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Article



Morphological and molecular characterization of *Theلودerma moloch* (Anura: Rhacophoridae) from Indo-Burma biodiversity hotspot of northeast India

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Specimens of a rare rhacophorid frog of the genus *Theلودerma* were collected from Hmuifang, Mizoram, India. Based on their morphology and molecular analysis (16S rRNA), the specimens were identified as *Theلودerma moloch*, a rare species previously recorded only from the Himalayan foothills of India and China. The present record significantly extends the known range of the species and is a first record for the state of Mizoram and Indo-Burma biodiversity hotspot. The uncorrected p-distance between the specimen from Mizoram, NE India and the specimen from Arunachal Pradesh, India (KU169993) and Tibet, China (KU243081) are 0.0% and 1.2% respectively.

Key words: 16S rRNA; DNA barcoding; Hmuifang; phylogeny; changbawl; conservation.

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Introduction

The genus *Theلودerma* Tschudi, 1838 is composed of 25 species distributed from Sri Lanka; northeastern India to Myanmar and southern China through Indochina to Malaya, Sumatra, and Borneo.¹ *Ixalus tuberculatus* Anderson, 1879 is tentatively placed under the genus.² The genus is characterised by their rough, tuberculate skin, and Y-shaped terminal phalanges,^{3,4} but no synapomorphy is found for the genus.^{5,6} With the advances in molecular phylogenetic studies, several species from other rhacophorid genus had been placed under *Theلودerma*.^{2,7} Out of the 24 species of *Theلودerma* recognised, four species

viz. *Theلودerma andersoni* (Ahl, 1927); *T. albopunctatum* (Liu & Hu, 1962); *T. moloch* (Annandale, 1912) and *T. nagalandense* Orlov, Dutta, Ghate & Kent, 2006 are recorded from northeast India.^{8,9,10,11} *T. baibungense* (Jiang et al., 2009) is also expected to occur in northeast India,¹ as the previous records of *T. asperum* (Boulenger, 1886) from northeast India may likely represent this species.^{2,12}

T. moloch was described as *Phrynoderma moloch* from Abor country, Arunachal Pradesh of India.¹³ Since its description, it had been placed under different genus like *Rhacophorus* Kuhl & Van Hasselt, 1822, *Nyctixalus* Boulenger, 1882 and *Theلودerma*.^{10,14-16} Until its recent rediscovery

in China, the phylogenetic placement of *T. moloch* is unclear as it renders the genus *Theلودerma* non-monophylatic and formed a clade with *Nasutixalus* Jiang, Yan, Wang & Che, 2016 (= *Frankixalus* Biju, Senevirathne, Garg, Mahony, Kamei, Thomas, Shouche, Raxworthy, Meegaskumbura & Van Boclaer, 2016), a recently described genus.^{12,17,18} A recent rediscovery of the species in China proved that the genus *Theلودerma* is monophyletic,¹ and the specimen previously identified as *T. moloch* used in earlier studies is *Nasutixalus medogensis* Jiang, Wang, Yan & Che, 2016. During a herpetological survey conducted in Mizoram, northeast India, specimens of *T. moloch* were collected for the first time which formed the basis of this paper.

DNA barcoding had been proposed as a fast and reliable tool for species level identification

of biodiversity¹⁹ and had been used in the detection of cryptic species.²⁰ The 16S rRNA gene of the mitochondria had been used as a reliable tool for species identification in amphibians²¹ and its performance outweighs COI gene as a DNA barcoding marker in amphibians due to its higher success rate and the availability of sequences in gene banks.^{22,23}

Materials and Methods

Sampling

Specimens were collected from Hmuifang Community forest at Hmuifang village (23.454047°N 92.751808°E, 1486 m asl.), Aizawl district, India. Specimens were euthanised with

Table 1 | Specimens and Genbank sequences of *Theلودerma* used in molecular analyses.

