremsanga et al., unpublished

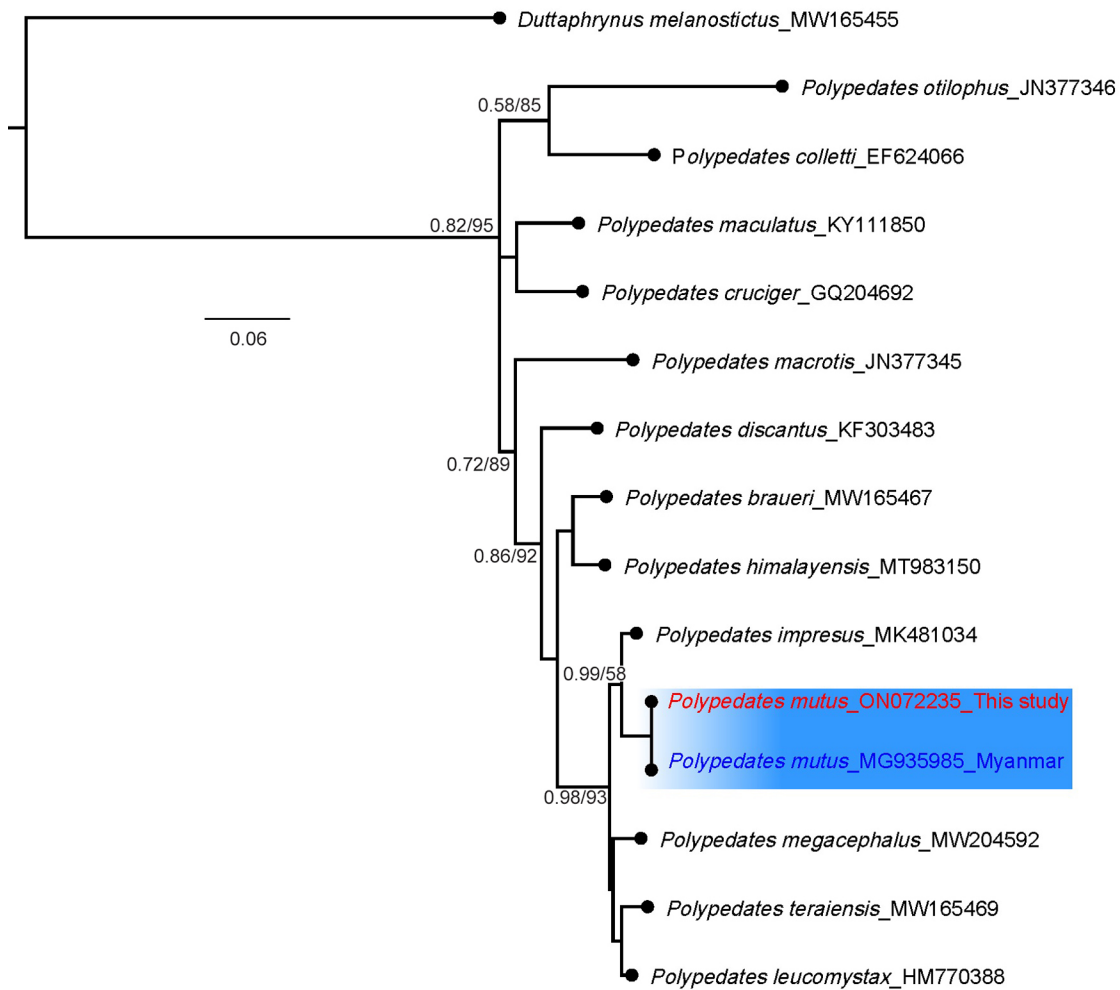


Figure 3. Phylogenetic position of the Burmese Whipping Frog (*Polypedates mutus*) from Mizoram, based on partial 16S rDNA. Numbers at nodes represent support values for that node in both Bayesian and maximum likelihood (BPP/BS). Colors denote this study; the red font indicates study species and blue denotes type material with its GenBank accession number followed by the distribution.

calculated interspecific genetic distances within the genus *Polypedates* ranged from 0% to 12% from *P. mutus* to *P. otlophus*. The genetic distance (uncorrected p-distance) between the specimen from Sialhawk, Mizoram (India), and *P. mutus* from Mandalay (Myanmar), was 0.002%. The genetic distances between two congeners found within the state (i.e., *P. braueri* and *P. teraiensis*) were 6% and 3%, respectively. Members of the *P. leucomystax* complex are morphologically very similar and notoriously difficult to classify (Sutthiwisea et al. 2020; Yuan et al. 2021), however, they clearly differ genetically.

