DNA barcoding elucidates the new altitude record and range-extension of lesser-known bullfrog (*Hoplobatrachus litoralis*) in northeast India

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**ABSTRACT**

The molecular information of lesser-known bullfrog, *Hoplobatrachus litoralis* is restricted from Bangladesh (type locality) and Myanmar. The morphological observation further evidenced the range-extension of this species from India and Myanmar. Here, we collected the *H. litoralis* specimen from the Dampa Tiger Reserve, Mizoram state and provides a new altitude record (268 m) and range-extension in northeast India. The DNA barcoding with mitochondrial Cytochrome b gene discriminates *H. litoralis* from other congeners with sufficient genetic distance (13.9\% to 27.8\%). The Bayesian phylogeny revealed monophyletic clustering of all five *Hoplobatrachus* species and showed the sister relationship of *H. litoralis* and *H. tigerinus*. The generated sequences of *H. litoralis* from northeast India depicted shallow divergence (1.5\%) from the sequences generated from Bangladesh, indicates the possible genetic variability due to the alteration of the ecological niche. We suggested further physiological and molecular study with extensive sampling of this species to confirm their actual range distribution, high-altitude adaptation, and gene flow.

**Introduction**

The genus *Hoplobatrachus* (Family Dicroglossidae) consists of five species globally and widely distributed in Sub-Saharan Africa and southern and southeastern Asia (Frost 2020). Among them, two species *Hoplobatrachus crassus* and *Hoplobatrachus tigerinus* are antecedently known to be distributed in India. Except *Hoplobatrachus litoralis*, all other species are bearing Least Concern status in the International Union for the Conservation of Nature (IUCN) Red List of Threatened species. However, they are confronted the illegitimate threat of hunting and trapping for consumption as food and medicine in Asian countries (IUCN 2020). Nevertheless, owing to the rapid growth of urbanization; their suitable habitats are largely shrinking in the wild. Among all extant congeners, *H. litoralis* is lately described species from the Cox’s Bazar district, coastal belt of Bangladesh (Hasan et al. 2012). Later on, multiple specimens from different geographical locations further extended the range distribution of *H. litoralis* beyond its type locality. Based on the morphological observation the specimen was recorded from three different states (Assam, Tripura and West Bengal) in eastern and northeastern India (Purkayastha and Basak 2018; Mondal et al. 2018; Bohra et al. 2019) (Figure 1(A)). Besides the taxonomic studies, the molecular data of *H. litoralis* was also generated from Bangladesh and Myanmar (Hasan et al. 2012; Sultana et al. 2017; Mulcahy et al. 2018). The morphological characters often create confusion to identify the *H. litoralis* from their close relative *H. tigerinus* in different life stages. In this instance, the utility of mitochondrial genes (16S rRNA and Cytb) were evidenced to identify the species accurately and effectively discriminated *H. litoralis* from the sister species *H. tigerinus* (Mulcahy et al. 2018).

The integrated approach in combination with morphology, molecular, bioacoustics, and zoogeography is manifested to be best practice in anurans systematic studies (Padial et al. 2010; Ortega-Andrade et al. 2015; Köhler et al. 2017). The partial or complete mitochondrial genes were evidenced to discriminate variety of animals including anurans (Verma and Singh 2002; Hebert et al. 2003; Biju et al. 2014; Chambers and Hebert 2016; Kundu et al. 2020). Apart from the accurate species level identification, this molecular tool is also utilized to recognize the phylogenetic and evolutionary relationship (Biju and Bossuyt 2009; Van Boeckelaer et al. 2012; Mahony et al. 2017), cryptic diversity (Bickford et al. 2007; Tyagi et al. 2017), and origin as well as diversification of amphibians (Van Boeckelaer et al. 2006; Röelants et al. 2007; Garg and Biju 2019). Although the previous morphological studies witnessed the presence of *H. litoralis* in India, their genetic information was lacking from these regions. Considering the young adult specimen and lack of bioacoustics information,
the present study aimed to generate the DNA sequences of this lesser-known bullfrog to affirm the morphology-based identification. This is the first molecular information of this species from India contributed to the global database for future reference.

