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DNA Barcoding Elucidates a Range-extension of the Bangladesh Skittering Frog, *Euphlyctis kalasgramensis* (Dicroglossidae), in Northeastern India

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The Kalasgram Skittering Frog (*Euphlyctis kalasgramensis*) is a dicroglossid described from Bangladesh by Howlader et al. (2015). Due to its conserved morphology, *E. kalasgramensis* had been formerly assigned to the Indian Skittering Frog (*E. cyanophlyctis*; Sen 2004; Ahmed et al. 2009; Mathew and Sen 2010; Lalremsanga 2011; Saikia and Lyngdoh 2014). With the use of DNA barcoding approaches, especially the mitochondrial gene 16S ribosomal RNA (16S rRNA), this species has been identified and subsequently reported throughout Bangladesh, from western Punjab; Pakistan (Ali et al. 2020); Mizoram, northeastern India (Muansanga 2020); various locations below 2,500 m elevation in Nepal (Khatiwada et al. 2021); and likely as far as Rakhine State, Myanmar (Zug 2022). Lalremsanga (2011) reported 30 different localities of *E. kalasgramensis* (as *E. cyanophlyctis*) at elevations ranging from 40–1,460 m throughout Mizoram. The present work, based on morphological studies and DNA barcoding of these specimens, provides an updated distribution for *E. kalasgramensis* in Mizoram and its first record for Manipur State.

Methods

During an ongoing herpetological survey conducted in the states of Mizoram and Manipur, northeastern India, we collected *Euphlyctis* specimens which were morphologically cryptic. Elevation and GPS coordinates of collection sites were recorded with a Garmin Montana 650-GPS Navigator global positioning system. Specimens were euthanized following Conroy et al. (2009), preserved in 70% ethanol, and deposited and catalogued in the Departmental Museum of Zoology, Mizoram University (MZMU). Measurements were taken using Mitutoyo dial Vernier Calipers (Model 505–671) to the nearest 0.1 mm (Table 1). Morphological measurements include individuals collected from new localities from the Chakpi River, Manipur, India (24.318930°N, 93.991177°E; elev. 881 m asl; Fig. 1); Dampa Tiger Reserve, Mamit District (23.68794°N, 92.45498°E; elev. 275 m asl; Pualreng Wildlife Sanctuary, Kolasib District (24.22989°N, 92.80502°E; elev. 670 m asl); Tiau River, Lunglei District (22.81675°N, 93.10908°E; elev. 270 m asl); and Kawlchaw Village, Siaha District (22.39722°N, 92.96528°E; elev. 343 m asl). We attempted to identify the individuals to the species level based on the diagnosis provided by Howlader et al. (2015).

To verify our species identification using DNA, whole genomic DNA was isolated from the liver tissue of *E. kalasgramensis* according to the manufacturer's protocol (QIAamp DNA Mini Kit, Cat No.ID: 51306). Partial fragments of 16S rRNA were amplified using polymerase chain reaction (PCR) using 16S rRNA primers: forward (L02510 –

Fig. 1. Male Kalasgram Skittering Frog (*Euphlyctis kalasgramensis*) MZMU2700E from Manipur, India. Photograph by H.T. Lalremsanga

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Table 1. Measurements of Bangladesh Skittering Frog (*Euphlyctis kalasgramensis*) from Manipur, India. Abbreviations: SVL = snout-vent length, HL = head length (from rear of jaw to tip of snout), HW = head width (Head width at angle of jaw), EN = eye to nostril distance, NS = nostril to snout, SL = tip of snout to anterior eye distance, TRL = trunk length (distance from posterior base of forelimb at its joining with body to anterior base of hindlimb at its joining with body), TYM = horizontal diameter of tympanum, TE = tympanum-eye distance, IOD = interorbital distance, UEW = upper eyelid width, ED = eye diameter, IND = internarial distance, FLL = length of forelimb from tip of disc of finger III to axilla, HAL = hand length (from the base of outer palmar tubercle to tip of finger), F_1 = length of first finger (from the base of palm to tip of first finger), F_2 = length of second finger (from the base of palm to tip of second finger), F_3 = length of third finger (From the base of palm to tip of third finger), F_4 = length of fourth finger (from the base of palm to tip of fourth finger), TL = tibia length, FL = femur length, FOT = length of hindlimb from tip of disc of toe IV to posterior edge of tibia, T_1 = length from base of foot to tip of first toe, T_2 = length from base of foot to tip of second toe, T_3 = length from base of foot to tip of third toe, T_4 = length from base of foot to tip of fourth toe, T_5 = length from base of foot to tip of fifth toe.

