

A new species of *Bufoides* Pillai and Yazdani 1973 (Amphibia: Bufonidae) from Mizoram (India) and the delimitation of the distribution range of *Bufoides meghalayanus* (Yazdani & Chanda 1971) to the Khasi hills, Meghalaya (India)

R.S. NAVEEN^{1,2}, BENJAMIN TAPLEY³, S.R. CHANDRAMOULI^{5,*}, PHILLIP A. JERVIS⁴, S. BABU^{1, **},
A.B. MEETEI⁶, P.V. KARUNAKARAN¹

¹Sálim Ali Centre for Ornithology and Natural History, Anaikatty, Coimbatore, Tamil Nadu, India. **email: sanbabs@gmail.com

²EDGE of Existence Programme, Conservation and Policy, Zoological Society of London, London, NW1 4RY, UK

³Zoological Society of London, Regent's Park, London, NW1 4RY, United Kingdom

⁴ZSL Institute of Zoology, Regent's Park, London, NW1 4RY, United Kingdom

⁵Department of Ecology and Environmental Sciences, School of Life Sciences, Pondicherry University, Puducherry, India. *email: findthesnakeman@gmail.com

⁶North Eastern Regional Centre, Zoological Survey of India, Risa Colony, Shillong, Meghalaya 793003, India

Manuscript received: 14 May 2023. Revision accepted: 1 September 2023

Abstract. Naveen RS, Tapley B, Chandramouli SR, Jervis PA, Babu S, Meetei AB, Karunakaran PV. 2023. A new species of *Bufoides* Pillai and Yazdani 1973 (Amphibia: Bufonidae) from Mizoram (India) and the delimitation of the distribution range of *Bufoides meghalayanus* (Yazdani & Chanda 1971) to the Khasi hills, Meghalaya (India). *Biodiversitas* 24: 4617-4627. The Oriental toad genus *Bufoides* currently comprises two species: *Bufoides meghalayanus* and *B. kempfi*. Populations of *Bufoides* from Mizoram were previously considered to be conspecific with *Bufoides meghalayanus*, although it has been hypothesized that these populations could represent an undescribed species. An uncorrected p-distance at the 16S rDNA gene between the Mizoram population and each of the two congeneric species was 2.74-3.0% and 3.5% for *B. meghalayanus* and *B. kempfi* respectively. We describe the population from Dampa Tiger Reserve, Mizoram, as new based on molecular data from two specimens and morphological data from two adult males and one adult female. We confirm that *B. meghalayanus* is endemic to the Khasi Hills in Meghalaya and it does not occur in Mizoram. The new species from Mizoram differs from congeneric species by differences in interdigital webbing, coloration, skin tuberculation and the presence of ovoid, tuberculated and depressed parotoid glands. Like other *Bufoides* species, it is a microhabitat specialist and utilizes streamside rock crevices as refugia, which might make it vulnerable to changes in habitat. The new species is currently only known to occur in Dampa Tiger Reserve and it is probably range-restricted and likely meets the International Union for Conservation of Nature's criteria for being assessed as Critically Endangered.

Keywords: Amphibian, conservation, cryptic diversity, Indo-Burma region, taxonomy, systematics

INTRODUCTION

The family Bufonidae is a cosmopolitan group represented by 646 species belonging to 52 genera, spread almost across every continent (Frost 2023). The distribution of several taxonomic groups within this family is restricted to certain parts of biodiversity hotspots. Among these range-restricted genera, the genus *Bufoides* holds a special mention for being confined to a very narrow belt in the Northeast Indian region (Stuart et al. 2008). The genus currently includes two species, *B. meghalayanus* and *B. kempfi* (Frost 2023). Currently, the type localities of the recognized species are confined to the state of Meghalaya in the Garo and Khasi hills (Naveen et al. 2022). This genus was erected to accommodate the morphologically distinct species of semi-arboreal toad, *Ansonia meghalayana* (see Pillai and Yazdani 1973). *Bufoides kempfi* (Boulenger 1919) was previously assigned to the genus *Pedostibes* Günther, 1876 but was later transferred to the genus *Bufoides* after a detailed morphological examination (Chandramouli & Amarasinghe 2016). The

recent rediscovery of *B. kempfi* by Naveen et al. (2022) further corroborated and confirmed its generic placement in *Bufoides*. Historically, *B. meghalayanus* has been reported from Dampa Tiger Reserve and Ngengpui Wildlife Sanctuary in Mizoram State (Pawar and Birand 2001). Both these locations are more than 200 km away from Mawblang, the type locality of *B. meghalayanus*. Until recently, *B. meghalayanus* was assessed as Endangered by the International Union for Conservation of Nature as it was thought to occur at more than one location, including Dampa Tiger Reserve in Mizoram (IUCN SSC 2022). It was previously suggested that the populations of *Bufoides* found in Mizoram might represent a novel species (Das et al. 2009). However, this hypothesis has never been tested. During a recent IUCN Red List workshop, the range of *B. meghalayanus* was restricted to three locations, all within 1.5 km from one another in Cherrapunjee, East Khasi Hills in Meghalaya and the species was reassessed as Critically Endangered (IUCN SSC 2022). As a result, primarily the systematic status and consequently, the conservation status of the Mizoram population of *Bufoides* has not been

assessed until now and these populations are in conservation limbo.

A recent extensive study on the amphibian diversity in Dampa Tiger Reserve (Lalremsanga et al. 2021) reported several new records of amphibians from the region but did not report the presence of any *Bufo* species, likely because *Bufo* are cryptic and seasonally active (Pillai and Yazdani 1973). There have not been any further reports of the species from Ngengpui Wildlife Sanctuary since the original report of *Bufo* occurring there (Pawar and Birand 2001). To resolve taxonomic confusion, we conducted field surveys in Dampa Tiger Reserve, and we encountered a population of *Bufo*, superficially resembling *B. meghalayanus*. Herein, we assess the systematic status of the *Bufo* population that was encountered in Dampa Tiger Reserve of Mizoram and describe it as a new species.

MATERIAL AND METHODS

To resolve the taxonomic uncertainty surrounding *Bufo* in Mizoram (Figure 1), we undertook targeted searches for *Bufo* in their preferred microhabitats in moist, cool canopy-shaded rock outcrops in subtropical forests (Pillai and Yazdani 1973; Deuti et al. 2012; Naveen et al. 2022). A total of three *Bufo* specimens, comprising one adult female and two adult males, were collected from rock crevices of dry streams from two different locations within Dampa Tiger Reserve. These specimens were collected under the following permits (No.B.19060/1/2020-CWLW/) and ethical approval was granted by the Zoological Society of London's ethics committee (project ZDZ201). Specimens were photographed in life before being euthanized using about 0.10 ml of 20% solution of benzocaine applied to the

ventral surface of the toad. Liver tissues were extracted for molecular analyses from freshly euthanized specimens and stored in 99% molecular grade ethanol prior to fixation of specimens with 10% buffered formalin and storage in 75% ethanol. All specimens were deposited at the herpetological collection facility at Salim Ali the Centre for Ornithology and Natural history (SACON).

