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Research Article

Larval description of Coastal bullfrog *Hoplobatrachus litoralis* (Anura: Dicroglossidae) from Manipur, India

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ABSTRACT

Despite the ecological and taxonomic importance of larval morphological features, the tadpole of *Hoplobatrachus litoralis* has remained undescribed. This study presents the first detailed account focusing on their external morphology—especially the detailed structure of the oral apparatus as revealed through Scanning Electron Microscopy (SEM). The present study highlights a distinctive characteristic of the *H. litoralis* tadpoles—the presence of a prominent medial projection on the upper labium and four continuous rows of needle like teeth arrangement in the lower labium. The labial tooth row formula (LTRF) observed is 1/4. Phylogenetical analysis positions *H. litoralis* as a distinct lineage, forming a sister clade to *H. tigerinus.*

Key words: LTRF, morphology, phylogeny, Scanning Electron Microscopy (SEM), tadpoles.

INTRODUCTION

Hoplobatrachus litoralis Kuramoto, Islam, Alam, Khan, and Sumida, 2012, was described from Bangladesh (Hasan et al. 2012). This species was later reported in Myanmar (Mulcahy et al. 2018 and Zug, 2022), and its presence in northeast India was confirmed from multiple states, including Tripura (Purkayastha and Besak 2018), Assam (Bohra et al. 2019), Mizoram (Kundu et al. 2020; Decemson et al. 2021), and Manipur (Lalremsanga et al. 2022). Additionally, *H. litoralis* has been recorded from West Bengal (Mondal et al. 2018) and Bhutan (Wangyal et al. 2020) further expanding its known geographic range (Frost, 2024).

According to Altig and McDiarmid (1999), tadpoles and their adult forms differ significantly in their morphology. Tadpoles are frequently identified by examining the external shape and appearance of their mouthparts (Altig and Pace 1974; Lee 1976; Inthara et al. 2005). Knowledge about anuran tadpoles is crucial to understand their natural history and development. Tadpole morphology needs to be described in more detail to further our understanding of the species identity, biology and their ecology.

The *Hoplobatrachus* family comprised of six species: *H. chinensis* (Osbeck 1765), *H. crassus* (Jerdon 1853), *H. litoralis* Hasan, Kuramoto, Islam, Alam, Khan, and Sumida 2012, *H. occipitalis* (Günther 1858), *H. salween* Thongproh, Chunskul, Sringurngam, Waiprom, Makchai, Cota, Duengkae, Duangjai, Hasan, Chuaynkern, and Chuaynkern 2022, and *H. tigerinus* (Daudin 1802). However, the morphology of tadpoles and their oral structures have not been extensively studied. Our study provides the first larval description of *Hoplobatrachus litoralis* tadpole and compare with previously described tadpoles.

MATERIAL AND METHODS

Specimen collection: Tadpoles were collected from Kangchup Hills, Manipur (23°88'63''N and 93°80'64''E) using dip net (1m×1m). A total of 15 *Hoplobatrachus litoralis* tadpoles were caught from the paddy field in the month of June-July. Two tadpole specimens were kept in molecular grade alcohol at -40°C for molecular study, and the remaining specimen were preserved in 10% formalin for morphological study. A total of 14 parameters were measured (Table 1) using Mitutoyo digimatic calipers (CD-6''ASX) (accuracy ±0.01 mm).

Image capture: *Hoplobatrachus litoralis* tadpoles were photographed from dorsal, lateral, and ventral sides using a Nikon Z50 mirrorless camera equipped with a Nikkor ZDX 50–250 mm lens. The oral discs were captured on camera using a Stereozoom microscope that was connected to a Micron Optic Digital Camera. The labial tooth row formula (LTRF) and oral disc morphology were explained following Altig (1970) and Lateral Tooth Row Formula (LTRF) follows Dubois 1995. Terminology related to oral and exterior morphology is based on Altig and McDiarmid (1999).

SEM: Tadpole mouthparts was fixed in 3% glutaraldehyde, washed with 0.1M sodium cacodylate buffer, and dehydrated in increasing acetone grades before drying in tetra methyl silane. The specimens were mounted on brass stubs with adhesive tape and coated with gold

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using a JFC 1100 ion sputter to enhance conductivity and prevent radiation damage. Observations were conducted at 20 KV using a Jeol JSM 6360 Scanning Electron Microscope in secondary electron emission mode at SAIF, North Eastern Hill University, Shillong.