Voucher No.	MZMU 2700A	MZMU 2700B	MZMU 2700C	MZMU 2700D	MZMU 2700E	MZMU 1820	MZMU 1836	MZMU 1995A	MZMU 2710A
Sex	Female	Male	Female	Female	Male	Juvenile Female	Female	Female	Juvenile Female
SVL	48.4	31.9	41.1	43.6	33.3	29.7	56.5	52.6	34.9
HL	17.9	12.4	12.5	13.2	10.1	10.9	18.2	15.5	11.2
HW	19.8	14.4	15.3	15.8	12.4	11.5	20.1	19.8	13.5
EN	4.8	2.8	3.8	3.9	3.1	2.3	4.4	4.3	3.0
NS	2.3	1.5	1.9	1.9	1.7	1.1	2.8	2.5	1.5
SL	6.9	5.0	6.3	6.4	5.6	4.3	7.1	7.0	4.5
TRL	21.7	10.9	16.9	17.6	12.2	9.4	22.2	21.9	11.3
TYM	4.1	3.4	3.7	3.9	3.5	3.3	5.0	4.6	3.5
TE	1.5	1.2	1.4	1.4	1.3	1.0	1.6	1.6	1.1
IOD	2.8	2.1	2.6	2.6	2.2	2.0	3.1	2.9	2.3
UEW	3.6	2.5	2.9	2.9	2.6	2.3	3.6	3.6	2.7
ED	4.8	3.9	3.9	4.3	4.0	3.9	5.6	5.5	4.6
FLL	27.8	22.7	21.6	26.5	17.5	16.5	29.3	29.1	18.8
HAL	13.3	10.5	11.7	12.0	10.9	8.6	14.5	13.6	10.8
F1	11.2	8.8	8.9	10.9	6.7	6.5	12	11.6	6.7
F2	10.9	8.2	8.5	10.8	6.6	6.3	11.9	11.5	6.4
F3	13.3	9.9	10.7	11.8	8.5	7.7	14	13.6	9.9
F4	11.9	7.3	9.8	10.2	7.4	6.9	12.8	12.4	8.1
TL	23.8	14.0	20.5	21.7	14.1	13.8	25.8	24.1	16.5
FL	22.7	13.3	19.8	21.3	13.4	13.1	25.6	23.8	15.7
FOT	34.7	26.8	24.0	27.2	27.0	22.8	39.4	37.2	26.5
T1	11.9	9.8	9.2	10.6	8.0	7.8	13.1	12.5	8.4
T2	16.2	11.8	13.2	15.6	10.6	10.8	19.0	17.9	12.1
T3	22.1	17.0	19.1	19.8	14.6	14.1	25.5	23.3	16.5
T4	27.3	19.8	20.0	22.6	16.2	17.3	30.8	28.4	19.4
T5	19.8	15.7	17.3	19.3	14.5	13.7	25.3	23.1	16.3