Molecular data

Total genomic DNA was extracted from two specimens of *Bufo* from Mizoram (SACON VA 400 and SACON VA 402) 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 16sB R-H (5'-CCGGTCTGAACTCAGATCACGT 3') respectively (Kocher et al. 1989). Amplifications were performed in an Applied Bio Systems Veriti 96 well thermal cycler: 20 µl reactions with 4 µl of 5X Phusion HF buffer, 0.4 µl of 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 min, annealing at 55°C for 1 min, extension at 72°C for 1 min 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 at Centre for Cellular And Molecular Platforms (C-CAMP), National Centre for Biological Sciences (NCBS), Bangalore, India. The new sequences were then checked on the Basic Local Alignment Search Tool, BLAST (The National Center for Biotechnology Information) (Altschul et al. 1990) to verify their approximate identity.

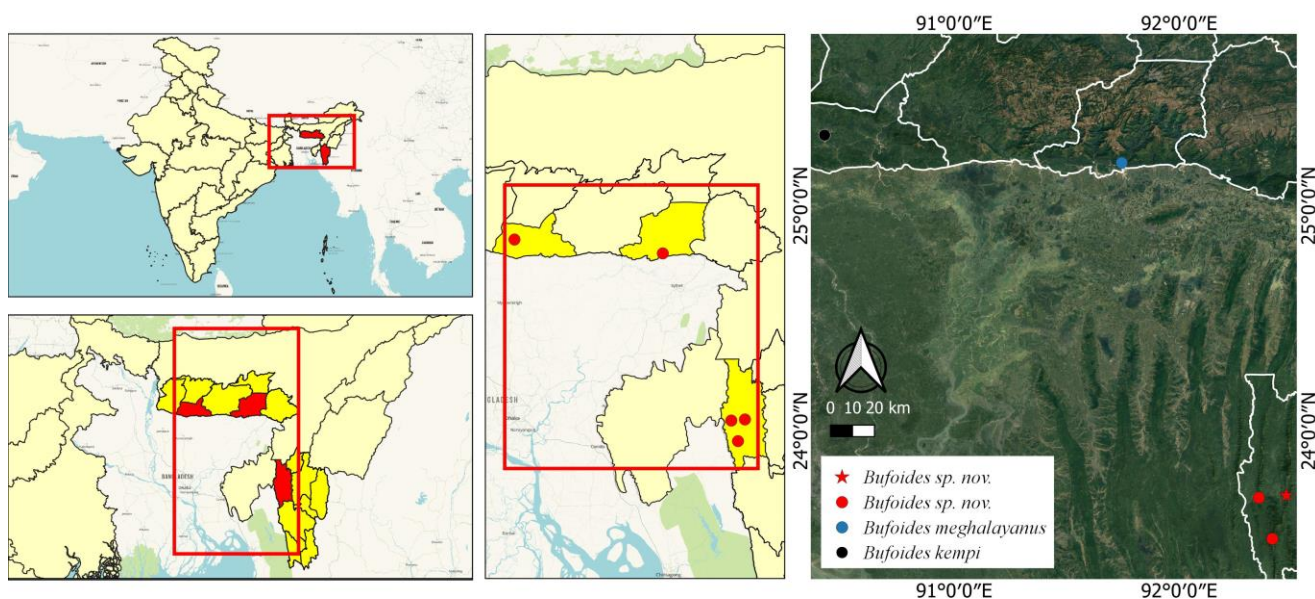


Figure 1. Map showing the distribution of *Bufo* species in India, Red star and red circles showing the type locality and occurrence points of *Bufo* sp. nov. respectively. Black and blue circles show the type localities of *Bufo kempii* and *Bufo meghalayanus* respectively. Green shaded area denotes the EOO of the new species of *Bufo* from Mizoram

Phylogenetic analysis

Results of the BLAST analyses, including sequences from the two specimens we collected from Dampa Tiger Reserve, showed that all of the least divergent samples were from species within the genus *Bufoides*. The sequences were aligned along with 14 other taxa belonging to 12 genera from Bufonidae, comprising eight of the genera reported from India and Sri Lanka (Frost 2023), with *Hyla arborea* as the outgroup taxon, see Table 1. For the sequences obtained from the *Bufoides* collected in Dampa Tiger Reserve, forward and reverse sequences were aligned and checked for any discrepancies. Consensus sequences were then trimmed to 413 bp to match the rest of the sequence dataset. The sequences were aligned with ClustalX.

The resulting alignment was subjected to a Bayesian phylogenetic analysis in MrBayes version 3.1.2 (Ronquist and Hueslenbeck 2003). The analysis was run for three million MCMC iterations initially until the standard deviation of split frequencies reached a value 0.001. Otherwise, the analysis was run for another 10,000-10,000,0 generations until the standard deviation of 0.001 was obtained for the split frequencies. Initial 20% of the trees were discarded as ‘Burn-in’. The tree files generated were then visualized using Fig Tree v. 1.4.4 (Rambaut 2009). The distance matrix from the sequence alignment was extracted using Mega 6.0 (Tamura et al. 2013).

Morphological data

We recorded morphological data from fixed specimens to the nearest 0.02 mm with INSIZE dial calipers. All specimens were measured by a single author (R.S. Naveen) for consistency and to minimize inter-observer bias. Measurements follow Naveen et al. (2022); snout-vent length, from the tip of the snout to the anterior margin of the cloaca (SVL), axilla-groin distance, measured from the posterior margin of the forelimb at its insertion point in the body to the anterior margin of the hind limb at its insertion point in the body (AG), head length, measured from the posterior edge of the mandible to the tip of the snout (HL), head width, the maximum width of the head at the angle of the jaws (HW), head depth, the maximum depth of the head (HD), body width, the maximum width of the body on the trunk (BW), eye diameter, the greatest horizontal diameter of the orbit (ED), eye-nostril distance, measured from the anterior border of the orbit to the middle of the nostril (EN), eye-snout distance, measured from the anterior border of the orbit to the tip of the snout (ES), upper eyelid width, the maximum width of the upper eyelid (UEW), interorbital distance, the distance between the upper eyelids (IO), internarial distance, the distance between the nostrils (IN), upper arm length, measured from the axilla to elbow (UAL), lower arm length, measured from the posterior margin of the elbow to the base of the outer metacarpal tubercle (LAL), palm length, from the posterior border of the outer metacarpal tubercle to tip of the 3rd finger (PAL), femur length, measured from the cloaca to the knee (FEL), tibia length, measured from knee to heel (TBL), tarsus length, measured from posterior margin of the tibia to anterior margin of the foot (TSL), foot length, measured

from inner metatarsal tubercle to the tip of the 4th toe (FOL). Interdigital webbing formulae follow Savage & Heyer (1997). Sex was determined by the presence of nuptial pads in males and by the presence of eggs that were visible through the ventral abdominal surface of females.

We compared the newly collected specimens from Dampa Tiger Reserve with the two congeneric species; *B. meghalayanus* and *B. kempi* that were collected from their respective type localities. In addition, digital images of the dorsal, lateral and ventral views of the holotype and paratype specimens of *B. meghalayanus* (ZSI A 6969 and ZSI A 6970) were studied for comparison.