Species	Voucher	Locality	GenBank Accession Nos.
<i>Theلودerma asperum</i>	ZRC 1.1. 9321	Fraser's Hill, Pahang, Malaysia	GQ204725
<i>Theلودerma asperum</i>	Pet trade	Malaysia	KT461929
<i>Theلودerma albopunctatum</i>	KIZ0608201	Jinping County, Yunnan, China	EF564521
<i>Theلودerma baibungense</i>	YPX31940	Medog, Tibet	KU981089
<i>Theلودerma corticale</i>	AMNH A 161499	Tam Dao, Vin Phu, Vietnam	DQ283050
<i>Theلودerma corticale</i>	VNMN 3556	Tam Dao, Vietnam	LC012841
<i>Theلودerma gordonii</i>	VNMN 4407	Ngoc Linh, Kon Tum, Vietnam	LC012852
<i>Theلودerma horridum</i>	KUHE 52582	Kenaboi, Negeri Sembilan, Malaysia	LC012861
<i>Theلودerma laeve</i>	VNMN 4403	Mang Yang, Gia Lai, Vietnam	LC012846
<i>Theلودerma lateriticum</i>	VNMN 1216	Yen Tu, Bac Giang, Vietnam	LC012851
<i>Theلودerma leporosum</i>	LJT W46	Malaysia	KC465841
<i>Theلودerma licin</i>	KUHE 19426	Nakon Sri Tamarat, Thailand	LC012859
<i>Theلودerma moloch</i>	YPX31941	Medog, Tibet	KU243081
<i>Theلودerma moloch</i>	SDBDU 2011.345	Arunachal, India	KU169993
<i>Theلودerma moloch</i>	SL328	Mizoram, India	Pending
<i>Theلودerma nebulosum</i>	AMS R 173409	Kon Tum, Vietnam	JN688168
<i>Theلودerma palliatum</i>	AMS R 173130	Lam Dong, Vietnam	JN688172
<i>Theلودerma petilum</i>	HNUE MNA 2012.0001	Muong Nhe, Dien Bien, Vietnam	KJ802925
<i>Theلودerma phrynoderma</i>	CAS 243920	Tanintharyi, Myanmar	KJ128282
<i>Theلودerma rhododiscus</i>	CIB GX200807017	Guangxi, China	LC012842
<i>Theلودerma ryabovi</i>	VNMN 3924	Mang Canh, Kon Tum, Vietnam	LC012860
<i>Theلودerma stellatum</i>	KUHE 22056	Mae Yom, Thailand	LC012862
<i>Theلودerma truongsongense</i>	VNMN 4402	Khanh Hoa, Vietnam	LC012847
<i>Gracixalus gracilipes</i>	060821196Rao	Pingbian, Yunnan, China	GQ285668
<i>Liuixalus hainanus</i>	LJT V15	Hainan, China	KC465826
<i>Nasutixalus medogensis</i>	KIZ016395	Medog county, China	KU243082
<i>Nasutixalus medogensis</i>	6255Rao	Motuo, Xizang (= Medog, Tibet)	GQ285679
<i>Fejervarya asmatai</i>	SL121	Mizoram, India	MF319219

benzocaine, fixed in 4% formaldehyde and later transferred to 70% ethanol for longer preservation. Livers were extracted and kept in absolute ethanol for DNA analysis. Specimens were deposited at the Zoological Museum, Pachhunga University College (PUCZM).

Morphological analysis

Morphometric measurements were taken on the left side of the specimens. Measurements were taken with a digital caliper with the nearest 0.1 mm accuracy. The following morphological parameters were taken: Snout-vent length (SVL); head length, from tip of snout to the articulation of the jaw (HL); head width, at greatest width of the jaw (HW); snout length, from the anterior portion of the eye to the tip of the snout (SL); internarial distance (IND); distance from anterior corner of eye to posterior edge of nostril (NEL); eye diameter (ED); interorbital distance (IOD); maximum width of upper eyelid (UEW); tympanum diameter (TD); forearm length, from elbow to base of outer palmar tubercle (FLL); hand length, from base of outer palmar tubercle to tip of finger III (HAL); thigh length, from vent to knee (FL); tibia length (TIB); maximum shank

width (TBW); foot length, from distal end of tibia to tip of distal phalanx of III (FOT).

