

RESEARCH ARTICLE



DNA barcoding reveals a new country record for three species of frogs (Amphibia: Anura) from India

Samuel Lalronunga^{1,2*}, Vanramliana³, C. Lalrinchhana⁴, Vanlalhrima², Vanlalhriatzuala Sailo², Lalnunhlua², Lalkhawngaiha Sailo², Isaac Zosangliana^{1,2}, K. Lalmangaiha^{1,2}, Esther Lalhmingliani^{1,2}

¹Systematics and Toxicology Laboratory, Department of Zoology, Mizoram University, Aizawl 796004, Mizoram, India

²Biodiversity and Nature Conservation Network, Aizawl 796004, Mizoram, India

³Department of Zoology, Pachhunga University College, Aizawl 796001, Mizoram, India

⁴Holy Child School, Nalkata, Dhalai, Tripura 799263, India

We present a first record for three species of frogs, viz. *Amolops indoburmanensis*, *Euphlyctis kalisgraminensis* and *Polypedates braueri* in India based on DNA barcoding data. We also discussed on the records of *Amolops* spp. in Mizoram and delisted all previous recorded species, viz. *A. afghanus*, *A. kaulbacki* and *A. marmoratus* from the amphibian fauna of Mizoram until further research confirmed their presence, since the data were not sufficient enough for the confirmation of their occurrence and are likely based on misidentification of *A. indoburmanensis*.

Key words: Biodiversity hotspot, conservation, 16S rRNA, northeast India, genetic distance.

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*For correspondence:
samuellna@gmail.com

Contact us:
sciencevision@outlook.com

Introduction

Four of the thirty six global biodiversity hotspots partly fall within the territory of India, viz. Western Ghats, Indo-Burma, Himalaya and Sundaland.¹⁻³ India harbours high species diversity and endemism in both flora and fauna.⁴⁻⁷ Amphibian conservation is a global concern as a third of the amphibian species on the planet are endangered due to emerging diseases, climate change and ozone depletion,⁸ and their extinction rate is estimated to be as much as 200 times more than background extinction rate.⁹ Geographical range of a species is an important aspect in biodiversity conservation as it is among the parameters used for assessing the threat status of a species.¹⁰ The mitochondrial gene, 16S ribosomal RNA (16S rRNA) had been used successfully for

amphibian species identification and detection of cryptic species.¹¹⁻¹⁴

Most part of northeast India falls within the Indo-Burma and Himalaya biodiversity hotspots. The amphibian diversity of the area is highly underestimated and several new species were discovered within the last decade alone.¹⁵⁻³⁰ In an ongoing herpetological survey conducted in Mizoram state of northeast India, specimens of *Amolops indoburmanensis* Dever, Fuiten, Konu and Wilkinson, 2012 (family Ranidae); *Euphlyctis kalisgraminensis* Howlader, Nair, Gopalan and Merilä, 2015 (family Dicoglossidae) and *Polypedates braueri* (Vogt, 1911) (family Rhacophoridae) were confirmed using DNA barcoding. We herein report the