CGCCTGTTTATCAAAAACAT [Palumbi 1996]) and reverse (H03063 – CTCCGGTTTGAACTCAGATC [Rassmann 1997]). Amplification was carried out in 20-µL reactions, with the following thermocycler steps: 5 min at 95 °C for initial denaturation, followed by 35 cycles of 1 min at 95 °C for denaturation, 30 sec for annealing at 50.3 °C, elongation for 1 minute at 72 °C, and then a final elongation for 5 min at 72 °C. PCR products were visualized on 1.5% agarose gels and sequenced at Barcode Bioscience, Bangalore, India. Raw DNA reads were checked for quality score, assembled and edited in sequence scanner v2 (Applied Biosystems), and deposited in GenBank under

the accession numbers: OM574590 (MZMU2700A) and OM574591 (MZMU2700B) from Chakpi River, Manipur, and MW165471 (MZMU1820) and MW165474 (MZMU1836) from Kawlchaw Village, Mizoram.

We incorporated eight newly generated sequences and ten retrieved from the NCBI Genbank: *E. kalasgramensis*, (KP091862), *E. cyanophlyctis* (AB290418), *E. hexadactylus* (AB272608), *E. aloysii* (KU870382; AB530594), *E. karaavali* (KU870373; KU870372), *E. mudigere* (AB530599), *E. ehrenbergi* (AY014367) (Kosuch et al. 2001; (Alam et al. 2008; Hasan et al. 2014; Howlader et al. 2015; Priti et al. 2016) and *Hoplobatrachus occipitalis* (AB272600) as an outgroup from the NCBI database into our dataset. We generated a sequence alignment using the MUSCLE algorithm (Edgar 2004), manually editing to remove sequence errors, and estimated Kimura 2-parameter (K2P) genetic distances (Kimura 1980) in MEGA 7 (Kumar et al. 2016). We identified the best-fitted model of nucleotide substitution using the Bayesian Information Criterion in ModelTest-NG (Darriba et al. 2019). We estimated the gene tree using a maximum likelihood (ML) apprach with RaxmlGUIv2.3 with 10,000 bootstrap (BS) replicates (Silvestro and Michalak 2012), and Bayesian Inference (BI) in MrBayes 3.2.7 (Ronquist et al. 2012) for 1 million generations (25% burn-in) under a GTR+G model of nucleotide evolution. Bayesian posterior probability (BPP) values are given in the BI tree to assess nodal support. We considered the nodal support value ≥ 0.95 as strong support (Ojha et al. 2022; Simmons et al. 2004). The analysis was terminated when the standard deviation of split frequencies was less than 0.01. Phylogenetic trees were visualized using FigTree v1.4.4 (http://tree.bio.ed.ac.uk/software/figtree/).

Results

Morphological remarks.—Although there are no genetic data available for *E. ghoshi* to include in the present genetic analysis, *E. kalagramensis* is morphologically distinct from *E. ghoshi*, which is endemic to Manipur, by having indistinct canthus rostralis (concave in *E. ghoshi*), nostrils much closer to tip of snout than to eyes (closer to eyes than to tip of snout in *E.ghoshi*) and presence of an oval-shaped, small, distinct outer metatarsal tubercle (outer metatarsal tubercle absent in *E. ghoshi*).

Phylogenetic results.—Our final DNA alignment contained 535 base pairs, and GTR+G was supported as the best-fitting substitution model. The topology of the ML and BI trees were congruent (Fig. 2), recovering the *E. kalasgramensis* specimens from Manipur and Mizoram States (India) with the holotype sequence from Bangladesh (KP091862) with weak support (BPP/BS=0.86/78). *Euphlyctis kalasgramensis* is also sister to a clade consisting of *E. ehrenbergi*, *E. cyanophlyctis*, and *E. mudigere.* Intraspecific distances of *E. kalasgramensis* ranged from 0–0.2%. We also determined that the lowest inter-specific genetic distance (4.1%) from *E. kalasgramensis* is with *E. cyanophlyctis* (AB290418) (Table 2).

Table 2: The uncorrected K2P distances of 16S rRNA of *Euphlyctis* used in this study (and outgroup *Hoplobatrachus*). Genbank accession numbers are listed after the names of the taxa.