Molecular analysis

Genomic DNA was extracted from the liver tissue of one specimen (PUCZM/IX/SL 328) using PureLink® Genomic DNA Kit (Thermo Fisher Scientific, USA) following the manufacturer's instructions. We amplify a partial sequence of mitochondrial 16S rRNA to assess the phylogenetic relation of the taxon using the primer pair L2188 -5'TCAACCAACCACAAAGACATTGGCAC3²⁴ and 16H1 -5'TAGACTTCTGGGTGGCCAAAGAATCA3²⁵ using a ProFlex™ 3 x 32-Well PCR System (Applied Biosystems). Amplified DNA was produced in 25 mL reactions after 30 cycles of denaturation for 30 sec at 94°C, annealing for 30 sec at 56°C, and extension for 1 min at 72°C. PCR products were isolated through electrophoresis using 1% agarose gels. The PCR products were directly sequenced in both directions at Agrigee, Kochi, Kerala, India.

The resulting sequences were edited using FinchTV 1.4.0 software packages (Geospiza, Inc.; Seattle, WA, USA; <http://www.geospiza.com>) and aligned using Clustal X 1.64.²⁶ The phylogenetic

Table 2 | Morphometric data of *Theلودerma moloch* from Mizoram, India.

Parameter	PUCZM/IX/SL 371	PUCZM/IX/SL 328	PUCZM/IX/SL 329
	Male	Male	Male
SVL	36.6	39.8	36.5
HL	10.8	11.7	11.9
HW	12.5	13.5	13.4
SL	4.5	4.6	4.3
IND	2.5	2.8	3.0
NEL	3.3	3.5	3.2
ED	3.4	4.1	4.1
IOD	6.5	7.5	6.7
UEW	2.7	2.9	3.3
TD	2.6	2.6	2.7
FLL	9.5	9.7	8.9
HAL	10.5	11.0	11.2
FL	18.1	19.0	19.3
TIB	19.7	20.6	20.3
TBW	3.6	4.5	3.6
FOT	23.5	23.6	23.8
Relative length of fingers	I<II<IV<III	I<II<IV<III	I<II<IV<III
Relative length of toes	I < II < V < III < IV	I < II < V < III < IV	I < II < V < III < IV

relationship was established using Maximum Likelihood (ML) in SeaView version 4²⁷ with available sequences representing all valid species of the genus *Theلودerma* retrieved from GenBank and the newly generated sequence (Table 1). *Gracixalus gracilipes* (Bourret, 1937); *Liuixalus hainanus* (Liu & Wu, 2004); *Nasutixalus medogensis* and *Fejervarya asmata* Howlader, 2011 were used as outgroup. Uncorrected genetic distances (p-distances) between sequences were determined with MEGA 6.0.²⁸

Result

Theلودerma moloch (Annandale, 1912) (Figure 1)

Materials examined: PUCZM/IX/SL 328 (male); SVL 39.8mm: Collected by Samuel Lalronunga, Lalkhawngaiha Sailo, Vanlalchhuanga and Malsawmdawngliana from Community Forest Reserve, Hmuifang Village, Mizoram on 16th June, 2017. PUCZM/IX/SL 329 (male); SVL 36.5mm: Collected by Samuel Lalronunga, Lalkhawngaiha Sailo, Vanlalchhuanga and Malsawmdawngliana from Community Forest Reserve, Hmuifang Village, Mizoram on 16th June, 2017. PUCZM/IX/SL 371 (male); SVL 36.6mm: Collected by Samuel Lalronunga, C. Lalrinchhana, V.L. Hriatzala Sailo, Vanlalhrima and Esther Lahlmingliani from Hmuifang Village, Mizoram on 23rd April, 2015.