DNA extraction, sequencing and data analysis: The phenol-chloroform method was used to extract DNA from tadpole tail muscles that had been preserved in alcohol (Marmur 1961). The 16S rRNA gene was amplified using the forward primer AH-16S_S (5'–GGC CTG TTT ACC AAA AAC ATC GCC T–3') and the reverse primer AH-16S_R (5'–TGC GCT GTT ATC CCY RGG GTA ACT–3') following Grosjean et al. (2015). The PCR thermal cycling process included 30 seconds of initial denaturation at 95°C, 30 seconds of annealing at 50°C, 45 seconds of elongation at 72°C, and 3 minutes of final extension at 72°C. Using 0.8% agarose gel electrophoresis, the amplified DNA was examined under UV light. Sequencing in both directions was carried out using the same primers as in PCR, with an ABI3730xl automated sequencer from Applied Biosystems, USA, at Barcode Bioscience in Bangalore, India.

Sequence chromatograms were quality–checked, edited, and assembled into contigs using Sequence Scanner v1.0 (Applied Biosystems). Comparative 16S rRNA sequences for *Hoplobatrachus* species were retrieved from GenBank. Using MUSCLE (Edgar, 2004) in MEGA7 (Tamura and Nei 1993; Kumar et al. 2016) with default parameters, sequence alignment was carried out. The most effective partitioning strategy for the dataset was found using PartitionFinder v2 (Lanfear et al. 2017). A Maximum Likelihood (ML) phylogenetic tree was constructed in IQ-TREE (Nguyen et al. 2015) using an unpartitioned dataset, with the TIM2+F+G4 substitution model selected based on BIC scores from Model Finder (Kalyaanamoorthy et al. 2017). To assess clade support, ML analysis was conducted using 1,000 iterations of ultrafast bootstrap resampling (Minh et al. 2013). MEGA7 (Kumar et al. 2016) was used to compute the uncorrected pairwise p-distance after gaps and missing data were pairwise deleted.

RESULTS

Phylogenetic analysis: The Maximum Likelihood (ML) phylogenetic analysis based on the 16S rRNA gene robustly resolves *Hoplobatrachus litoralis* as a distinct clade with strong bootstrap support, clearly separating it from other congeners such as *H. tigerinus*, *H. crassus*, *H. chinensis*, and *H. occipitalis* (Fig. 1). The newly sequenced specimens YS150 and YS209 from Manipur, India cluster tightly within the H. litoralis clade, alongside sequences from Myanmar, Thailand, and Bangladesh, suggesting minimal intra-specific divergence with maximum uncorrected p -distance of 0.5% and confirming the conspecificity of these geographically separated populations (Table 2).

Larval description of tadpoles (Gosner Stage 36)

Tadpole morphology: The dorsal view of the tadpole shows an elliptical shaped body, body slightly depressed. The ventral side is translucent with four and a half circular coils of intestine, faintly visible through the body wall (Fig. 2). Total length (TL) and Snout Vent length (SVL) of tadpoles at Gosner Stage 35 are 42 mm and 14.04 mm respectively (Table 2). SVL is approximately 33% of the total length (SVL:TL =0.33±0.03). Body is widest in the middle, body width is 59% of the SVL (BW:SVL =0.59±0.02), 119% of the body height (BW:BH =1.19±0.10). Eyes are large and bulging (ED:SVL =0.13±0.01) positioned dorsally but directed dorso-laterally. Eye diameter is one-third of the interorbital distance (ED:IOD =0.33±0.33). The interorbital distance is 40% of the snout vent length, which indicates a relatively broad head (IOD:SVL =0.40±0.01). Nostrils are narrowly spaced, closer to snout tip than the eye (NS:EN =0.86±0.08), nares opening are small and rounded. Internarial distance is approximately one-fourth the length of interorbital distance (IND:IOD =0.25±0.02). Spiracle is single and sinistral, attached to the body wall. Vent tube medial and long, directed posteriorly linked to ventral tail fin. Tail is long which accounts for 72% of the total body length with moderate musculature (TAIL:TL =0.72±0.04), tail tip slightly rounded, dorsal fin originates at the tail junction where upper tail fin is slightly higher as compared to lower tail fin. Glands are absent.