Species distribution mapping

The coordinates of each sighting of each individual *Bufoides* encountered in Dampa Tiger Reserve were recorded using a hand-held GARMIN 64s GPS device (WGS 84 datum). The geographic range of the new species was estimated by plotting the known occurrences of the species from the current study on a distribution map generated using QGIS 3.24.1. The area under the minimum convex hull was computed by connecting the outermost occurrence points to calculate the Extent of Occurrence (EOO) as defined by the IUCN (2001)

RESULTS AND DISCUSSION

Molecular analyses. The two sequences we obtained from the *Bufoides* population from Dampa Tiger Reserve of Mizoram State were nested within the genus *Bufoides* and showed a close sister relationship to *Bufoides meghalayanus* in particular (Figure 2). The two new sequences formed a well-supported group with several other *Bufoides* sequences available on GenBank, which unfortunately lack any associated provenance information (MW741544.1 and MW741545.1 assigned to the organism *B. meghalayanus* and OL457694.1 assigned to the organism *B. kempi*). It is likely that the sequences that lack the provenance data were obtained from specimens also collected from Mizoram, as the title of the associated unpublished work relates to a project that aims to provide a storehouse of collection of the biodiversity of the region. The uncorrected *p*-distance between the two sequences from the two specimens collected in Dampa Tiger Reserve was 0.24% (Table. 2). The uncorrected *p*-distance between the two sequences from the two specimens collected in Dampa Tiger Reserve with those that lack provenance data (but also likely from Mizoram) ranged from 0.00-0.24%. This supports the hypothesis that these specimens represent a single operational taxonomic unit. Uncorrected *p*-distance between *Bufoides* sp. from Mizoram (including those that lack provenance data) and other taxa in the genus *Bufoides* ranged from 2.74-3.0% (*B. meghalayanus*) to 3.5% (*B. kempi*). A value of 3.0% has been considered an ideal threshold for a candidate species at the 16S gene (Vences et al. 2005). Molecular data combined with morphological differences (read below) provide additional support that the newly collected specimens of *Bufoides* sp. from Dampa Tiger Reserve represent a new species.

Table 1. List of specimens and GenBank accession numbers for all 16S rRNA sequences included in our molecular analysis

Taxa	Voucher No.	GenBank No.	Locality	Reference
<i>Bufoides bhupathyi</i> sp. nov.	SACON VA 400	OR417419	Dampa Tiger Reserve, Mizoram, India	<i>This study</i>
<i>Bufoides bhupathyi</i> sp. nov.	SACON VA 402	OR417420	Dampa Tiger Reserve, Mizoram, India	<i>This study</i>
<i>Bufoides meghalayanus</i>	ZSI A-12519	KT991342.1	Meghalaya, India	Chandramouli et al. 2016
<i>Bufoides kempi</i>	SACON VA 180	OP920605.1	Eman Asakgre, Meghalaya, India	Naveen et al. 2022
<i>Bufoides 'kempi'</i>	MZMU 2332	OL457694.1	No Locality data	Biakzuala et al. 2022
<i>Bufoides 'meghalayanus'</i>	MZMU 2078	MW741544.1	No Locality data	Biakzuala et al. 2022
<i>Bufoides 'meghalayanus'</i>	MZMU 2091	MW741545.1	No Locality data	Biakzuala et al. 2022
<i>Blythophryne beryet</i>	ZSI A-12521	KT991347.1	Mt. Harriet National Park, Andaman Islands, India	Chandramouli et al. 2016
<i>Blaira ornata</i>	AO5005	GU136099.1	India	Meenakshi et al. 2009
<i>Pedostibes tuberculosus</i>	SDB 4691	FJ882793.1	Western Ghats, India	Van Bocxlaer et al. 2009
<i>Duttaphrynus himalayanus</i>	SDB 4566	FJ882790.1	No Locality Data	Van Bocxlaer et al. 2009
<i>Duttaphrynus melanostictus</i>	VUB 0052	FJ882791.1	No Locality data	Van Bocxlaer et al. 2009
<i>Beduka koynayensis</i>	BS INH173	MF680074.1	India	Padhye et al. (unpublished)
<i>Adenomus kelaartii</i>	VUB 0171	FJ882780.1	Sri Lanka	Van Bocxlaer et al. 2009
<i>Bufotes viridis</i>	NP B-2-1	FJ882813.1	No Locality data	Van Bocxlaer et al. 2009
<i>Sigalegalephrynus mandailinguensis</i>	UTA A-63562	KX192094.1	Sumatra, Indonesia	Smart et al. 2017
<i>Sabahphrynus maculatus</i>	BORNEENSIS 08425	AB331718.1	Malaysia, Borneo	Matsui et al. 2007
<i>Pelophryne misera</i>	KUHE 37193	LC485456.1	Malaysia, Sabah	Matsui 2019
<i>Ansonia longidigita</i>	FMNH 242550	DQ283341.1	Sabah, Malaysia	Frost et al. 2006
<i>Hyla arborea</i>	HJ56	KP109597.1	Poland, Wroclaw	Gvozdk et al. 2014

Table 2. Uncorrected 16s pairwise distances among *Bufoides* nov. sp., allcongeneric species and representatives of bufonid genera

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	
1 KT991342.1 <i>Bufoides meghalayanus</i> _ZSI_A-12519																				
2 OR417419 <i>Bufoides bhupathyi</i>	2.74																			
3 OR417420 <i>Bufoides bhupathyi</i>	3	0.24																		
4 MW741544.1 " <i>Bufoides meghalayanus</i> "_MZMU2078	2.74	0	0.24																	
5 MW741545.1 " <i>Bufoides meghalayanus</i> "_MZMU2091	2.74	0	0.24	0																
6 OL457694.1 " <i>Bufoides kempi</i> "_MZMU2332	2.74	0	0.24	0	0															
7 OP920605.1 <i>Bufoides kempi</i> _voucher_SACON_VA_180	3.5	3.23	3.49	3.23	3.23	3.23														
8 KT991347.1 <i>Blythophryne beryet</i> _voucher_ZSI_A-12521	10.54	9.94	10.24	9.94	9.94	9.94	9.14													
9 MF680074.1 <i>Beduka koynayensis</i> _BS-INH173	6.65	5.83	6.1	5.83	5.83	5.83	5.32	10.56												
10 FJ882813.1 <i>Bufotes viridis</i> _NP_B-2-1	7.22	6.92	7.2	6.92	6.92	6.92	6.41	10.9	6.94											
11 FJ882790.1 <i>Duttaphrynus himalayanus</i> _SDB_4566	6.67	6.63	6.91	6.63	6.63	6.63	6.36	12.44	8.28	6.69										
12 FJ882791.1 <i>Duttaphrynus melanostictus</i> _VUB_0052	9.18	8.59	8.89	8.59	8.59	8.59	7.78	11.47	7.82	7.23	5.01									
13 FJ882793.1 <i>Pedostibes tuberculosus</i> _voucher_SDB_4691	5.85	5.82	6.1	5.82	5.82	5.82	5.58	12.23	5.85	5.58	6.39	7.51								
14 FJ882780.1 <i>Adenomus kelaartii</i> _voucher_VUB_0171	8.56	7.7	7.98	7.7	7.7	7.7	7.1	10.83	9.59	6.06	9.02	10.45	8.43							
15 KX192094.1 <i>Sigalegalephrynus mandailinguensis</i> _UTA_A-63562	10.25	9.66	9.95	9.66	9.66	9.66	10.22	12.34	9.07	9.92	9.97	11.15	10.18	10.76						
16 LC485456.1 <i>Pelophryne misera</i> _KUHE:37193	12.36	12.08	12.39	12.08	12.08	12.08	11.82	16.96	10.99	9.48	10.54	10.9	11.46	13.93	13.42					
17 AB331718.1 <i>Sabahphrynus maculatus</i>	12.77	11.79	12.1	11.79	11.79	11.79	11.6	14.62	12.49	10.33	10.73	11.93	10.94	11.95	13.59	13.26				
18 GU136099.1 <i>Blairia ornata</i>	10.88	11.13	11.44	11.13	11.13	11.13	11.43	13.84	10.9	9.8	9.76	11.22	10.31	11.41	10.52	12.38	12.77			
19 DQ283341.1 <i>Ansonia longidigita</i> _voucher_FMNH_242550	14.26	13.32	13.64	13.32	13.32	13.32	12.97	17.63	13.6	12.4	12.36	13.91	12.39	14.01	12.35	14.41	14.24	14.78		
20 KP109597.1 <i>Hyla arborea</i> _isolate_HJ56	17.43	16.03	16.37	16.03	16.03	16.03	17.39	20.14	16.4	15.52	16.89	17.84	15	18.41	19.56	22.13	19.19	17.95	21.51	