Morphological characters (Table 2): SVL males 36.5–39.8 mm (n =3); head slightly wider than long (HL 10.8–11.9 mm, HW 12.5–13.5 mm); snout truncate, longer than horizontal diameter of eye (SL 4.3–4.6 mm, ED 3.4–4.1 mm); nostrils oval, on lateral side, closer to the tip of snout than to the eye (SL 4.3–4.6 mm, NEL 3.2–3.5 mm); canthus rostralis angular; interorbital distance wider than upper eyelid (IOD 6.5–7.5 mm, UEW 2.7–3.3 mm); tympanum distinct, rounded, diameter lesser than eye diameter (TD 2.6–2.7 mm); supratympanic fold absent; vomerine teeth present.

Forelimbs. Forearm (FLL 8.9–9.7 mm) shorter than hand (HAL 10.5–11.2 mm); relative length of fingers: I<II<IV<III; tips of fingers dilated into

round flattened discs; webbing slightly developed; subarticular tubercles distinct, round, formula 1, 1, 2, 2.

Hind limbs. shanks about five to six times longer than wide (TIB 19.7–20.6 mm, TBW 3.6–4.5 mm); thigh length (FL 18.1–19.3 mm); foot length (FOT 23.5–23.8 mm); relative length of toes: I<II<III<V<IV except on the right limb of PUCZM/IX/SL 329 (I<II< IV<III<V) which may be due to ontogenetic deformation; tip of fingers enlarged into large flatted discs; subarticular tubercles present, formula 1, 1, 2, 3, 2; inner metatarsal tubercle distinct; outer metatarsal tubercle absent.

Skin. Dorsal skin very rough with large warts and granules; prominent ridges forming a \wedge -shape on foreback; dorsolateral folds absent; throat and underside of limbs smooth, belly granular.

Molecular analysis: In the Maximum Likelihood tree generated, the specimen of *T. moloch* from Mizoram (PUCZM/IX/SL 328) clustered with the *T. moloch* from Arunachal Pradesh, India (KU169993) and Tibet, China (KU243081) with high support value (Figure 2). The uncorrected p-distance between the specimen from Mizoram and the specimen from Arunachal Pradesh, India and Tibet, China is 0.0% and 1.2% respectively.

Ecological note: The first specimen (PUCZM/IX/SL 371, male) was spotted on a concrete retaining wall near Land Administrative Department (LAD) Guest House on 3rd April, 2015. On 16th June, 2017 two more specimens (PUCZM/IX/SL 328 -329, both males) were collected from the Community Forest Reserve, Hmuifang village, not far from where the first specimen was collected. One individual (PUCZM/IX/SL 328) was seen parching on the trunk of a tree at about 1m high (Figure 3A) and the second individual was collected from the roof of a toilet just near the tree where the first specimen was spotted. The collection site is dominated by *Quercus xylocarpa* Kurz, *Quercus* spp., *Dipterocarpus macrocarpus* Vesque, *Castanopsis tribuloides* (Sm.) A.DC., *Syzygium praecox* (Roxb.) Rathakr. & N.C.Nair and *Schima wallichii* (DC.) Korth., with a small patch of grassland (Figure 3B). The region falls under



Fig. 1 | Dorsal, ventral and lateral aspects of *Theلودerma moloch* (PUCZM/IX/SL 371; 36.6 mm SVL) from Mizoram, northeast India. Photographs by Lalronunga.