**Materials and methods**

During the recent survey of herpetofauna, a single young adult female specimen of dicroglossid frog was opportunistically collected from the Teirei Range in the Dampa Tiger Reserve.
Reserve (23.68 N 92.45 E), Mizoram state in northeast India (Figure 1(B)). Based on the morphological characters following the original description, the species was preliminarily identified as *H. litoralis* (Hasan et al. 2012). The survey and sampling were executed after acquiring the prior permission (No.A.33011/2/99-CWLW/225) from the Chief Wildlife Warden of Environment, Forests and Climate Change, Govt. of Mizoram, India. The specimen was vouchered (MZMU 1383) in the museum of Department of Zoology, Mizoram University, India. The tissue sample was collected and preserved in 70% ethanol for further molecular investigation.

The genomic DNA was extracted by using QIAamp DNA Mini Kit (Qiagen, Valencia, CA) with manufacturer's protocols. The genomic DNA was stored at −30 °C in the Center for DNA Taxonomy laboratory, Molecular Systematics Division, Zoological Survey of India, Kolkata. The published primer pair (Verma and Singh 2002): mcB 398: 5′-TACCATGAGGACAATAATCCTCTG-3′ and mcb 869 5′-CTCCTAGTTGTAGGATTGC-3′ was used to amplify the partial mitochondrial cytochrome c (Cyt c) gene on a Veriti® Thermal Cycler (Applied Biosystems, Foster City, CA). The 30 μl PCR mixture contains 10 pmol of each primer, 100 ng of DNA template, 1 × PCR buffer, 1.0–1.5 mM of MgCl2, 0.25 mM of each dNTPs, and 1 U of Taq polymerase (Takara BIO Inc., Japan). The thermal profile was set as 95 °C for 10 min; 35 cycles of 95 °C for 45 s, 51 °C for 1 min and 72 °C for 2 min and subsequent hold at 4 °C. The PCR amplified product was illustrated in 1% agarose gel containing Ethidium bromide (10 mg/ml). The PCR product was further purified using QIAquickR Gel extraction Kit (Qiagen, Valencia, CA). The cycle sequencing of the purified PCR product (15 ng) was performed with BigDye® Terminator ver. 3.1 Cycle Sequencing Kit (Applied Bio systems, Foster City, CA) by using 3.2 Picomoles of PCR primer pair. The thermal profile of the Cycle Sequencing was set as 96 °C for 1 min, followed by 25 cycles of 96 °C for 10 s, 50 °C for 5 s and a final extension at 60 °C for 1 min 15 s on a Veriti® Thermal Cycler. The cycle sequencing product was cleaned by BigDye X-terminator kit (Applied Biosystems, Foster City, CA) and Sanger sequencing was performed on 48 capillary ABI 3730 Genetic analyzer housed at Zoological Survey of India, Kolkata.

The generated sequence was checked by SeqScanner V1.0 (Applied Biosystems Inc., CA, USA), nucleotide BLAST (https://blast.ncbi.nlm.nih.gov/), and ORF finder (https://www.ncbi.nlm.nih.gov/orffinder/) to avoid the low quality reads and gaps. The generated sequence was submitted to the GenBank to acquire the accession number. Further, a total of 20 publicly available database sequences of *Hoplobatrachus* species were acquired from GenBank to form a combined dataset to estimate the genetic distance and phylogenetic analysis. The sequence of *Euphyctis hexadactylus* (AY014386) was used as an out-group in the Bayesian analysis (BA). The dataset was aligned using ClustalX (Thompson et al. 1997) and Kimura 2 parameter (K2P) genetic distances were calculated using MEGAX program (Kumar et al. 2018). The BA topology was constructed in Mr. Bayes 3.1.2 (Ronquist and Huelsenbeck 2003) by selecting rts = 6 and rate-s = invgamma for GTR + G + I model estimated through Mr.Modeltest v2 (Nylander 2004). The phylogeny was illustrated in a web-based iTOL tool (https://itol.embl.de/) (Letunic and Bork 2007).