Oral morphology: The oral disc is non-protruding, located antero-ventrally, and not emarginated. It is composed of anterior (upper) labium and posterior (lower) labium. It is semi-circular in ventral view, and much wider than it is long (ODL:ODW =0.23±0.05). Anterior labium is without marginal papillae; posterior labium with marginal papillae (only on one side) while sub-marginal papillae is absent. Upper jaw sheath black and broad; the lower jaw sheath is V-shaped and partially covered by the upper jaw sheath. Upper labial teeth are serrated and arranged in saw-toothed pattern .The lower labial teeth are arranged in a pattern resembling closely spaced needles. The oral apparatus possess a single upper labial tooth row with a distinct medial projection and four uninterrupted lower labial tooth rows (Fig. 3). LTRF 1/4.

Color in life: In life, tadpoles are light brown in color with dorsal side darker than ventral side.

Color in preservative: In preservative, body greyish brown in dorsal and ventral view. Tail fins are translucent creamy white in color with small brown dots. Hindlimbs translucent light brown, without pigmentation.

Habitat: *Hoplobatrachus litoralis* tadpoles occurred in shallow, stagnant waters of a muddy paddy field located near the foothills with muddy substratum and abundant aquatic vegetation.

DISCUSSION

Numerous studies have been carried out on *Hoplobatrachus* tadpoles (*H. occipitalis*: Lamotte and Zuber-Vogeli 1954; *H. tigerinus*: Annandale 1917; Annandale and Rao 1919; Boulenger and Annandale 1918; Khan 1969, 1982, 1996; Agarwal and Niazi 1980; Dutta and Mohanty Hejmadi 1984; Ray 1999; *H. chinensis*: Flower 1899; Smith 1917; Noble 1927; Pope 1931; Chou and Lin 1997). The labial tooth row formulas provided by these authors differ from what we observed in our present study.

We also compared the larval description of *H.litoralis* with *Hoplobatrachus* species collected from Vietnam (Grosjean et al. 2004) and Thailand (Chuaynkern et al. 2023). We found the descriptions

Table 1. Main morphometric parameters of tadpoles of *Hoplobatrachus litoralis*. Values are Mean±SD and expressed in mm.

Parameters	Mean±SD
TL(Total length, distance between snout tip to tail tip)	42.00±5.59
SVL(Snout Vent length, distance between snout tip to vent)	14.04±2.48
HLL(Hind limb length, from the vent to the tip of the longest toe)	13.69±5.66
TAIL(Tail length, from body and tail junction to tail tip)	30.32±4.33
BW(Body width, widest part of the body)	8.38±1.43
BH(Body height, maximum height of the body)	7.05±1.13
MTH(Maximum tail fin height, greatest distance between dorsal and ventral fin margin)	7.28±1.09
OSL(Oral disc length, from the anterior most portion to the posterior most margin)	0.37±0.09
OSW(Oral disc width, maximum distance between the two laterals of the oral disc)	1.66±0.43
EN(Eye to nostril, distance from the anterior corner of the eye to nostril)	3.40±0.85
NS(Nostril to snout, distance from the nostril to tip of snout)	2.95±0.87
INS(Internarial distance, distance between two nostrils)	1.39±0.20
IOS(Interorbital distance, distance between upper eyelids)	5.65±0.89
ED(Eye diameter, distance from posterior corner to anterior corner of eye)	1.90±0.42

Table 2. Estimates of evolutionary divergence based on uncorrected p-distance among species of *Hoplobatrachus* based on the 16S rRNA .

Species	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
YS209_Hoplobatrachus_litoralis																	
YS150_Hoplobatrachus_litoralis	0.000																
OQ535767.1_Hoplobatrachus_litoralis	0.006	0.006															
OQ535768.1_Hoplobatrachus_litoralis	0.006	0.006	0.000														
OQ535772.1_Hoplobatrachus_litoralis	0.006	0.006	0.000	0.000													
OQ535769.1_Hoplobatrachus_litoralis	0.006	0.006	0.000	0.000	0.000												
OQ535771.1_Hoplobatrachus_litoralis	0.006	0.006	0.000	0.000	0.000	0.000											
OQ535773.1_Hoplobatrachus_litoralis	0.006	0.006	0.000	0.000	0.000	0.000	0.000										
OQ535770.1_Hoplobatrachus_litoralis	0.006	0.006	0.000	0.000	0.000	0.000	0.000	0.000									
MW244089.1_Hoplobatrachus_litoralis	0.015	0.015	0.015	0.015	0.015	0.015	0.015	0.015	0.015								
MW244088.1_Hoplobatrachus_litoralis	0.015	0.015	0.015	0.015	0.015	0.015	0.015	0.015	0.015	0.000							
MW244087.1_Hoplobatrachus_litoralis	0.015	0.015	0.015	0.015	0.015	0.015	0.015	0.015	0.015	0.000	0.000						
MG935818.1_Hoplobatrachus_litoralis	0.021	0.021	0.021	0.021	0.021	0.021	0.021	0.021	0.021	0.006	0.006	0.006					
MG935820.1_Hoplobatrachus_litoralis	0.021	0.021	0.021	0.021	0.021	0.021	0.021	0.021	0.021	0.006	0.006	0.006	0.000				
OM501581.1_Hoplobatrachus_litoralis	0.021	0.021	0.021	0.021	0.021	0.021	0.021	0.021	0.021	0.021	0.021	0.021	0.028	0.028			
AB671175.1_Hoplobatrachus_litoralis	0.021	0.021	0.021	0.021	0.021	0.021	0.021	0.021	0.021	0.021	0.021	0.021	0.028	0.028	0.000		
AB671173.1_Hoplobatrachus_litoralis	0.021	0.021	0.021	0.021	0.021	0.021	0.021	0.021	0.021	0.021	0.021	0.021	0.028	0.028	0.000	0.000	
AB671174.1_Hoplobatrachus_litoralis	0.021	0.021	0.021	0.021	0.021	0.021	0.021	0.021	0.021	0.021	0.021	0.021	0.028	0.028	0.000	0.000	0.000