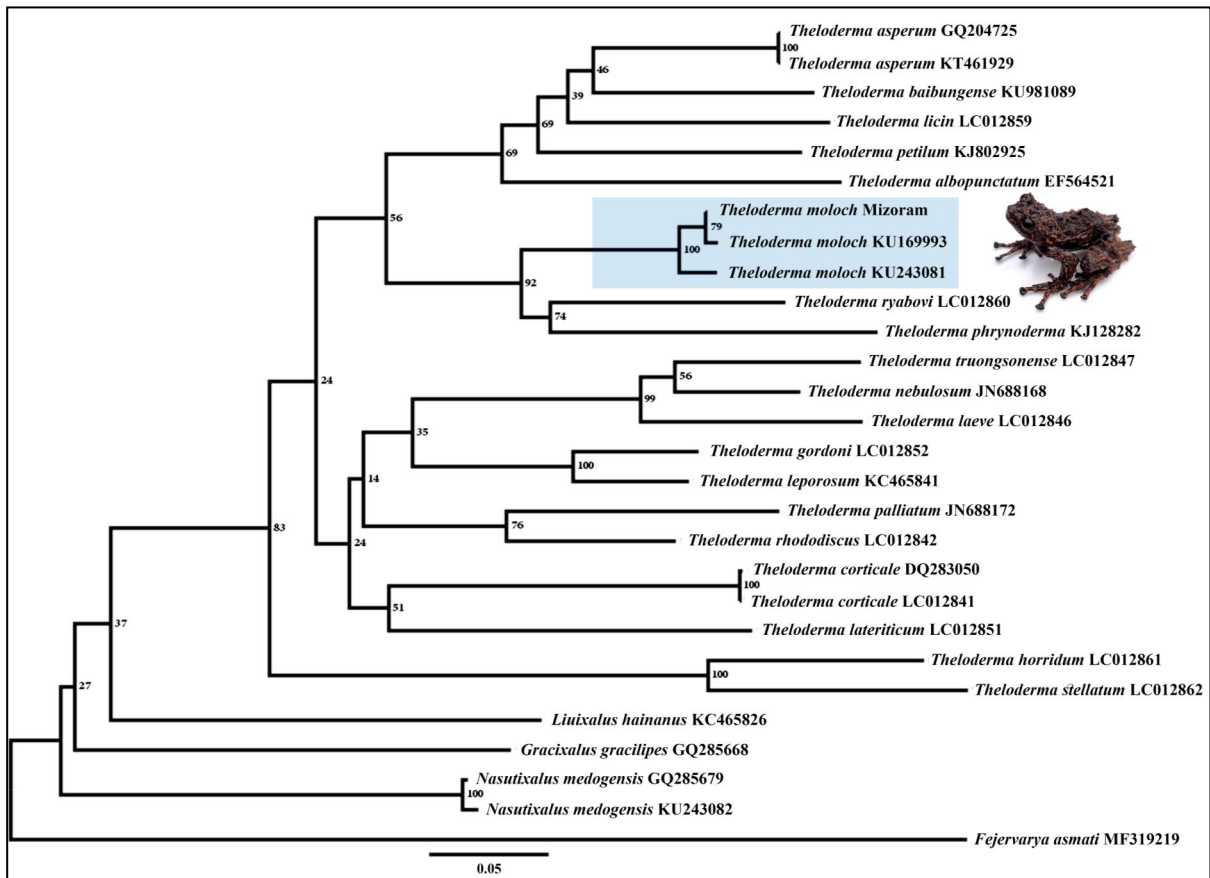


Fig. 2 | Maximum Likelihood tree of *Theلودerma* based on 16S rRNA fragments. Number in the tree nodes is the bootstrap value. The scale bar below the tree is in units of substitutions/site.