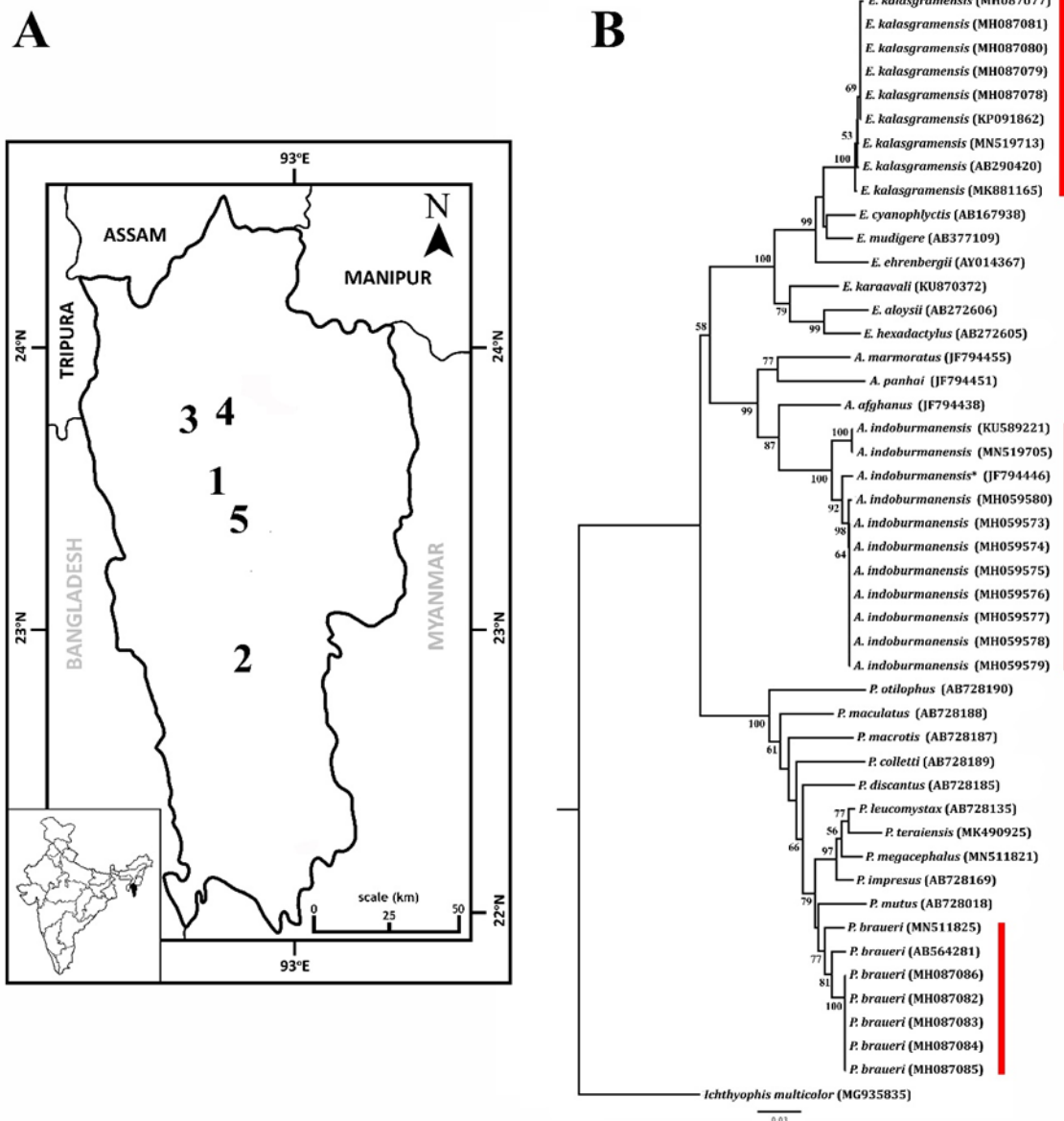


Figure 1 | A) Map showing the collection sites for samples used in this study: Hmuifang (1); Kikawn, Lunglei (2); Tlawng river at PHE pumphouse (3); Muthi river at Tuirial airfield (4); Sailam (5). **B)** Neighbour-Joining (NJ) tree of 16S rRNA dataset of 47 sequences using p-distance model with 1000 bootstraps.

occurrence of these species in India.

Materials and Methods

Study area

Collection sites of frog specimens are shown in **Figure 1A**. Specimens of *Amolops indoburmanensis* (**Figure 2**) were collected from Hmuifang community reserve forest at Hmuifang village, Aizawl district, Mizoram, India (23°27.213'N, 92°45.181'E, 1478 m asl); and Kikawn, Lunglei, Lunglei district, Mizoram

(22°53.929'N, 92°45.309'E, 1034 m asl). Specimens of *Euphlyctis kalisgraminensis* (**Figure 3**) were collected from Tlawng River near PHE pumphouse, Aizawl District, Mizoram (23°42.798'N, 92°39.803'E, 128 m asl); and Muthi River at the vicinity of Tuirial Airfield, Aizawl district, Mizoram (23°45.575'N, 92°48.013'E, 153 m asl). Specimens of *Polypedates braueri* (**Figure 4**) were collected from Hmuifang community reserve forest at Hmuifang village, Aizawl District, Mizoram (23°27.213'N, 92°45.181'E, 1478 m asl); and from Sailam community reserve forest at Sailam village, Aizawl District, Mizoram (23°20.532'N, 92°47.969'E,

