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NEW SITE RECORDS FOR TWO DICROGLOSSID FROG SPECIES BASED ON THEIR TADPOLES FROM MANIPUR, INDIA

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Yumkham S. Devi[1], Jayaditya Purkayastha[2] & Saibal Sengupta[1]*

[1] *Department of Zoology, Assam Don Bosco University, Tapesia Gardens, Sonapur, Assam 782402, India*
[2] *Help Earth, 16, Raghunath Choudhury Path, Lachitnagar, Guwahati, Assam 781007, India*
**Corresponding author.Email: saibal.sengupta@dbuniversity.ac.in*

Abstract
We document the occurrence of two species of the family Dicroglossidae from Kangchup Hills, Manipur, India, *Fejervarya limnocharis* and *Minervarya sengupti*. *Fejervarya limnocharis* is a widely distributed Asian dicroglossid frog with a complex taxonomy. This is the first time it has been reported in India, expanding its range significantly. The presence of *Minervarya sengupti* in Manipur also expands its distribution. We compare the morphology of their tadpoles, particularly focusing on their oral apparatus, which was examined using Scanning Electron Microscopy. We also report the results of a genetic analysis of the two species using 16S rRNA. Our findings underscore the importance of detailed morphological studies in clarifying species diversity and distribution within the family Dicroglossidae.

***Keywords*:** *Fejervarya limnocharis*, *Minervaya sengupti*, tadpole taxonomy, oral morphology

Introduction

Frogs of the genus *Fejervarya* (Bolkay, 1915), commonly referred to as rice frogs, have a complex taxonomy and geographical distribution. The genus is widely distributed across South and Southeast Asia (Frost 2024), inhabiting a variety of ecosystems ranging from lowland rice paddies to forests. Taxonomic classification within *Fejervarya* is particularly challenging, given the morphological similarities among species and the high degree of cryptic diversity revealed through molecular studies (Kotaki *et al*. 2010). India is home to five recorded species of *Fejervarya*: *F. moodiei* (Taylor, 1920); *F. orissaensis* (Dutta, 1997); *F. jhilmilensis* Bahuguna, 2018; *F. multistriata* (Hallowell, 1861); and *F. limnocharis* (Gravenhorst, 1829). Previous reports of *F. cancrivora* (Gravenhorst, 1829) from Bangladesh and India have been attributed to *F. moodiei* (Hasan *et al*. 2012). This species is distributed along the coastal regions of Odisha, West Bengal (Deuti *et al*. 2016, Gayen & Deuti 2021), and Pondicherry (Satheeshkumar 2011) in India. *F. orissaensis* is known to inhabit central, northern, and eastern India (Deuti *et al*. 2014, Das *et al*. 2020, Srinivasalu & Kumar 2022, Prasad *et al*. 2022) and in northeast India (personal

observation). *Fejervarya multistriata*, closely related to *F. limnocharis*, has been documented in Mizoram and Manipur (Lalbiakzuala & Lalremsanga 2019, Decemson *et al.* 2021). However, Kotaki *et al*. (2010) and Huang & Tu (2016) have suggested *F. multistriata* may be conspecific with *F. limnocharis*, and Chandramouli *et al*. (2020) advocated extensive sampling to confirm its taxonomic status. In India, *F. jhilmilensis* has been reported exclusively from its type locality in Haridwar, while *F. limnocharis* is reported from Nicobar Island (Chandramouli *et al*. 2020).

The systematic complexity within the genus *Fejervarya* arises from several factors, including their phenotypic characters, widespread distribution, and incomplete understanding of their lineage. Many species within this genus exhibit similar external traits, making distinguishing them based solely on physical characteristics difficult. For instance, their calls, which are crucial for species recognition, show subtle differences that are not always apparent without detailed acoustic analysis (Purkayastha & Matsui 2012). The phylogenetic relationships within *Fejervarya* have been subjected to extensive debate. Early taxonomic work relied heavily on morphological traits, but recent molecular studies have provided deeper insights into the genetic divergence and evolutionary history of this group. Phylogenetic analyses using mitochondrial and nuclear DNA sequences have identified distinct clades within *Fejervarya* and this genetic evidence has necessitated the re-evaluation of species boundaries discovering several cryptic species (Kotaki *et al*. 2010, Sanchez *et al*. 2018).