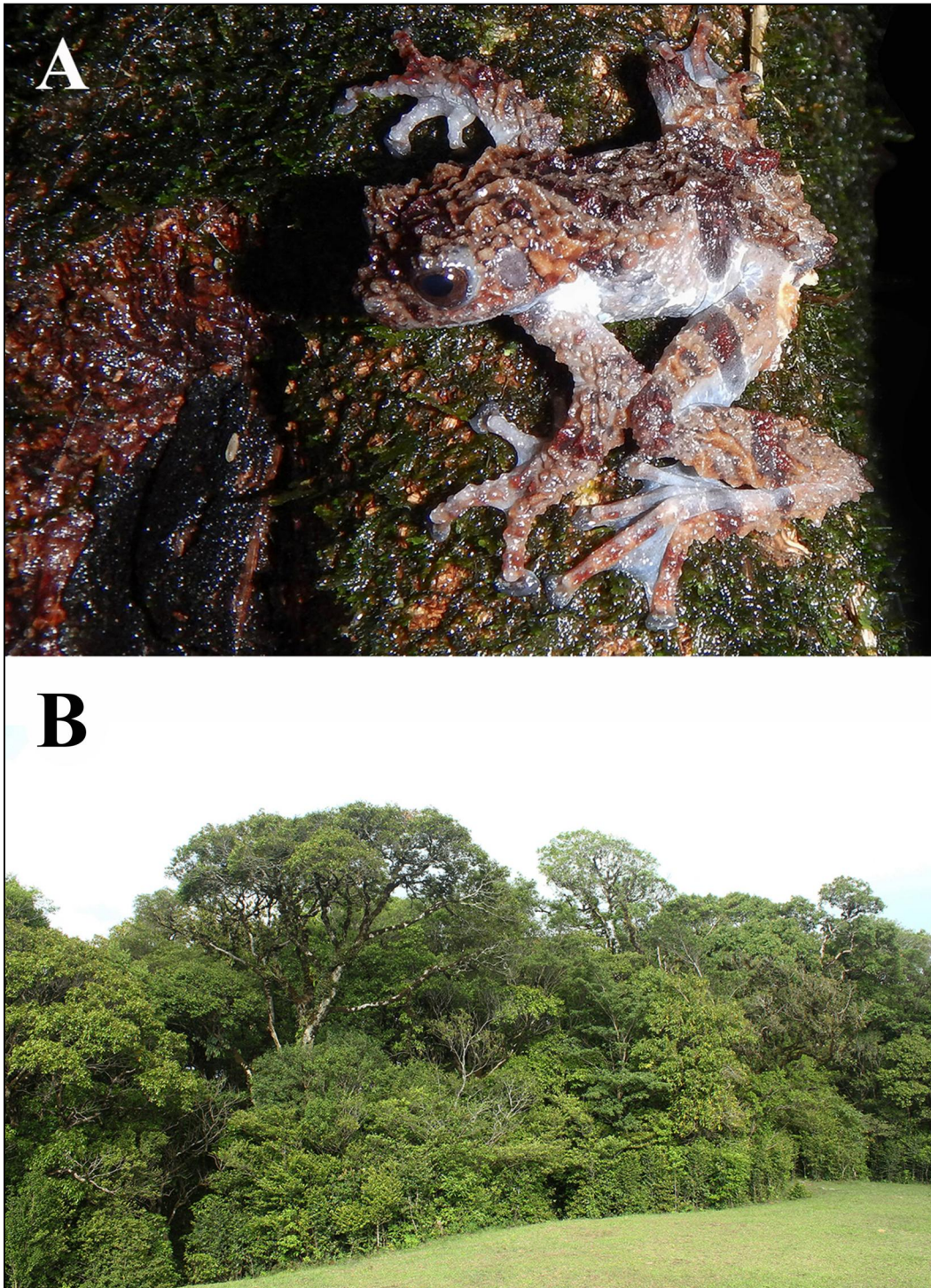


Fig. 3 | A. In-situ photo of male *Theloderma moloch* (PUCZM/IX/SL 328; 39.8 mm SVL) on tree trunk; **B.** Habitat of *T. moloch* at Hmuifang Community forest, Mizoram, India. Photographs by Lalronunga.