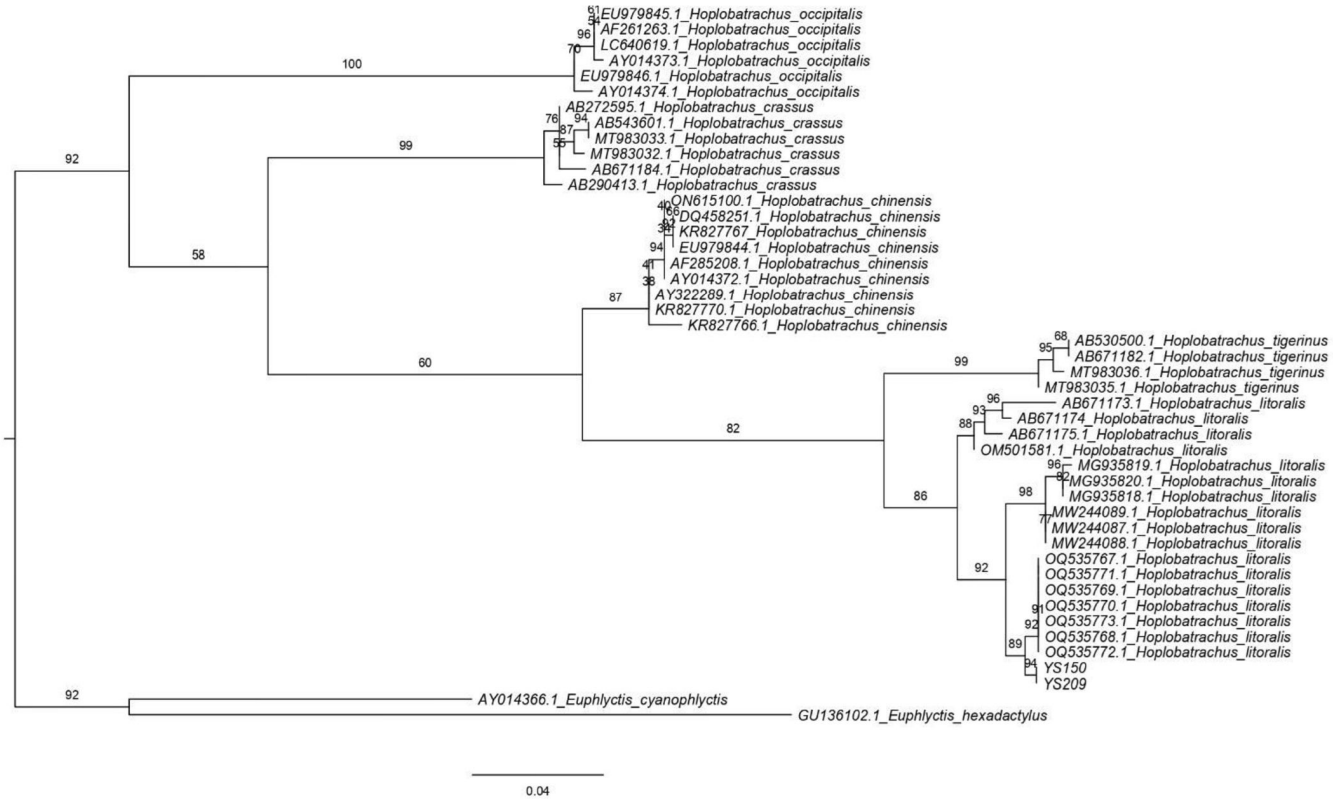


Figure 1. Maximum likelihood tree (16S rRNA) showing members of *Hoplobatrachus* used in the