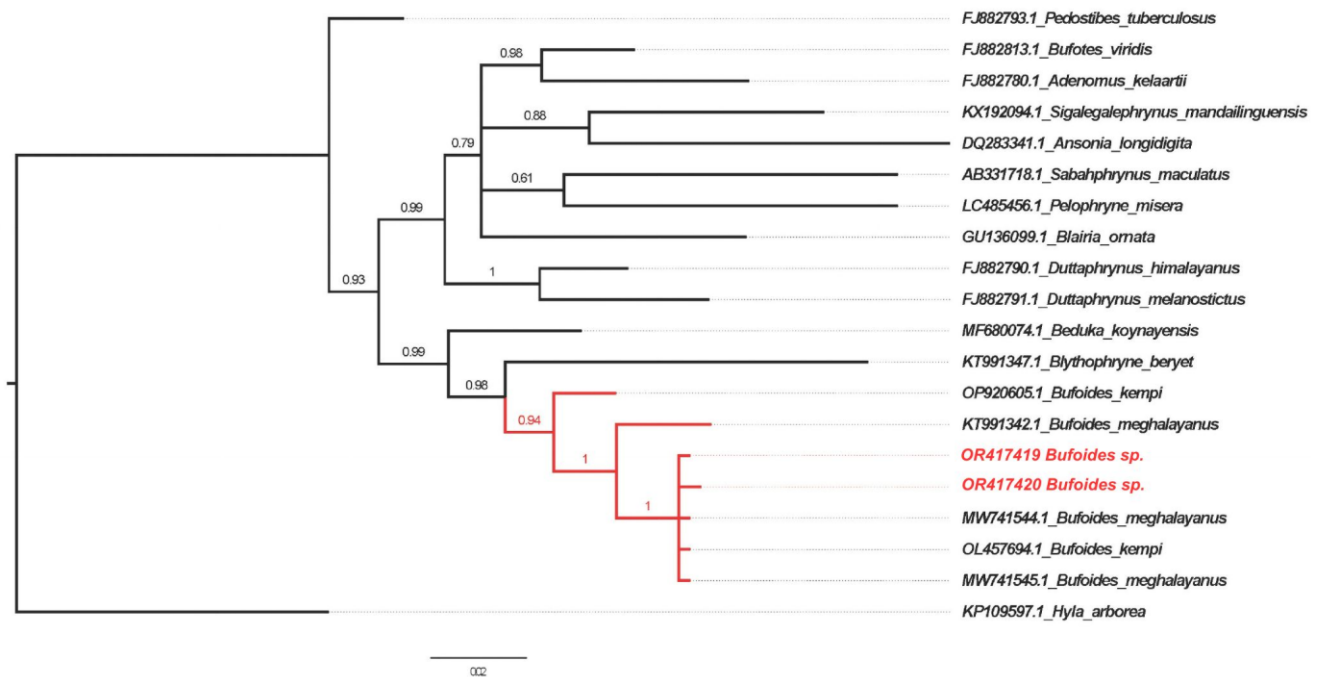


Figure 2. A Bayesian Inference tree for ~413 bp section of 16s (mtDNA) gene for *Bufoides* sp. nov. along with representatives of all *Bufoides* species. Bayesian Posterior Probabilities indicated on the tree

Systematics

Bufoides bhupathyi sp. nov.

Figures 3, 4, 5

Bufoides meghalayanus – Pawar and Birand (2001)
(partim “Mizoram”)

Holotype. SACON VA 400 (Figure 3 & 4), Adult male; found in a narrow rock crevice from a dried-out stream, locally known as “Dampa Luei” near Teirei village in Dampa Tiger Reserve, Mamit District, Mizoram, India (23.6874° N, 92.4552° E, 314 m asl). Collected at 11:30 hrs by R.S. Naveen on 12th March 2023.

Paratypes. SACON VA 402 (Figure 3 & 5), Adult female and SACON VA 401 adult male, found in a narrow rock crevice from a dried-out stream near Chaka Anti-Poaching Camp in Dampa Tiger Reserve, Mamit District Mizoram, India (23.6883° N, 92.35001° E 440 m asl). Collected at 14:30 hrs by R.S. Naveen on 1st April 2023.

Comparative material examined:

Bufoides meghalayanus (N=4) SACON VA 215, SACON VA 251 and SACON VA 252, three adult males collected by RSN from near Mawblang village 25.252°N, 91.471°E, 1050 m asl.), one unsexed adult ZSI A-12519 from the Khasi Hills, Meghalaya, India.

Bufoides kempi (N=10) ZSI 18481a, adult and ZSI 18481b subadult syntypes from “above Tura”, Garo Hills, Meghalaya; SACON VA 157 (adult female) and, VA 181(subadult female), and SACON VA156; VA 158 -160; VA 164 & VA 180 six adult males collected by RSN from Eman Asakgre (25.37°N, 90.54°E, 200 m asl.), Garo Hills, Meghalaya, India.

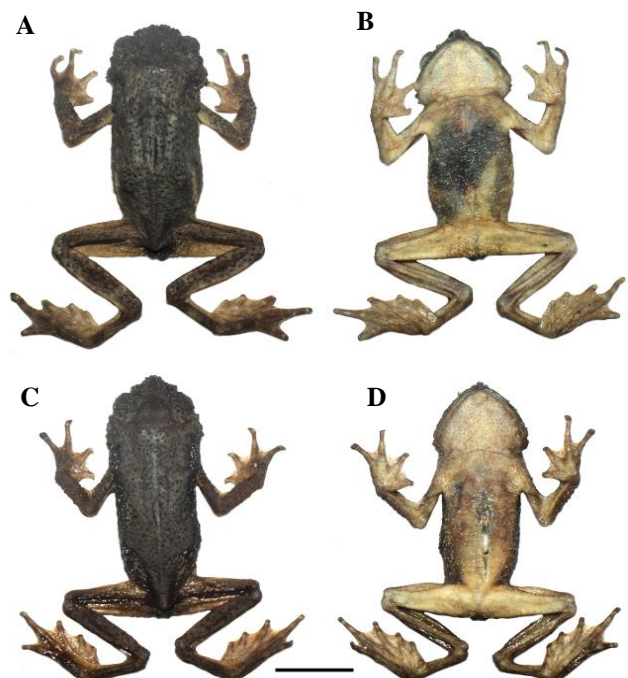


Figure 3. *Bufoides bhupathyi* sp. nov. in preservative. A) Dorsal view of adult male holotype (SACON VA 400), B) ventral view of adult male holotype (SACON VA 400), C) dorsal view of adult female paratype (SACON VA 402) and, D) ventral view of adult female paratype (SACON VA 402). 10 mm scale bar

Etymology. The species epithet honors the late Dr. S. Bhupathy, a noted scientist and a field herpetologist who served as a principal scientist at the Salim Ali Centre for Ornithology and Natural History (SACON).