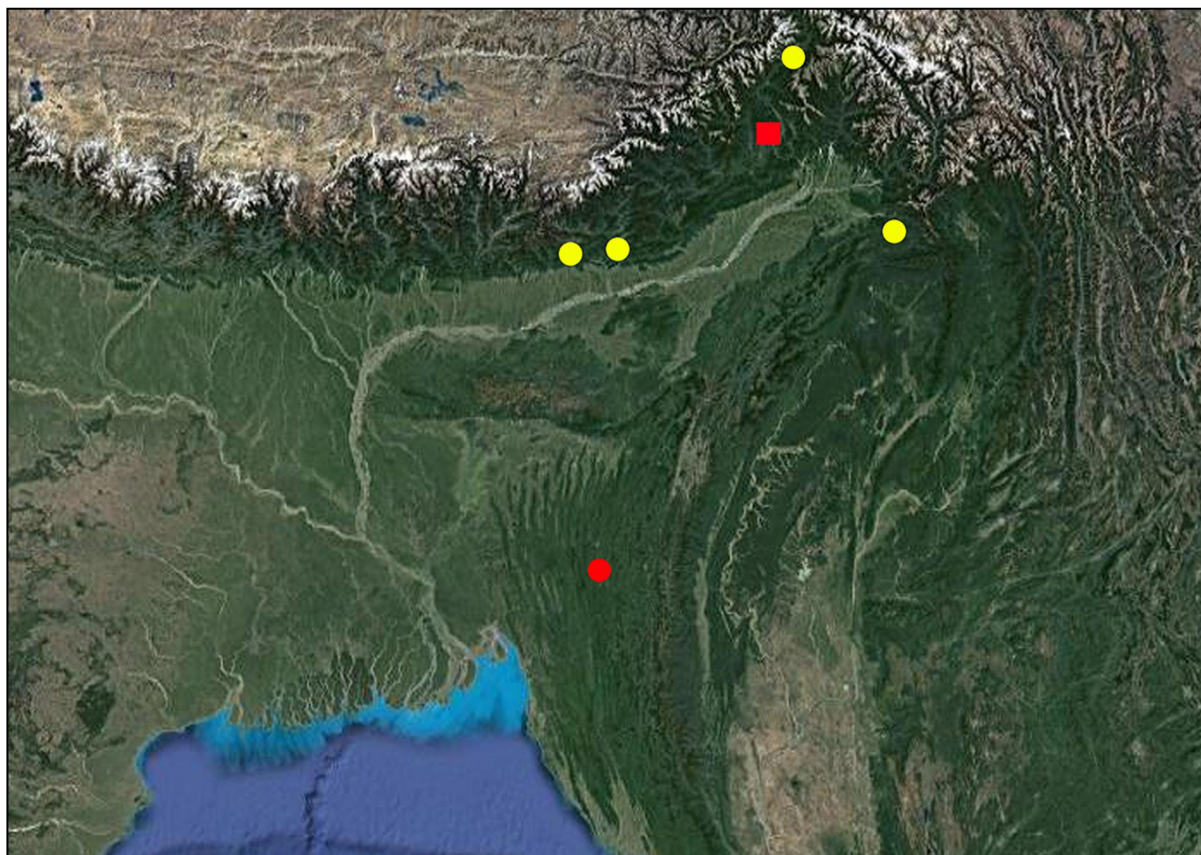


Fig. 4 | Satellite image showing the know locality of *Theلودerma moloch*. Red square indicates the type locality, yellow circles indicates other records, red circle indicates the present record.

Montane Sub-tropical Forest category.²⁹ Hmuidang Community forest had been preserved from time immemorial by the Mizo chiefs of the area. The virgin forest is a resort for many herpetofaunal species.

Due to their cryptic nature and distribution pattern (found in higher elevations), *T. moloch* is rarely known by the local communities of Mizoram and no local name is known for the species in published or popular media.

Suggested local name: “Changbawl” (*chang* meaning frog; *bawl* meaning warty in Mizo language) due to the presence of numerous warts on the skin.

Discussion

The extinction and decline of amphibian population is global concern, and almost one third of them are estimated to be threatened with extinction.³⁰ Knowledge on the diversity and distribution of the genus *Theلودerma* had increased in the recent years.^{2,12,18} The species is listed as vulnerable in the IUCN Red List since it is spotted from few localities covering a small geographical area, fewer than ten locations, and there is continuing decline in the extent and quality of its habitat in northeastern India.³¹ It was previously recorded from 5 localities in northeast India and China,^{13,18,32-34} all within the

Eastern Himalaya biodiversity hotspot (Figure 4). A recent record from Namdapha National Park, Arunachal Pradesh, India is the only known record of the species from the southern bank of Brahmaputra River.³³ The present record is a first record of the species from Mizoram, extending the distributional range of the species to the south of Indo-Burma biodiversity hotspot. Based on the current record of *T. moloch* from Mizoram, the species may be distributed in other localities of northeast India and adjacent areas with more intense survey.