Figure 2. Dorsal, lateral and ventral view of tadpoles of *Hoplobatrachus litoralis* (Gosner stage 36)

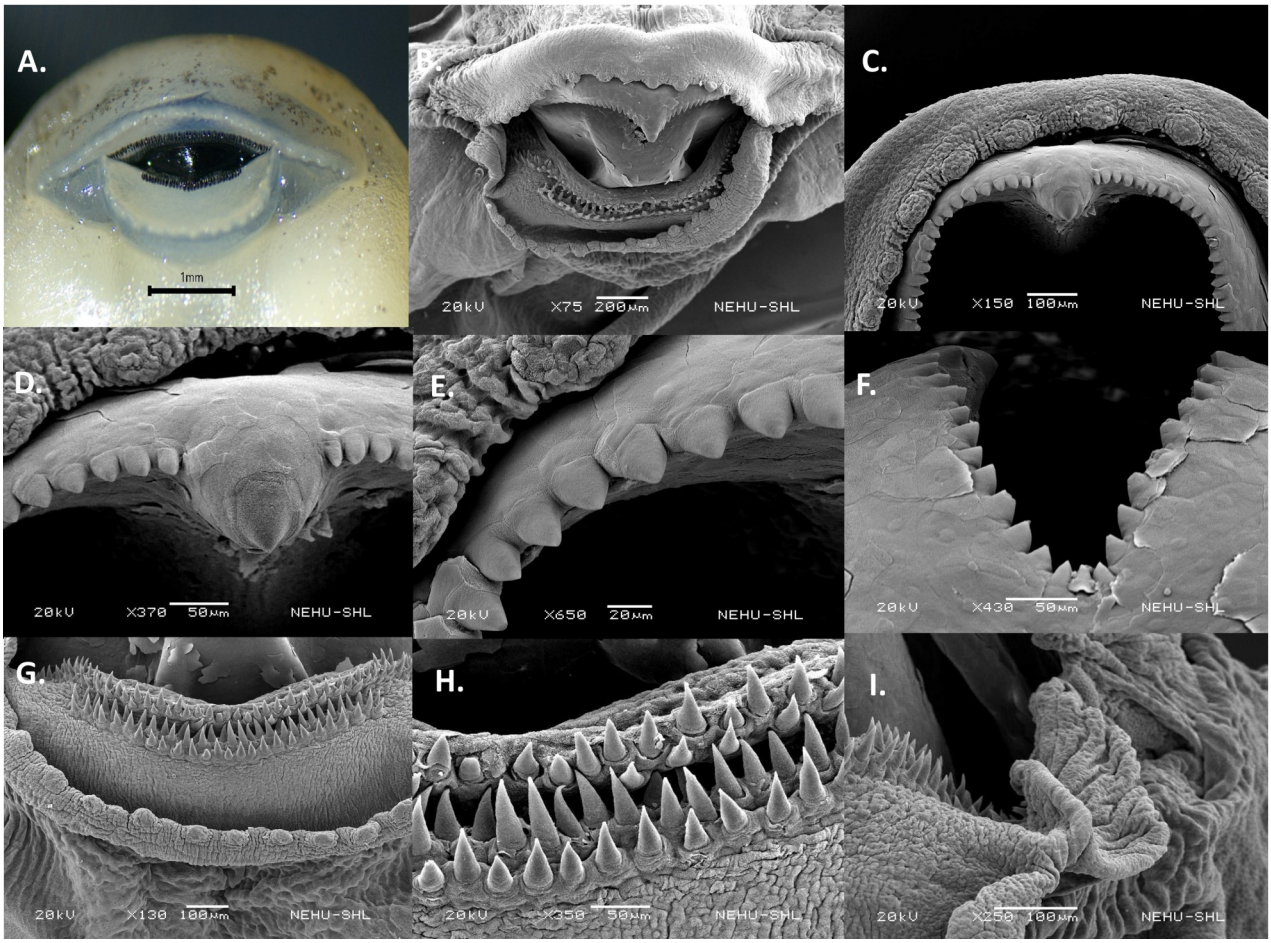


Figure 3. A) Oral disc taken under Stereo-zoom Microscope B)SEM micrograph showing the beak and jaw sheaths C) Upper labial teeth with strong medial projection D) Prominent Medial Projection E)Upper labial teeth (X650) F)V-shaped lower jaw sheath G)Lower labial teeth H)Needle like teeth arrangement in the lower labium I) Sub-marginal papillae