SNo.	Species	K2p distance																
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
1	*E. kalasgramensis*_OM574590																		
2	*E. kalasgramensis*_OM574591	0.000																	
3	*E. kalasgramensis*_MW165471	0.002	0.002																
4	*E. kalasgramensis*_MW165474	0.002	0.002	0.000															
5	*E. kalasgramensis*_OM363226	0.002	0.002	0.000	0.000														
6	*E. kalasgramensis*_OM363225	0.002	0.002	0.000	0.000	0.000													
7	*E. kalasgramensis*_OM363225	0.002	0.002	0.000	0.000	0.002	0.000												
8	*E. kalasgramensis*_OM363226	0.002	0.002	0.000	0.000	0.000	0.000	0.000											
9	*E. kalasgramensis*_KP091862_Holotype	0.002	0.002	0.000	0.000	0.000	0.000	0.000	0.000										
10	*E. cyanophlyctis*_AB290418	0.041	0.041	0.043	0.043	0.043	0.043	0.043	0.043	0.043									
11	*E. hexadactylus*_AB272608	0.103	0.103	0.101	0.101	0.101	0.101	0.101	0.101	0.101	0.114								
12	*E. aloysii*_KU870382	0.112	0.112	0.109	0.109	0.109	0.109	0.109	0.109	0.109	0.114	0.034							
13	*E. aloysii*_AB530594	0.114	0.114	0.112	0.112	0.112	0.112	0.112	0.112	0.112	0.109	0.039	0.004						
14	*E. karaavali*_KU870373	0.116	0.116	0.114	0.114	0.114	0.114	0.114	0.114	0.114	0.129	0.101	0.103	0.105					
15	*E. karaavali*_KU870372	0.118	0.118	0.116	0.116	0.116	0.116	0.116	0.116	0.116	0.127	0.099	0.101	0.103	0.006				
16	*E. mudigere*_AB530599	0.045	0.045	0.047	0.047	0.047	0.047	0.047	0.047	0.047	0.004	0.109	0.109	0.105	0.124	0.122			
17	*E. ehrenbergi*_AY014367	0.071	0.071	0.069	0.069	0.069	0.069	0.069	0.069	0.069	0.067	0.116	0.131	0.133	0.137	0.131	0.062		
18	*Hoplobatrachus occipitalis*_AB272600	0.148	0.148	0.146	0.146	0.146	0.146	0.146	0.146	0.146	0.148	0.159	0.163	0.165	0.135	0.137	0.148	0.165	

Fig. 2. Bayesian 16S gene tree of *Euphlyctis*. Numbers nodes indicate BPP/BS support values, respectively. Sequences generated in this study are indicated in bold font, and type material is indicated by an asterisk (*) with GenBank accession numbers followed by the localities.

Natural history notes.—All individuals were observed in temporary or permanent pools, back waters of streams and rivers, and rice and crop fields. Though individuals were seen floating and basking among aquatic plants during day time, male advertisement calls and mating were encountered during night time; thus, we consider this species as mostly nocturnal in activity. We observed breeding behavior from March to August. Eggs were deposited in shallow water, either standing or running, as well as paddy fields and ditches. Similar to previous studies (Lalremsanga 2011; as *E. cyanophlyctis*) on detailed breeding behavior, embryonic and larval development, we found that the time from egg fertilization to froglet is 64–65 days under natural conditions.

Discussion

Based on the confirmed distribution (Fig. 3) and description of this species (Howlader et al. 2015), it is likely that the previous northeastern India records of *E. cyanophlyctis* are referrable to *E. kalasgramensis*. DNA barcoding is an important tool for identification of organisms when dealing with cryptic species (Floyd et al. 2002). Here, we find that specimens previously referred to as *E. cyanophlyctis* from various parts of Mizoram and Manipur are in fact *E. kalasgramensis*, based on our results. Specimens collected from Chakpi River, Chandel District, Manipur (MZMU 2700A–E) represent the first report of this species from the state. The present study encourages extensive sampling of this species from northeastern India and its adjacent regions to elucidate its range distribution and phylogeography.