Table 1 | List of samples used for molecular analysis in this study with voucher numbers, localities and GenBank accession numbers. GenBank accession number given in bold letter indicates newly generated sequences in this study

	Species	Voucher number	Locality	Accession No.
1	<i>Amolops afghanus</i>	CAS 221313	Kachin Myanmar	JF794438
2	<i>Amolops indoburmanensis</i>	CAS 235070	Chin, Myanmar (Holotype)	JF794446
3	<i>Amolops indoburmanensis</i>	PUCZM/IX/SL634	Hmuifang, Mizoram, India	MH059573
4	<i>Amolops indoburmanensis</i>	PUCZM/IX/SL635	Hmuifang, Mizoram, India	MH059574
5	<i>Amolops indoburmanensis</i>	PUCZM/IX/SL636	Hmuifang, Mizoram, India	MH059575
6	<i>Amolops indoburmanensis</i>	PUCZM/IX/SL637	Hmuifang, Mizoram, India	MH059576
7	<i>Amolops indoburmanensis</i>	PUCZM/IX/SL372	Lunglei, Mizoram, India	MH059577
8	<i>Amolops indoburmanensis</i>	PUCZM/IX/SL373	Lunglei, Mizoram, India	MH059578
9	<i>Amolops indoburmanensis</i>	PUCZM/IX/SL374	Lunglei, Mizoram, India	MH059579
10	<i>Amolops indoburmanensis</i>	PUCZM/IX/SL375	Lunglei, Mizoram, India	MH059580
11	<i>Amolops indoburmanensis</i>	IASST AR85	Assam, India	KU589221
12	<i>Amolops indoburmanensis</i>	ZSIS-M50	Murlen, Mizoram, India	MN519705
13	<i>Amolops marmoratus</i>	CAS 240603	Mon, Myanmar	JF794455
14	<i>Amolops panhai</i>	CAS 229816	Tanintharyi, Myanmar	JF794451
15	<i>Euphylyctis aloysii</i>	BNHS 5123	Bajpe, Mangalore, Karnataka, India	AB272606
16	<i>Euphylyctis cyanophlyctis</i>	RBRL 03060702	Mudikari, Karnataka, India	AB167938
17	<i>Euphylyctis ehrenbergii</i>	MNHN 2000.649	Yemen	AY014367
18	<i>Euphylyctis hexadactylus</i>	RBRL 03060601	Mangalore, Karnataka, India	AB272605
19	<i>Euphylyctis mudigere</i>	BNHS 5127	Mudigere, Western Ghats, India	AB377109
20	<i>Euphylyctis kalasgramensis</i>	MZH-3376	Bangladesh (Holotype)	KP091862
21	<i>Euphylyctis kalasgramensis</i>	PUCZM/IX/SL29	Tlawng river, Mizoram, India	MH087077
22	<i>Euphylyctis kalasgramensis</i>	PUCZM/IX/SL62	Muthi river, Mizoram, India	MH087078
23	<i>Euphylyctis kalasgramensis</i>	PUCZM/IX/SL63	Muthi river, Mizoram, India	MH087079
24	<i>Euphylyctis kalasgramensis</i>	PUCZM/IX/SL64	Muthi river, Mizoram, India	MH087080
25	<i>Euphylyctis kalasgramensis</i>	PUCZM/IX/SL613	Tlawng river, Mizoram, India	MH087081
26	<i>Euphylyctis kalasgramensis</i>	GenBank	Assam, India	AB290420
27	<i>Euphylyctis kalasgramensis</i>	ZSIS-M17	Bornadi, Assam, India	MN519713
28	<i>Euphylyctis kalasgramensis</i>	ZMUVAS1	Punjab, Pakistan	MK881165
29	<i>Euphylyctis karaavali</i>	BNHS 5988	Sanikatta, Kumta, India	KU870372
30	<i>Polypedates braueri</i>	KUHE 32842	Taibei, Taiwan, China (Topotype)	AB564281
31	<i>Polypedates braueri</i>	PUCZM/IX/SL52	Hmuifang, Mizoram, India	MH087082
32	<i>Polypedates braueri</i>	PUCZM/IX/SL189	Sailam, Mizoram, India	MH087083
33	<i>Polypedates braueri</i>	PUCZM/IX/SL190	Sailam, Mizoram, India	MH087084
34	<i>Polypedates braueri</i>	PUCZM/IX/SL191	Sailam, Mizoram, India	MH087085
35	<i>Polypedates braueri</i>	PUCZM/IX/SL197	Hmuifang, Mizoram, India	MH087086
36	<i>Polypedates braueri</i>	YPX44208	Putao, Kachin State, Myanmar	MN511825
37	<i>Polypedates colletti</i>	MZB UN	Lampung, Sumatra, Indonesia	AB728189
38	<i>Polypedates discantus</i>	KUHE 52552	Temerloh, Pahang, Malaysia	AB728185
39	<i>Polypedates impresus</i>	KUHE 32448	Ban Saleui, Houa Phan, Laos	AB728169
40	<i>Polypedates leucomystax</i>	MZB UN	Temanggung, Java, Indonesia	AB728135
41	<i>Polypedates macrotis</i>	KUHE 42556	Kanowit, Sarawak, Malaysia	AB728187
42	<i>Polypedates maculatus</i>	KUHE 42336	Rajgir, Bihar, India	AB728188