Suggested vernacular name. Mizoram rock toad.

Diagnosis. The new species from Dampa Tiger Reserve is assigned to the genus *Bufo* on the basis of a robustly supported phylogenetic affinity (Figure 2) and the presence of the following combination of morphological characters: body dorsoventrally depressed and slender, parotoid glands ovoid, tuberculated and depressed, limbs slender, moderately webbed toes, and the absence of an externally visible tympanum (Boulenger 1919; Pillai and Yazdani 1973; Chandramouli & Amarasinghe 2016)

Bufo bhupathyi sp. nov. is a relatively small saxicolous species and can be distinguished from

congeneric species by a combination of the following characters: (1) adult male body size SVL 28.48 - 31.62 mm, (N=2); adult female body size SVL 33.78mm, (N=1); (2) presence of weakly defined irregular cranial ridges with pre-orbital, canthal and post-orbital ridges present and ovoid, depressed, and tuberculated parotoid glands; (3) dorsal surfaces, snout, inter-orbital region, lateral surfaces of head, upper part of flanks, forelimbs, hands, hind limbs and feet covered with small to medium-sized black conical tubercles; (4) dorsum dark brownish black with yellow blotches and reticulations in males and uniformly brownish black with only faint traces of yellow in flanks in females; (5) interdigital webbing between fingers I₀₋₁II₁₋₁III₂₋₁IV; and, (6) interdigital webbing between toes I₀₋₀II₀₋₀III_{1.5-2}IV_{2-1.5}V.



Figure 4. *Bufo bhupathyi* sp. nov. in life (holotype SACON VA 400). A) Dorsolateral view, B) dorsal view, and C) ventral view. Not to scale

Table 3. Measurements (mm) of *Bufo bhupathyi* sp. nov.

Species	<i>Bufo bhupathyi</i> sp. nov. *	<i>Bufo bhupathyi</i> sp. nov. **	<i>Bufo bhupathyi</i> sp. nov. **
Voucher	SACON VA 400	SACON VA 401	SACON VA 402
Sex	Male	Male	Female
SVL	31.62	28.48	33.78
AG	13.8	11.54	13.5
BW	10.94	8.76	10.54
HL	9.06	9.42	10.62
HW	12.56	10.82	12.78
HD	5.08	4.56	5.94
ED	3.48	3.12	4.12
EN	2.62	2.46	3.52
ES	4.68	3.64	4.86
UEW	2.9	2.68	3.18
IO	3.86	3.7	4.82
IN	2.42	2.62	2.82
UAL	6.72	5.3	7.46
LAL	7.62	7.5	9.9
PAL	9.02	7.24	8.78
FEL	12.58	11.12	13.24
TBL	13.3	12.3	13.74
TSL	8.9	8.64	9.5
FOL	12.88	10.2	13.44
Interdigital webbing fingers	I ₀₋₁ II ₁₋₁ III ₂₋₁ IV	I ₀₋₁ II ₁₋₁ III ₂₋₁ IV	I ₀₋₁ II ₁₋₁ III ₂₋₁ IV
Interdigital webbing toes	I ₀₋₀ II ₀₋₀ III _{1.5-2} IV _{2-1.5} V	I ₀₋₀ II ₀₋₀ III _{1.5-2} IV _{2-1.5} V	I ₀₋₀ II ₀₋₀ III _{1.5-2} IV _{2-1.5} V

Note: Abbreviations defined in Materials and Methods section. *Holotype, **paratypes



Figure 5. *Bufooides bhupathyi* sp. nov. in life. A) Dorsolateral view of adult male paratype (SACON VA 402), B) dorsolateral view of uncollected gravid female, C) dorsal view of uncollected gravid female, D) ventral view of uncollected gravid female, E) dorsal view of an uncollected male observed in September 2022, and F) dorsolateral view of an uncollected male observed in September 2022. Not to scale

Description of holotype. An adult male measuring (31.62 mm SVL), body dorsoventrally compressed. Trunk fairly short and relatively gracile (AG:BW 1.26), trunk less than half as long as the total body length (AG:SVL 0.44). Head wider than long (HL:HW 0.72) and longer than deep (HL:HD 1.78). Pupil, oval, horizontally elongated when dilated. Snout obtusely pointed dorsally and rounded laterally, tip of snout slightly projecting beyond lower jaw. Canthus rostralis distinct and curved, lores vertical, slightly concave; rounded nostrils open laterally, just below canthus, positioned slightly closer to tip of snout than to the eye (EN:ES 0.56). Eyes large, their diameter less than half the length of the head (ED:HL 0.38), eye diameter less than the distance between the eye and the snout (ED:ES 0.74). Upper eyelids narrower than interorbital space (IO:UEW 1.33). Interorbital distance greater than internarial distance (IO:IN 1.60). Tympanum not visible externally. Vomerine ridges, vomerine, maxillary and mandibular teeth absent. Tongue oval, not bifid and lacking papilla. Upper arm short (UAL:SVL 0.21). Lower arm longer than upper arm (UAL:LAL 0.88), palm longer than upper arm (UAL:PAL 0.75). Fingers long and slender, relative finger lengths III>IV>II>I, tips of digits small but obviously expanded

into discs. Fingers with moderate interdigital webbing, interdigital webbing formula $I_{0-1}II_{1-1}III_{2-1}IV$. Metacarpal tubercles present, fairly distinct and ovoid, subarticular tubercle present at the base of FII and supernumerary, and palmar tubercles absent. Thigh robust and short (FEL:SVL 0.40), nearly as long as tibia (FEL:TBL 0.95), tarsus shorter than tibia (TSL:TBL 0.67). Foot length subequal to thigh (FEL:FOL 0.98). Toes elongate with tips expanded into small discs, discs on toes smaller than discs on fingers; toes partially webbed, interdigital webbing formula $I_{0-0}II_{0-0}III_{1.5-2}IV_{2-1.5}V$; prominent inner and outer metatarsal tubercle and an ovoid shaped palmar tubercle present.