**Results and discussion**

Northeast India has gained worldwide attention due to the pristine natural condition, zoogeography, climates, and diverse forest cover. This region is a part of two global biodiversity hotspots with remarkable faunal diversity with endemism (Tordoff et al. 2011). However, apart from the checklist and taxonomic information, limited attempts were aimed to deal with molecular data to assess the herpetofaunal diversity in this region. The collected specimen was preliminarily identified as *H. litoralis* based on the morphological characters. The previous study reported that the mean snouts vent length (SVL) of *H. litoralis* is less than *H. tigerinus* (89.96 ± 5.92 mm in male and 101.42 ± 12.01 mm in female). The SVL of the collected female specimen was 94 mm which is within the range of *H. litoralis* and different from the female of *H. tigerinus* (113.67 ± 15.43 mm). The diagnostic feature for *H. litoralis* was observed in the collected individual. Two continuous distinct bands, first from anterior corner of eye through the nostrils to anterior edge of upper jaw and second along the lateral margin of upper jaw were observed in the studied specimen. Further, a distinct black margin in the inner side of the upper arm, inner metatarsal tubercle is black, and inter-orbital distance is narrower than eyelid width and inter-nostril distance were also noticed in the collected specimen. Due to the lack of bioacoustics information and avoid to wait for the upcoming seasons, we adopted the support of molecular tool to confirm the species identity. Besides, the species was described from the coastal belt of Bangladesh of about 3 m ASL and also reported from India of about 80 m ASL (Kamrup district Assam). This is the first record of the species from Mizoram state as well as from the Dampa Tiger Reserve that links from eastern India (West Bengal) to Myanmar through Bangladesh and northeast India. We provide the range extension (ca.255 km towards northeast from the type locality) and new altitude record (268 m) of *H. litoralis* from India.

The high-altitude adaptation allows an excellent phenomenon for examining how the animals cope with environmental stressors and accumulating genetic alterations and other physiological systems (Hutchison et al. 1976; Weber et al. 2002). The frogs are evident to acquire different genetic mechanisms correlated with the elevation distribution and high-altitude adaptation compared to other endotherms (Chen et al. 2013; Yang et al. 2016; Chen et al. 2018). Due to the high-altitude adaptation the physiological traits (body size and body mass), behavior such as vocal response are also switched among anurans and often hypothesized with different ecogeographical rules (Hu et al. 2011; Meenderink et al. 2017; von May et al. 2017). The range expansion of *H. litoralis* from coastal area of Bangladesh to the erratic environmental conditions at high-altitude in northeast India manifested strong and opposing selection pressures on their different life stages and influence fitness at the same time. Our results furnished further scope for additional empirical data to recognize the physiology and gene modification of this species.
The combined approach of both morphology and molecular data also evidenced that, the amphibians are one of the unique biotic components which can provide new insights on the phylogeny and diversification hypothesis (Biju and Bossuyt 2003; Bossuyt et al. 2006; Kamei et al. 2012). However, the knowledge of amphibian species and their genetic diversity is still under progress in northeast India. The study provides the first molecular data (470 bp mtCytb gene) of *H. litoralis* from India. The generated sequences showed 98.51% similarity in nucleotide BLAST search with the published database sequences generated from the type locality in Bangladesh (Hasan et al. 2012). The overall mean K2P genetic distance of five *Hoplobatrachus* species was 19% in the studied dataset. The intra-species genetic distance was ranging from 0.2% (*H. tigrinus*) to 4.9% (*H. crassus*). The inter-species genetic distance was ranging from 13.9% (*H. litoralis* and *H. tigrinus*) to 28.8% (*H. crassus* and *Hoplobatrachus occipitalis*). The Sub-Saharan African species, *H. occipitalis* revealed 23.6% to 28.8% genetic distance with other four species distributed in southern and southeastern Asia. All the studied *Hoplobatrachus* species showed monophyletic clustering in the BA phylogeny with high posterior probability support (Figure 1(C)). The targeted species, *H. litoralis* depicted sister relationship with *H. tigrinus*, as suggested in the previous studies (Hasan et al. 2012; Sultana et al. 2017; Mulcahy et al. 2018). The generated sequence of *H. litoralis* from northeast India showed 1.5% genetic distance from the generated sequences from Bangladesh. *H. litoralis* might have acquired these genetic changes due to the alteration of the ecological niche, which can be further demonstrated through further studies. By observing the genetic variability of *H. litoralis* within its range distribution, the present study encouraged extensive sampling of this species from India, Bangladesh and Myanmar to elucidate the phylogeography and gene flow.

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**Disclosure statement**

No potential conflict of interest was reported by the author(s).

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**Data availability statement**

The data that support the findings of this study are openly available in NCBI GenBank database at (https://www.ncbi.nlm.nih.gov) with the accession number (MT474118) which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

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