43	<i>Polypedates megacephalus</i>	KIZ01569	Hong Kong, China	MN511821
44	<i>Polypedates mutus</i>	CIB 2851K	Jinxu, Guangxi, China	AB728018
45	<i>Polypedates otitophus</i>	KUHE 42555	Kanowit, Sarawak, Malaysia	AB728190
46	<i>Polypedates teraiensis</i>	GenBank	Assam, India	MK490925
47	<i>Ichthyophis multicolor</i>	USNM 576283	Mwe Hauk, Ayeyawady, Myanmar	MG935835

1364 m asl).

Sample collection and preservation

Field collection was conducted under the permission issued by the Chief Wildlife Warden, Department of Environment, Forest and Climate Change, Government of Mizoram, India (A.38011/5/2011-CWLW/338). Specimens were euthanized with benzocaine, fixed in 4% formaldehyde and later transferred to 70% ethanol for longer preservation. Prior to fixation, a muscle tissues from the right thigh were incised and stored in absolute ethanol for DNA extraction. Specimens were deposited in the Zoological Museum, Department of Zoology, Pachhunga University College, Mizoram, India. Specimen voucher numbers are listed in **Table 1**.

DNA extraction, sequencing and data analysis

DNA extraction, amplification and sequencing were done following Lalronunga and Lalrinchhana.¹⁴ All the sequences generated in this study were deposited in GenBank (see **Table 1** for accession numbers). Sequences were edited using FinchTV 1.4.0 software packages (Geospiza, Inc.; Seattle, WA, USA; <http://www.geospiza.com>). The generated sequences were blasted in NCBI (<http://www.ncbi.nlm.nih.gov>) for the nearest matches. All the newly generated sequences of this study were deposited in GenBank. To confirm the identity of the specimens via DNA barcoding and to evaluate their relationships, we analyzed our new sequences along with congener sequences available in GenBank (**Table 1 & 2**). Sequences were aligned using ClustalW integrated in MEGA 7 (Molecular Evolutionary Genetics Analysis) software,³¹ with the default settings. Phylogenetic trees were constructed by Neighbour-Joining (NJ) method with p-distance parameter using MEGA 7,³¹ with 1000 bootstrap support. *Ichthyophis multicolor* Wilkinson, Presswell, Sherratt, Papadopoulou and Gower, 2014 was assigned as outgroup for the analysis. Uncorrected pairwise genetic distances (p-distance) between sequences were determined with MEGA 7.³¹

Result and Discussion

Amolops indoburmanensis

The sample sequences of *A. indoburmanensis* from Mizoram clustered with the holotype of *A. indoburmanensis* in the NJ tree (**Figure 1B**). The genetic distance between the samples from Mizoram and the holotype is only 0.013 (**Table 2**). Two sequences submitted to GenBank as *A. indoburmanensis* from Assam, India (KU589221) and Murlen, Mizoram, India (MN519705) also claded with the holotype of *A. indoburmanensis* with a genetic distance of 0.013 and 0.015 respectively (**Table 2**). While reviewing the *Amolops marmoratus* species complex of Myanmar, Dever *et al.*³² described *A. indoburmanensis* from southern Myanmar; and is also expected to occur in northeast India.^{32,33} Dinesh *et al.*³⁴ included the species in their amphibian checklist of India, but did not mention any voucher sample or locality. To the best of our knowledge, there is no literature that deals with the record of this species in India except Dinesh *et al.*³⁴ Therefore, the present record is the first confirmed record of this species from India.