Skin texture and coloration of holotype in life. Skin of dorsal surfaces of snout, inter-orbital region, sides of head, upper part of flanks, forelimbs, hands, hind limbs and feet covered with small to medium-sized black conical tubercles. Lower portion of flanks with few scattered, small tubercles. Cranial ridges present, discontinuous and of closely arranged keratinized tubercles on top of the head with irregular, weakly defined pre-orbital, canthal and post-orbital ridges visible. Parotoid glands small, depressed, tuberculated and ovoid extending from the anterior edge of eye to just beyond the axilla. Lower half of eye bordered by

a single row of large tubercles. Upper lip lined with fine small black tubercles. Dorsal skin coloration uniformly dark brownish black with yellowish reticulations which are more intense on the flanks, thighs and lateral aspects of the head; ventral skin less tuberculated with dark brown and bright yellow granules, granules larger and higher around the cloaca and flanks. Ventral region pale brown and grey in the belly region with blotches of yellow on the throat and bright yellow spots on the belly and thighs. Row of large yellow tubercles on the outer edge of the tarsus extending from the heel to the inner metatarsal tubercle. Ventral surfaces of the palm and feet greyish brown and palmar aspects of hands covered in many small tubercles. Plantar aspect of feet with very few, small tubercles. Iris golden yellow with black reticulations; pupil black.

Color of holotype in preservative. Dorsal surface uniformly dark brownish black with faint traces of pale-yellow reticulations on flanks. Ventral surfaces of body, head, hands and thighs cream brown with greyish black belly and flanks. Ventral surface of tibio-fibula and feet pale brown in color.

Variation. (Detailed measurements of paratypes are provided in Table 3).

The female paratype, SACON VA 402 is larger than the two males collected. Dorsum coloration uniformly black. Venter, dark brown speckled with small yellow, pink and brown tubercles, fewer tubercles relative to holotype. The plantar aspects of the feet are more tuberculated than in the holotype (Figure 3). An uncollected female that was observed at 23.6878° N, 92.3491° E (Figure 5) was larger than the males (SVL 34.06 mm), dorsum brownish black with faint traces of yellow in flanks, venter dark brown to black, speckled with yellow and brown tubercles. The uncollected female was gravid and clusters of milky white eggs, each about 2 mm in diameter were visible through the ventral skin of the abdomen.

The male paratype, SACON VA 401 mostly matched the color description of the holotype with the exception of having more prominent yellow reticulations on the dorsal body surface. An uncollected male (Figure 5) was observed in the month of September 2022. This individual was dark black and bright yellow in color, with a diamond-shaped dark marking on the dorsal surface of mid-body and haddistinct dark bars on the dorsal surfaces of the hind limbs and forearms.

Secondary sexual characters Both male specimens had slightly raised nuptial pads covered with black microspinules; nuptial pads covering most of the dorsal surface of the base of FI.

Natural history. A total of six individuals were observed at three sites in Dampa Tiger Reserve, Mamit District, Mizoram.

Dampa luei stream: The stream originates from inside the core area of Dampa Tiger Reserve. At the time of collection (March 2023), the stream was mostly dry except for a few small stagnant pools. *Bufoides* tadpoles were not seen at this site. The adult male holotype (SACON VA 400) was found inside a 5 cm wide horizontal rock crevice of a medium-sized rock. The crevice was about one meter high off the ground and was sandy and dry, the temperature

inside the crevice was 22°C. The toad was found resting about 20 cm inside the crevice. The area was dominated by subtropical moist deciduous forests, with a canopy cover of approximately 80% and the ambient temperature over a 24-hr period ranged from 15-26°C, the lowest recorded at around 04:00 hrs and highest at around 14:00 hrs. The following anuran species were encountered at the site *Ingerana borealis*, *Leptobrachella tamdil*, *Odorrana chloronota*, *Raorchestes rezakhani*, *Kurixalus yangi* and *Theلودerma baibungense*.

Stream near Chaka Anti-Poaching Camp: A narrow dried-out stream near Chaka Anti-Poaching Camp was surveyed in March 2023. The paratypes (SACON VA 401 and 402), an adult male and female were collected from a rock crevice in this stream bed. The rock was surrounded by moist deciduous forests with an estimated 75% canopy cover. The crevice was about one meter high off the ground and 5 cm deep, the paratypes along with another uncollected male were found to be resting close together in the same crevice. The ambient temperature in the area was 27°C and the temperature inside the crevice was recorded to be 25°C when the toads were observed at 14:30 hrs. Another uncollected gravid adult female with 10-15 pearly white eggs visible through her abdomen was found inside a different rock crevice further along the stream. The ambient temperature in the area was 23°C and the temperature inside the crevice was recorded to be 23°C at 9:00 hrs when the toad was observed. Tadpoles of *Bufoides* species were not seen at this site. The following anuran species were encountered at the site in March 2023, *Ingerana borealis*, *Amolops indoburmanensis*, *Odorrana chloronota*, *Raorchestes rezakhani* and *Kurixalus yangi*. In September 2022, an adult male (Figure 5) was photographed from a dry stream near the southern entrance of Dampa Tiger Reserve, which was resting on a leaf about 0.4 m above the ground.

Distribution and conservation status. *Bufoides meghalayanus* and *B. kempfi* are known to be microhabitat specialists inhabiting narrow niches limited to moist, cool canopy-shaded rock outcrops in subtropical forests with elevational ranges of 1000-1200 m asl and 100-250 m asl respectively, where their cryptic behavior, morphology and body coloration provide camouflage in this environment (Pillai and Yazdani 1973; Deuti et al. 2012; Naveen et al. 2022). *Bufoides bhupathyi* sp. nov. was recorded from elevations between 314 m asl and 440 m asl. in Dampa Tiger Reserve and exclusively from areas with abundant rock boulders in well-shaded undisturbed moist deciduous forest. All individuals were recorded from narrow rock crevices and these observations indicate that this species also requires cool and damp environments indicative of a narrow ecological niche like other *Bufoides* species. The specific microhabitat requirements may make this species vulnerable to habitat modification and disturbance. Although *Bufoides bhupathyi* sp. nov. is relatively well protected from common anthropogenic threats because of its known distribution range occurring within protected tiger reserve, vegetation within Dampa Tiger Reserve has changed drastically in the last quarter of a century. There has been a marked reduction in moist deciduous forest

cover (Devi et al. 2011) which is the dominant habitat type currently known to harbor *Bufoides bhupathyi* sp. nov. and this indicates that this species could be threatened despite its habitat being protected. Rock quarrying was observed in a location about three km near from where the toads were observed (Figure 6), this activity is likely to imperil populations of *Bufoides bhupathyi* sp. nov. Rock quarrying and blasting are a known threat to the congeneric species *B. meghalayanus* (Deuti et al. 2012). The Extent of Occurrence based on sightings of the species from the current work is 96 km² and the species is known from a single location, we suggest that this species qualifies for being assessed as Critically Endangered in accordance with the IUCN Red List of Threatened Species categories and criteria B1ab (iii) (see IUCN 2012).

Comparisons. *Bufoides bhupathyi* sp. nov. can be distinguished from all congeners on the basis of morphology and molecular data. The following comparisons are based on two adult males and one adult female *Bufoides bhupathyi* sp. nov.