of LTRF different from *H.litoralis*:- *H. occipitalis*- 2:2+2/2+2:2 (Gosner stage 36), *H. chinensis* - 2:2+2/4+4:1 (Gosner Stage 36), *H. tigerinus*- 2:3+3/4+4:2 (Gosner Stage 36), H.salween- 2:2+2/3+3:2 (Gosner Stage 37). However, the oral structure of *H. litoralis* was found to be 1/4 in the current study.

We observed that *Hoplobatrachus litoralis* tadpoles show substantial morphological similarities to tadpoles of *H. tigerinus* and *H. salween* by having rounded snout, strong medial projection, moderate tail musculature and nares positioned closer to eye than snout. *H. litoralis* tadpoles differ from *H. chinensis* and *H. occipitalis* where medial projection is absent (Grosjean et al. 2004).

The needle-like labial teeth and their double-row arrangement appear to be unique traits among anuran tadpoles (Dubois 1992). As a result, they have not been included in comprehensive studies of tadpole morphology (Duellman and Trueb 1986; Altig and McDiarmid 1999) and have not been understood as such.

The three rows of straight, upright spikes on the lower labium of *Mantidactylus lugubris* (Altig and McDiarmid 2006) and the teeth of *Hoplobatrachus* (Altig et al. 2009) most likely share a similar structure. The most prominent character of *Hoplobatrachus litoralis* larvae we observed in the present study is the presence of strong medial projection in the upper labium and multiple rows of needle-like teeth arrangement in the lower labium.

The tadpoles of other *Hoplobatrachus* species, including *H. chinensis*, *H. tigerinus*, and *H. occipitalis*, have multiple rows of needle-like teeth arrangement (Grosjean et al. 2004). The labial teeth of the present specimens resembled those of other previously documented *Hoplobatrachus* tadpoles. The double row arrangement of needle-like labial teeth appears to be specific to anuran tadpoles (Dubois 1992). According to Grosjean et al. (2004), this unique characteristic is likely not similar to the multiserial rows in *Ascaphus truei* and family Discoglossidae, but rather homologous of *Hoplobatrachus*.

Finally, the presence of medial cornified protrusion in *H. litoralis* tadpole, a feature that is also seen in *H. tigerinus* (Grosjean et al. 2004) and *H. Salween* (Chuaynkern et al. 2023) is absent in *H. chinensis* and *H. occipitalis* (Grosjean et al. 2004). Although there is little information available, the tadpole of *H. crassus* appears to have the same characteristic beak as reported by Kirtisinghe (1957).

The molecular findings, in conjunction with distinct larval morphological traits such as a strong medial projection on the upper labium and the unique needle-like labial teeth arrangement, further affirm the identity and distribution of *H. litoralis* as a valid and widespread species across Northeast India and neighbouring countries.

This study provides the first detailed morphological traits of *Hoplobatrachus litoralis* tadpoles contributing to a better understanding of larval distinction within the genus *Hoplobatrachus*. The labial tooth row formula (LTRF) of *H. litoralis* (1/4) is notably diverge from those previously documented in congeners, including H. occipitalis, *H. chinensis*, *H. tigerinus*, and *H. Salween*. Additionally, unique oral features like strong medial projection in the upper labium and two rows of needle like labial teeth on the lower labium represent unique morphological characters among anuran larvae. The medial cornified protrusion, a character shared with *H. tigerinus* and *H. Salween* but not with *H. chinensis* and *H. occipitalis* and specialised labial teeth are the diagnostic features, which may be considered reliable morphological marker for species identification. Moreover, integration of these larval features with molecular evidence strengthens the recognition of *H. litoralis* as a valid species with a broader distribution across north-eastern India and adjacent Southeast Asian regions. Given the taxonomic complexity and overlapping adult morphology in *Hoplobatrachus*, larval features such as those documented here are invaluable for species identification and phylogenetic interference.

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Conflict of interest

The authors declare that there is no conflict of interest.

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Author contributions

S.S. contributed to the conceptualization, supervision, and methodology of the study. J.P. was responsible for software and formal analysis. Y. S. D. handled visualization and wrote the original draft of the manuscript. All authors have read and approved the final version of the manuscript. All authors are responsible for the review and editing of the manuscript.

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