Three species of the genus *Amolops* were previously recorded from Mizoram, India viz. *A. afghanus* (Günther, 1858), *A. kaulbacki* (Smith, 1940) and *A. marmoratus* Blyth 1885.³⁵⁻⁴⁰ *Amolops afghanus* had been recorded from Serchhip and Hmuifang,^{36,39,40} but this species had not been recorded from any other parts of India.³³⁻³⁴ Sailo *et al.*³⁷ recorded *A. kaulbacki* from Theiriat, Lunglei, Mizoram but Dever *et al.*³² doubted the record and stated the need to verify this record as the species is known only from the original description.⁴¹ Moreover, the collection site of Sailo *et al.*,³⁷ Theiriat and the collection site of *A. indoburmanensis* in the present study, Kikawn are within Lunglei township, and are not far from each other. *Amolops marmoratus* is recorded from southern Myanmar and northern Thailand,³² and populations from eastern Himalayan region of India and adjoining China, Nepal and Bangladesh assigned to this species needs confirmation.³³ All previous records of *Amolops* species from Mizoram needs confirmation



Figure 2 | In-situ photo of *Amolops indoburmanensis* from Hmuifang, Mizoram, India (PUCZM/IX/SL636).



Figure 3 | In-situ photo of *Euphlyctis kalasgramensis* from PHE pump house at Tlawng river, Mizoram, India (PUCZM/IX/SL29).



Figure 4 | In-situ photos of *Polypedates braueri* from Mizoram, India. **A)** Gravid female (PUCZM/IX/SL189) from a seasonal lake at Sailam. **B)** Male individual from Hmuifang (not collected).

Table 2 | Uncorrected pairwise distance (p-distance) of the 16s rRNA sequences of *Amalops* used in this study. Asterisk after species name indicates the holotype

	1	2	3	4	5	6	7	8	9	10	11	12	13
1 <i>A. afghanus</i> (JF794438)													
2 <i>A. indoburmanensis</i> * (JF794446)	0.101												
3 <i>A. indoburmanensis</i> (MH059573)	0.095	0.013											
4 <i>A. indoburmanensis</i> (MH059574)	0.095	0.013	0.000										
5 <i>A. indoburmanensis</i> (MH059575)	0.095	0.013	0.000	0.000									
6 <i>A. indoburmanensis</i> (MH059576)	0.095	0.013	0.000	0.000	0.000								
7 <i>A. indoburmanensis</i> (MH059577)	0.095	0.013	0.000	0.000	0.000	0.000							
8 <i>A. indoburmanensis</i> (MH059578)	0.095	0.013	0.000	0.000	0.000	0.000	0.000						
9 <i>A. indoburmanensis</i> (MH059579)	0.095	0.013	0.000	0.000	0.000	0.000	0.000	0.000					
10 <i>A. indoburmanensis</i> (MH059580)	0.093	0.015	0.002	0.002	0.002	0.002	0.002	0.002	0.002				
11 <i>A. indoburmanensis</i> (KU589221)	0.106	0.013	0.017	0.017	0.017	0.017	0.017	0.017	0.017	0.019			
12 <i>A. indoburmanensis</i> (MN519705)	0.107	0.015	0.019	0.019	0.019	0.019	0.019	0.019	0.019	0.021	0.006		
13 <i>A. marmoratus</i> (JF794455)	0.118	0.117	0.114	0.114	0.114	0.114	0.114	0.114	0.114	0.117	0.123	0.124	
14 <i>A. panhai</i> (JF794451)	0.153	0.130	0.128	0.128	0.128	0.128	0.128	0.128	0.128	0.130	0.131	0.132	0.102

Table 3 | Uncorrected pairwise distance (p-distance) of the 16s rRNA sequences of *Euphlyctys* used in this study. Asterisk after species name indicates the holotype