Bufoides bhupathyi sp. nov. differs from *B. kempfi* and *B. meghalayanus* by the presence of small, ovoid, and depressed parotid glands (vs. more protruding and divided in *B. kempfi* and elongated in *B. meghalayanus*); less extensive interdigital webbing between fingers I₀₋₁II₁₋₁III₂₋₁IV (vs. I₀₋₁II₂₋₃III₃₋₂IV in *B. kempfi* and I₀₋₁II₀₋₂III₁₋₁IV in *B. meghalayanus*), more extensive interdigital webbing between toes I₀₋₀II₀₋₀III_{1.5-2}IV_{2-1.5}V (vs. I₀₋₀II₀₋₅III_{0.5-2}IV_{2-1.0}V in *B. kempfi* and I₀₋₀II₀₋₀III_{0.0-0.5}IV_{0.5-0.0}V in *B. meghalayanus*) and coloration in life where the dorsum is

dark brownish-black to black with yellow blotches and reticulations in males and uniformly brownish black with only faint traces of yellow on the flanks in females (vs. blotches and reticulations predominantly mossy green in color in *B. kempfi* and blotches and reticulations predominantly dark green in color in *B. meghalayanus*; Naveen et al. 2022). *Bufoides bhupathyi* sp. nov. can be further distinguished from *B. meghalayanus* by differences in skin texture. The upper part of the flanks and the dorsal surfaces of forelimbs, hands, hind limbs and feet are covered with small to medium-sized black conical tubercles and the lower portion of flanks with few scattered, small tubercles in *Bufoides bhupathyi* sp. nov. (vs. profuse covering of large tubercles on these surfaces and on lower flanks in *B. meghalayanus*; Figure 7).

Discussion

The description of *Bufoides bhupathyi* sp. nov. brings the total number of *Bufoides* to three nominal species. Genetic data from a few previously deposited *Bufoides* sequences under various species names, such as *B. kempfi* and *B. meghalayanus* from the GenBank shows 0-0.24% genetic divergence from our samples which reveals that they belong to neither of those species and are conspecific with the new species described here. This points to the cryptic nature of *Bufoides* species, which show only subtle differences in morphology that require a finer examination of detailed morphological characters to effectively distinguish them in the field.



Figure 6. Habitat of *Bufoides bhupathyi* sp. nov. A) type locality at 314 m asl, Dampa Leui, Dampa Tiger Reserve, Mamit District, Mizoram, India, B) collection site of paratypes 440 m asl, near Chaka Anti-Poaching Camp in Dampa Tiger Reserve, Mamit District, Mizoram, India, and C) rock quarrying about three km away from paratype collection site of *Bufoides bhupathyi* sp. nov., Mamit District, Mizoram, India

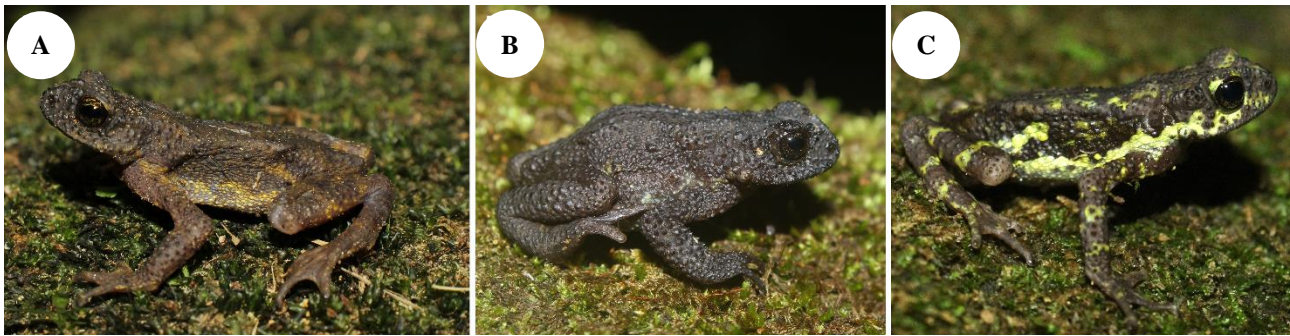


Figure 7. A) Dorsolateral view of *Bufoidea bhupathyi* sp. nov. (Holotype SACON VA 400) and B) Dorsolateral view of an adult female *Bufoidea meghalayanus* (SACON VA 252) clearly illustrating the differences in the extent of skin tuberculation between the species and C) Dorsolateral view of an adult female *Bufoidea kempi*

The fact that the taxonomic uncertainty around this population of *Bufoidea* from this part of Mizoram remained unresolved for so long, despite having been highlighted fourteen years ago (Das et al. 2009) reflects that a more intensive field sampling is required across several parts of the country to understand the true diversity of amphibians in India. This underscores the significance of the need for further extensive studies on amphibians, even in areas that are assumed to have been well-studied.

Currently, the larvae and the call of *Bufoidea bhupathyi* sp. nov. are undescribed. Describing the call and the larvae would not only address an important knowledge gap but could also provide additional means to identify *Bufoidea* species in the field. The Extent of Occurrence based on sightings of the species from the current work is 96 km². However, we speculate that the species might have a wider distribution in and around Dampa Tiger Reserve and potentially into adjacent forests of Mizoram and Bangladesh. Further work is required to determine the exact distribution and elevational range of this species so that its conservation status can be assessed accurately. Rock quarrying is a known threat to *Bufoidea* and we recommend that rock quarrying activities be halted in areas that could support populations of *Bufoidea bhupathyi* sp. nov.

This work does not address the taxonomic status of the population of *Bufoidea* reported from Ngengpui Wildlife Sanctuary in Mizoram State (See Pawar and Birand 2001) and resolving the taxonomic uncertainty of this population should be a priority for further study.

ACKNOWLEDGEMENTS

We are extremely grateful for the assistance provided during fieldwork by the Anti-Poaching Watcher staff, Mr. Mamuana from Dampa Tiger Reserve. This work would not have been possible without his help and assistance. We also thank Mr. Mamuana for the images of *Bufoidea* sp. taken in September 2022. We are grateful for the assistance provided by Mr. Lising G. Momin throughout the course of this study. We thank the Additional Principal Chief Conservator of Forests (Wildlife) and Chief Wildlife

Warden (CWLW) for issuing permissions (No.B.19060/1/2020-CWLW/). We also thank Kevin Hopkins for his assistance with the phylogenetic analysis. Ethical approval was granted by the Zoological Society of London's ethics committee (project ZDZ201). This project is funded by Stiftung Artenschutzamphibian Conservation Fund.