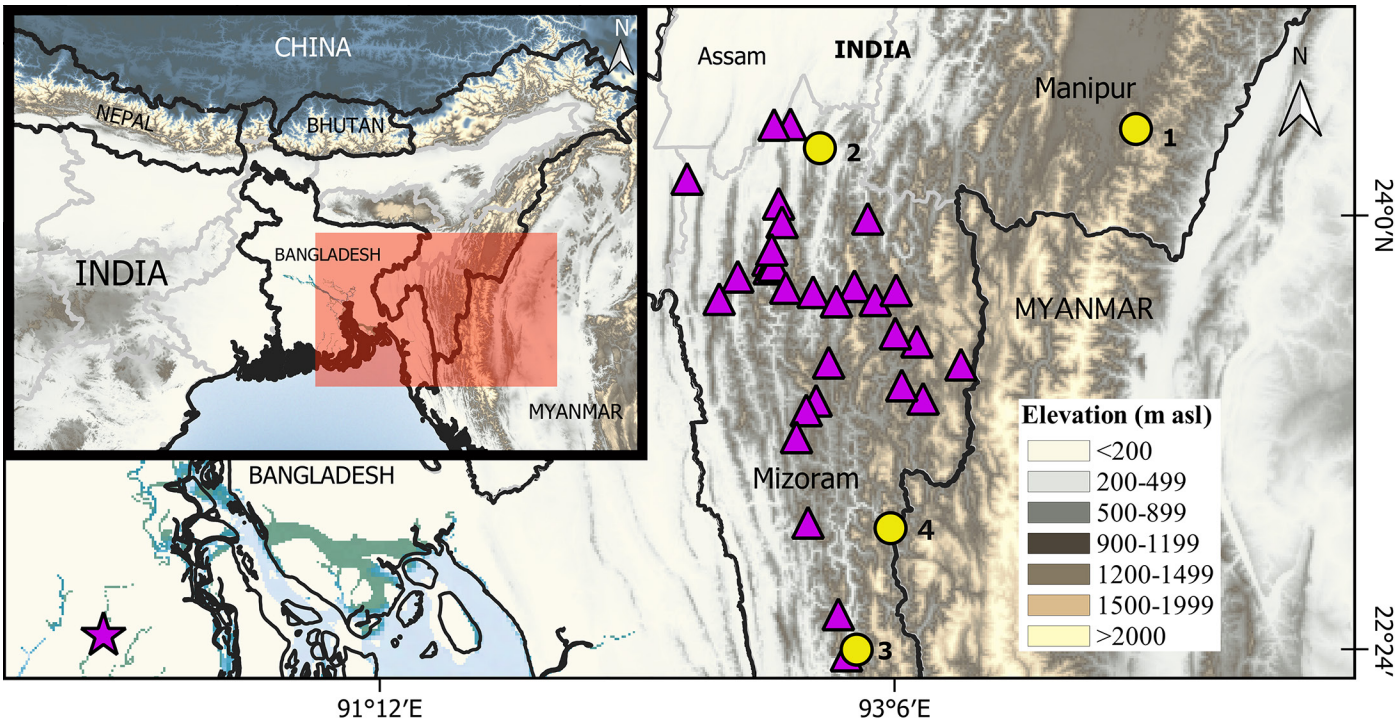

Fig. 3. Map showing the locality records of *Euplyctis kalasgramensis* from the Indo-Bangladesh Region. The type locality is marked by a purple star; previous records from the Dampa Tiger Reserve (Decemson et al. 2021) and various localities from Mizoram (Lalremsanga 2011) are represented by purple triangles; and the new records from this study are indicated by yellow circles: 1. Chakpi River, Manipur; 2. Pualreng Wildlife Sanctuary, Mizoram; 3. Kawlchaw, Mizoram; 4. Tiau, Mizoram.

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