	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1 <i>E. aloysii</i> (AB272606)														
2 <i>E. cyanophlyctis</i> (AB167938)	0.118													
3 <i>E. ehrenbergi</i> (AY014367)	0.115	0.060												
4 <i>E. hexadactylus</i> (AB272605)	0.052	0.112	0.113											
5 <i>E. mudigere</i> (AB377109)	0.099	0.040	0.050	0.097										
6 <i>E. kalasgramensis</i> * (KP091862)	0.109	0.051	0.067	0.103	0.047									
7 <i>E. kalasgramensis</i> (MH087077)	0.109	0.056	0.066	0.103	0.051	0.002								
8 <i>E. kalasgramensis</i> (MH087078)	0.107	0.054	0.065	0.101	0.049	0.000	0.002							
9 <i>E. kalasgramensis</i> (MH087079)	0.107	0.054	0.065	0.101	0.049	0.000	0.002	0.000						
10 <i>E. kalasgramensis</i> (MH087080)	0.107	0.054	0.065	0.101	0.049	0.000	0.001	0.001	0.001					
11 <i>E. kalasgramensis</i> (MH087081)	0.107	0.054	0.065	0.101	0.049	0.000	0.001	0.001	0.001	0.000				
12 <i>E. kalasgramensis</i> (AB290420)	0.109	0.051	0.070	0.103	0.047	0.004	0.006	0.004	0.004	0.004	0.004			
13 <i>E. kalasgramensis</i> (MN519713)	0.109	0.056	0.068	0.103	0.052	0.010	0.011	0.009	0.009	0.009	0.009	0.002		
14 <i>E. kalasgramensis</i> (MK881165)	0.107	0.056	0.077	0.103	0.052	0.017	0.018	0.016	0.016	0.016	0.016	0.006	0.014	
15 <i>E. karaavali</i> (KU870372)	0.091	0.114	0.123	0.087	0.110	0.115	0.114	0.112	0.112	0.112	0.115	0.114	0.114	0.114

Table 4 | Uncorrected pairwise distance (p-distance) of the 16s rRNA sequences of *Polypedates* used in this study. Asterisk after species name indicates the topotype material

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1	<i>P. braueri*</i> (AB564281)															
2	<i>P. braueri</i> (MH087082)	0.021														
3	<i>P. braueri</i> (MH087083)	0.020	0.001													
4	<i>P. braueri</i> (MH087084)	0.020	0.001	0.000												
5	<i>P. braueri</i> (MH087085)	0.020	0.001	0.000	0.000											
6	<i>P. braueri</i> (MH087086)	0.020	0.001	0.000	0.000	0.000										
7	<i>P. braueri</i> (MN511825)	0.027	0.027	0.027	0.027	0.027	0.027									
8	<i>P. colletti</i> (AB728189)	0.117	0.119	0.118	0.118	0.118	0.118	0.080								
9	<i>P. discantus</i> (AB728185)	0.086	0.088	0.087	0.087	0.087	0.087	0.060	0.123							
10	<i>P. impresus</i> (AB728169)	0.063	0.065	0.064	0.064	0.064	0.064	0.054	0.121	0.091						
11	<i>P. leucomystax</i> (AB728135)	0.059	0.065	0.064	0.064	0.064	0.064	0.054	0.136	0.098	0.043					
12	<i>P. macrotis</i> (AB728187)	0.094	0.098	0.096	0.096	0.096	0.096	0.072	0.125	0.100	0.102	0.109				
13	<i>P. maculatus</i> (AB728188)	0.103	0.103	0.103	0.103	0.103	0.103	0.072	0.127	0.114	0.116	0.120	0.112			
14	<i>P. megacephalus</i> (MN511821)	0.056	0.058	0.058	0.058	0.058	0.058	0.064	0.091	0.076	0.034	0.026	0.101	0.085		
15	<i>P. mutus</i> (AB728018)	0.064	0.070	0.069	0.069	0.069	0.069	0.047	0.136	0.097	0.085	0.094	0.102	0.066		
16	<i>P. ottilophus</i> (AB728190)	0.153	0.149	0.150	0.150	0.150	0.150	0.118	0.148	0.161	0.150	0.157	0.146	0.131	0.160	
17	<i>P. teraiensis</i> (MK490925)	0.066	0.066	0.066	0.066	0.066	0.066	0.079	0.129	0.091	0.043	0.111	0.109	0.045	0.076	0.157

and seems to be a case of misidentification as most publications were before the review of *Amolops marmoratus* species complex. Therefore, we propose to remove *A. afghanus*, *A. kaulbacki* and *A. marmoratus* from the amphibian list of Mizoram until further research is carried out to confirm their presence. Based on the confirmation of *A. indoburmanensis* from Mizoram and Assam, northeast India; we hypothesize that *A. indoburmanensis* to be a widespread species in northeast India and most records of other species of *Amolops marmoratus* complex of the region maybe based on samples of this species.