REFERENCES

- Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. 1990. Basic local alignment search tool. *J Mol Biol* 215, 403-410. DOI: 10.1016/S0022-2836(05)80360-2.
- Boulenger GA. 1919. Descriptions of three new batrachians from the Garo Hills, Assam. *Rec Indian Mus* 16, 207-208. DOI: 10.5962/bhl.part.25921.
- Biakzuala, L Muansanga, L, Decemson, HT, Hmar, G, Malsawmdawngliana, F, Vabeiryureilai, M, Lalremsanga, H.T. 2022. New distributional records of the Baibung Small Treefrog, *Theloderma baibungense* (Jiang, Fei, and Huang 2009) (Anura: Rhacophoridae), from Mizoram, India with comments on taxonomy, natural history and conservation status. *Reptiles & Amphibians*. 29. 66-70. 10.17161/randa.v29i1.15961.
- Chandramouli SR, Amarasinghe AAT. 2016. Taxonomic reassessment of the arboreal toad genus *Pedostibes* Günther 1876 (Anura: Bufonidae) and some allied oriental bufonid genera. *Herpetologica* 7, 137-147. DOI: 10.1655/HERPETOLOGICA-D-15-00053.
- Chandramouli SR, Vasudevan K, Harikrishnan S, Dutta SK, Janani SJ, Sharma R, Das I, Aggarwal RK. 2016. A new genus and species of arboreal toad with phytotelmonous larvae, from the Andaman Islands, India (Lissamphibia, Anura, Bufonidae). *ZooKeys* 555, 57-90. DOI: 10.3897/zookeys.555.6522.
- Das I, Rangad D, Tron RKL, Deut K, Hooroo RNK. 2009. Rediscovery of the endangered Khasi Hills rock toad, *Bufoidea meghalayana* in Meghalaya, Northeastern India. *Froglog* 92: 1-4.
- Deuti K, Ray S, Dey SK. 2012. Status survey of the Khasi Hills rock toad (*Bufoidea meghalayana*) at Cherrapunjee, Meghalaya. *Rec Zool Surv India* 111 (3): 21-25. DOI: 10.26515/rzsi/v111/i3/2011/158851.
- Devi HS, Hmingthangpuii, Sarma KK. 2011. Change in vegetation cover of Dampa Tiger Reserve, Mizoram, North East India: A serious threat to tiger population. *J Exp Sci* 2 (9): 1-6.
- Frost, DR. 2023. *Amphibian Species of the World: an Online Reference*. Version 6.1 (Date of access 10 Feb 2023). Electronic Database accessible at <https://amphibiansoftheworld.amnh.org/index.php>. American Museum of Natural History, New York, USA. <https://doi.org/10.5531/db.vz.0001>
- Frost, D, Grant, T, Faivovich, J, Bain, RH, Haas, A, Haddad, CFB, Sá, Rafael, Channing, A, Wilkinson, M, Donnellan, S & Blotto, BL. 2006. The Amphibian Tree of Life. *Bulletin of the American Museum*

- of Natural History. 297. 1-291. 10.1206/0003-0090(2006)297[0001:TATOL]2.0.CO;2.
- Günther ACLG. 1876. "1875". Third report on collections of Indian reptiles obtained by the British Museum. Proceedings of the Zoological Society of London 1875, 567-577.
- Gvoždík, V, Canestrelli, D, García-París, M, Moravec, J, Nascetti, G, Recuero, E, Teixeira, J, Kotlík, P. 2014. Speciation history and widespread introgression in the European short-call tree frogs (*Hyla arborea sensu lato*, *H. intermedia* and *H. sarda*). Molecular phylogenetics and evolution. 83. 10.1016/j.ympev.2014.11.012.
- IUCN 2012. IUCN Red List Categories and Criteria: version 3.1. 2nd ed. Gland, Switzerland and Cambridge, UK.
- IUCN SSC Amphibian Specialist Group 2022. *Bufoides meghalayanus*. The IUCN Red List of Threatened Species 2022.
- Kocher TD, Thomas WK, Meyer A, Edwards SV, Pääbo S, Villablanca FX, Wilson AC. 1989. Dynamics of mitochondrial DNA evolution in animals: amplification and sequencing with conserved primers. Proceedings of the National Academy of Sciences of the United States of America 86, 6192-6200. DOI: 10.1073/pnas.86.16.6196.
- Lalremsanga, HT & Biakzuala, Lal & Muansanga, Lal & Vabeiryureilai, Mathipi & Hmar, Gospel & Decemson, Ht. (2021). An annotated checklist of amphibians in and around Dampa Tiger Reserve, Mizoram, India. Journal of Threatened Taxa. 13. 17918 - 17929. 10.11609/jott.6319.13.3.17918-17929.
- Matsui, M. 2019. A New Species of *Pelophryne* from Malay Peninsula (Anura, Bufonidae). Current Herpetology. 38. 128. 10.5358/hsj.38.128.
- Matsui M, Yambun P, Sudin A. Taxonomic relationships of *Ansonia anotis* Inger, Tan, and Yambun, 2001 and *Pedostibes maculatus* (Mocquard, 1890), with a description of a new genus (Amphibia, Bufonidae). Zoolog Sci. 2007 Nov;24(11):1159-66. doi: 10.2108/zsj.24.1159. PMID: 18348617.
- Meenakshi S, Gnanambigai, Mozhi ST, Arumugam M, Balasubramanian T. 2009. Total flavanoid and in vitro antioxidant activity of two seaweeds of Rameshwaram coast. Global J Pharmacol 3 (2): 59-62.
- Naveen RS, Chandramouli SR, Kadam G, Babu S, Karunakaran PV, Kumara HN, Parthasarathy N. 2022. Systematics of the enigmatic and narrowly endemic toad genus *Bufoides* Pillai & Yazdani, 1973: Rediscovery of *Bufoides kempi* (Boulenger, 1919) and expanded description of *Bufoides meghalayanus* (Yazdani & Chanda, 1971) (Amphibia: Anura: Bufonidae) with notes on natural history and distribution. J Threatened Taxa 14, 22277-22292. DOI: 10.11609/jott.8040.14.12.22277-22292.
- Pawar S, Birand A. 2001. A Survey of Amphibians, Reptiles, and Birds in Northeast India. Centre for Ecological Research and Conservation.
- Pillai RS, Yazdani GM. 1973. *Bufoides*, a new genus for the rock-toad, *Ansonia meghalayana* Yazdani and Chanda, with notes on its ecology and breeding habits. J Zool Soc India 25, 65-70.
- Rambaut A. 2009. FigTree. Tree Figure drawing tool. <http://tree.bio.ed.ac.uk/software/figtree/>.
- Ronquist F, Huelsenbeck JP. 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics 19 (12): 1572-1574. DOI: 10.1093/bioinformatics/btg180.
- Savage JM, Heyer WR. 1997. Digital webbing formulae for anurans: a refinement. Herpetol Rev 28, 131.
- Smart, U, Sarker, G, Arifin, U, Harvey, M, Sidik, I, Hamidy, A, Kurniawan, N, Smith, E. 2017. A new genus and two new species of arboreal toads from the highlands of Sumatra with a phylogeny of Sundaland toad genera. Herpetologica. 73. 63-75. 10.1655/Herpetologica-D-16-00041.
- Stuart S, Homann M, Chanson JS, Cox NA, Berridge RJ, Ramani P, Young BE. 2008. Threatened amphibians of the world. Lynx Ediciones, Barcelona/IUCN. E World Conservation Union, Conserv Intl Nat Serv. Washington, D.C.
- Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. 2013. MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. Mol Biol Evol 30 (12): 2725-2729. DOI: 10.1093/molbev/mst197.
- Van Bocxlaer I, Biju SD, Loader SP, Bossuyt F. 2009. Toad radiation reveals into-India dispersal as a source of endemism in the Western Ghats-Sri Lanka biodiversity hotspot. BMC Evol Biol 9 (1): 1-10. DOI: 10.1186/1471-2148-9-131.
- Vences M, Thomas M, Van der Meijden A, Chiari Y, Vieites DR. 2005. Comparative performance of the 16S rRNA gene in DNA barcoding of amphibians. Front Zool 2 (1): 1-12. DOI: 10.1186/1742-9994-2-5.