Morphological characters for specimen MZMU2612 were small size (SVL 26.8 mm); head longer than wide (HL 10.1 mm, HW 9.2 mm); snout pointed, longer than eye (SL 4.4 mm, ED 3.8 mm); canthus rostralis distinct, slightly concave; loreal region vertical; nostrils closer to tip of snout than to eye (EN 3.2 mm, SN 1.5 mm); interorbital distance wider than internarial distance and upper eyelid (IOD 3.3 mm, IND 2.2 mm, UEW 2.7 mm); tympanum distinct, approximately half of eye diameter (TYE 0.6 mm,

TYD 1.9 mm); vomerine teeth present; tongue notched posteriorly; fingers free of webbing; relative length of fingers I<II<IV<III; tips of fingers with enlarged discs; relative length of toes I<II<III<V<IV; subarticular tubercles present; inner metatarsal tubercle present (IMT 1 mm); tibio-tarsal articulation reaching tip of snout; dorsum and upper flanks smooth; lower flanks, belly, and ventral surfaces of thighs granular; supratympanic fold present. Coloration in life was a yellowish brown dorsum with dark blotches, limbs with dark transverse bars, blackish brown stripes bordering supratympanic folds from eyes to arms, backs of thighs with small white spots, and belly yellowish white.

Discussion

This work confirms the occurrence of *Polypedates mutus* in Mizoram, India, which represents the first record of this species and the fourteenth species of *Polypedates* in the country. This frog was collected along the fringe of a homestead garden characterized by vegetation that included *Acacia pennata*, *Albizia chinensis*, *Amomum maximum*, *Artocarpus herophyl-*

Table 2. Estimates of evolutionary divergence based on uncorrected K2p distance among species of *Polypedates* based on the 16S rRNA partial gene.

No.	Species	K2p distance														
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1	<i>Polypedates mutus</i>															
2	<i>Polypedates mutus</i>	0.00														
3	<i>Polypedates megacephalus</i>	0.03	0.03													
4	<i>Polypedates leucomystax</i>	0.03	0.03	0.02												
5	<i>Polypedates impresus</i>	0.02	0.02	0.03	0.02											
6	<i>Polypedates teraiensis</i>	0.03	0.03	0.03	0.02	0.03										
7	<i>Polypedates braueri</i>	0.06	0.06	0.06	0.05	0.05	0.05									
8	<i>Polypedates discantus</i>	0.07	0.07	0.07	0.07	0.06	0.07	0.05								
9	<i>Polypedates himalayensis</i>	0.06	0.06	0.06	0.06	0.06	0.06	0.04	0.05							
10	<i>Polypedates maculatus</i>	0.08	0.08	0.08	0.09	0.09	0.09	0.08	0.08	0.07						
11	<i>Polypedates cruciger</i>	0.09	0.09	0.08	0.09	0.09	0.08	0.08	0.08	0.07	0.06					
12	<i>Polypedates macrotis</i>	0.09	0.09	0.10	0.09	0.09	0.10	0.09	0.08	0.08	0.09	0.09				
13	<i>Polypedates colletti</i>	0.09	0.09	0.10	0.10	0.08	0.11	0.09	0.09	0.09	0.08	0.09	0.11			
14	<i>Polypedates otitophus</i>	0.12	0.12	0.12	0.12	0.12	0.13	0.12	0.12	0.12	0.10	0.12	0.12	0.11		
15	<i>Duttaphrynus melanostictus</i>	0.20	0.20	0.20	0.20	0.19	0.19	0.18	0.19	0.18	0.19	0.19	0.20	0.20	0.20	

lus, *Mangifera indica*, and *Psidium guajava*. Other rhacophorids found in the area are *Kurixalus yangi*, *P. braueri*, and *P. teraiensis*.

This new locality is about 560 km aerial distance southwest of the type locality in Myanmar and represents the westernmost record of the species. According to Champion and Seth (1968), the area is in Assam Subtropical Pine Forest (9/C2), which has a moderate climate. Winter temperatures vary from 10 to 20 °C and summer temperatures are between 15–30 °C. Mean annual rainfall is 2,037.6 mm. Mizoram is an important part of the Indo-Burma biodiversity hotspot, as it contains a diverse flora and fauna. Studies within the last decade have revealed new records of amphibians in the state (e.g., Chaitanya et al. 2017; Lalremsanga 2017a, 2017b, 2017c; Lalbiakzuala and Lalremsanga 2019; Decemson et al. 2020, 2021; Kundu et al. 2020; Lalremsanga et al. 2020, 2021a, 2021b; Malsawmhriatzuali et al. 2020; Muansanga 2020; Muansanga et al. 2020, 2021a, 2021b). Additional surveys combined with more stable systematics are likely to identify additional species in the region.

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