A recent study on the anuran fauna of Hmuifang community forest recorded the occurrence of 18 species from the area.³⁵ However, the overall structure of the paper, numerous taxonomic errors and inaccurate information made the paper in need of review, even though a full review of the paper beyond the scope of this study. Most of the species accounts (descriptions, comparisons and colourations) in the paper are reproduced verbatim of several research papers, even to the parts where new genus and species were mentioned in the original publications without giving citations.^{36–38} A dicroglossid species, *Fejervarya limnocharis* (Gravenhorst, 1829) which is restricted to Indonesia, Malaysia, Laos, Myanmar, Thailand, Cambodia, and Vietnam was recorded from the study area.¹ They further made a remark on the distribution of *F. limnocharis* without giving a justification as to why they allocate their specimens to this species. *Duttaphrynus chandaii* Das, Chetia, Dutta & Sengupta, 2013 lacks species authors throughout the whole paper, whereas Article 51 of the International Code of Zoological Nomenclature (ICZN) recommends that species name should be accompanied with original author(s) and year at least once dealing with the taxon denoted by that name. *Polypedates himalayensis* Annandale, 1912 a junior subjective synonym of *P. maculatus* (Gray, 1830) was included in the list without any justification and the species authority was erroneously credited as Gray, 1830. The authors comment that nine species were recorded for the first time from Mizoram. However, more than half of them viz. *Amolops marmoratus* (Blyth, 1855); *Duttaphrynus chandaii*; *Odorrana mawphlan-*

gensis (Pillai & Chanda, 1977); *Polypedates himalayensis* and *Rhacophorus suffry* Bordoloi, Bortamuli & Ohler, 2007 were already recorded from Mizoram,^{39–42} and the remaining species viz. *Frankixalus cf. jerdonii*, *Kurixalus* sp., *Philautus* sp. and *Theloderma cf. asperum* were not ascertained to the species level. Based on priority, the genus *Frankixalus* is a junior synonym of *Nasutixalus*. The two species *Nasutixalus cf. jerdonii* and *Theloderma cf. asperum* needs confirmation as the taxon names assigned to them are uncertain and provisional. The genus *Kurixalus* Ye, Fei & Dubois, 1999 and *Philautus* Gistel, 1848 were already recorded from Mizoram.^{39,43}

The description of *T. cf. asperum* in the recent paper is too brief and moreover without any photograph.³⁵ The distribution of *T. asperum* (Boulenger, 1886) is restricted to Malaysia and three species resembling it in colouration viz. *T. albopunctatum*, *T. baibungense* and *T. pyaukkya* Dever, 2017 are known from northeast India and adjoining countries.^{2,44} We had come across photographs of *T. asperum* sensu lato from localities near Hmuifang, but the recent record from Hmuifang³⁵ may be a case of misidentification as the ventral colouration (dark blue) does not conform to *T. asperum* sensu lato and may actually be a specimen of *T. moloch* which have dark blue ventral colouration. Closer examination of their specimen (MZMU- 1051) is required to ascertain the identity of the species.

Hmuifang community forest is conserved by the local community since time immemorial, and this small patch of virgin forest harbours some unique flora and fauna. The location is one of the hottest tourist destination and activities like felling of trees for construction of roads and buildings for attracting tourists pose a threat for this conserved area. Recently, aware of their biological wealth, the local community setup a Biodiversity Management Committee (BMC) in 2016 under the Biological Diversity Rules, 2004 to carry out conservation initiatives within their jurisdiction.

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