Euphlyctis kalasgramensis

The sample sequences of *E. kalasgramensis* from Mizoram clustered with the holotype of *E. kalasgramensis* in the NJ tree (**Figure 1B**). The genetic distance between the samples from Mizoram and the holotype ranges from 0.000 to 0.002 (**Table 3**). Two sequences submitted to GenBank as *E. kalasgramensis* from Bornadi, Assam, India (MN519713) and Punjab, Pakistan (AB290420) also clustered with the holotype of *E. kalasgramensis* in the NJ tree. Another sample from Assam, India (AB290420) submitted to GenBank as *E. cyanophlyctis* clustered with sequences of *E. kalasgramensis*, with a genetic distance of only 0.004 compared to the holotype of *E. kalasgramensis*. *Euphlyctis kalasgramensis* was described from Kalasgram, Barisal, Bangladesh with a wide distribution in Bangladesh.⁴² The species is also expected to occur in adjacent areas of India.^{33,42} It was recently recorded from Punjab, Pakistan suggesting the distribution of this species is much wider than previously expected.⁴³ *Euphlyctis kalasgramensis* was included in the amphibian checklist of India,³⁴ but the authors did not mention any voucher sample or specific locality. Therefore, the present record constitute the first confirmed record of this species from India.

Prior to this study, *Euphlyctis cyanophlyctis* (Schneider, 1799) had been recorded from Mizoram.³⁸ *Euphlyctis cyanophlyctis* is a species complex,⁴⁴ and the species now assigned to *E. kalasgramensis* was also identified as *E. cyanophlyctis* in older literatures.^{42,44,45} With the discovery of *E. kalasgramensis* from parts of northeast India and even into Pakistan, we believe that the species previously identified as *E. cyanophlyctis* from Mizoram and other parts of northeast India were based on samples of *E. kalasgramensis*.

Polypedates braueri

The sample sequences of *P. braueri* from Mizoram clustered with other *P. braueri* sequences (**Figure 1B**). The genetic distance between our samples and a sample from Taiwan (AB564281), the

type locality of *P. braueri* ranges from 0.020 to 0.021 (**Table 4**). *Polypedates braueri* is a species of the *Polypedates leucomystax* complex, described from Taiwan, China.⁴⁶ Subsequent authors treated it as a junior synonym of *P. megacephalus*,⁴⁷⁻⁵⁰ until it was validated using integrative taxonomic approach.⁵¹ The species is reported from Taiwan, tropical and subtropical China, and Myanmar.^{33,52} *Polypedates braueri* had not been recorded in India, but the occurrence of this species in northeast India is not surprising as it was recently reported near the Indo-Myanmar border in Kachin state, Myanmar and Medog, Tibet.⁵² The present record extends the known distribution of *P. braueri* more than 600km SW from the nearest locality record near Ziradum, Kachin state in Myanmar and represents the first record from India.

It is important to note that the samples of *P. braueri* from Mizoram were from a population previously identified as *P. himalayensis*,⁴⁰ a species which was recently removed from a synonym of *P. maculatus* and *P. leucomystax*.⁵³ Annandale⁵⁴ described *P. himalayensis* as a subspecies of *P. maculatus* from the Abor hill, in present day Arunachal Pradesh state of India and is reported from the northeast states of Arunachal Pradesh, Assam, Meghalaya and Mizoram.^{38,40,53} While resurrecting *P. himalayensis*, Gogoi and Sengupta⁵³ did not compare it with *P. braueri* which had been validated from the synonym of *P. megacephalus*.⁵¹ The molecular confirmation of *P. braueri* from a population previously reported as *P. himalayensis* raised a question on the conspecificity of these taxons.

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Conflict of interest

The authors declare that there is no conflict of interest.

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