One taxonomic revision within *Fejervarya* was proposed by Sanchez *et al*. (2018), who advocated for the recognition of the genus *Minervarya* (Dubois *et al*. 2001). This revision was based on robust phylogenetic evidence that supported the monophyly of two separate clades: one centered in Asia and the other in Southeast Asia. The reclassification was driven by several taxonomic criteria, including the support for monophyly, biogeographic distribution, and morphological distinctiveness. *Minervarya* was established to accommodate species primarily distributed in South Asia, whereas *Fejervarya* was restricted to species from Southeast Asia. This taxonomic revision has helped clarify the evolutionary relationships within the group and provided a more coherent framework for studying their biodiversity (Sanchez *et al*. 2018).

In this study, we report new records of two amphibian species of the genera *Fejervarya* and *Minervarya* from the state of Manipur. Herein we also cast doubt on the presence of *F. multistriata* (Hallowell, 1861) in Northeast India as recorded by Decemson *et al*. (2021a) and (2021b) in Mizoram and Manipur, respectively.

Material and Methods

Tadpoles were collected from Kangchup Hills, Manipur (23°88′63″N, 93°80′64″E) and segregated into two groups. The first group was preserved in 10% formalin for morphological study; the second group was kept in molecular grade alcohol at -40◦C for molecular study. The first group consisted of a total of eleven tadpoles, out of which seven were identified as *F. limnocharis* (Gosner stage 36) and four as *M. sengupti* (Gosner stage 30). To assess the morphological differences among the collected specimens, a total of 13 parameters were measured using Mitutoyo digimatic calipers (CD-6”ASX) (accuracy ±0.01 mm).

Image capture. Dorsal, lateral, and ventral views of the tadpoles of *F. limnocharis* were photographed with Stereozoom Microscope attached to a Micron Optic Digital camera. Dorsal, lateral, and ventral views of the tadpoles of *M. sengupti* were photographed with Nikon Z50 mirrorless camera fitted with Nikkor ZDX 50–250 mm. The oral discs of the specimens spread with a needle were photographed with a Stereozoom Microscope attached to a Micron Optic Digital Camera. The oral disc morphology and labial tooth row formula (LTRF) were described following Altig (1970). Terminology concerned with external and oral morphology follows Altig & McDiarmid (1999).

DNA analysis. The tail muscles of tadpoles preserved in alcohol were used for DNA extraction using the phenol-chloroform method, as detailed by Shangpliang *et al.* (2024). The amplification of the 16S rRNA gene was accomplished through the utilization of 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’). The PCR thermal cycling conditions included an initial denaturation at 95C° for 30 seconds, 50C° for 30 seconds, 72C° for 45 seconds, and a final extension at 72C° for 3 minutes. The resulting amplified DNA was subjected to electrophoresis on a 0.8% agarose gel and subsequently visualized under UV light. Sequencing in both directions was performed

using the same primers employed in PCR, utilizing 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 comprising members of *Fejervarya and Mineryarya* were obtained from GenBank. Sequence alignment using MUSCLE (Edgar 2004) in MEGA7 (Tamura & Nei 1993, Kumar *et al*. 2016) with default parameter settings. The best partitioning schemes for the dataset were searched through PartitionFinder v2 (Lanfear *et al*. 2017). A Maximum Likelihood (ML) phylogenetic tree was reconstructed using an unpartitioned dataset in IQ-TREE (Nguyen *et al*. 2015) with the substitution model TIM2+F+G4 selected based on the BIC scores by Model Finder (Kalyaanamoorthy *et al*. 2017). The ML analysis was run with an ultrafast bootstrap option (Minh *et al*. 2013) for 1,000 iterations to assess clade support. The Uncorrected pairwise *p*-distance was calculated in MEGA7 (Kumar *et al*. 2016) with pairwise deletions of missing data and gaps.

Results

The specimens from this study represent two distinct species. Specimens ADBUA0097, 0098, and 0099 from Manipur, India, bearing Genbank accession numbers of PQ489332, PQ489333, and PQ489334, respectively, were nested within *F. limnocharis* + *F. multistriata* and differed by an uncorrected *p*-distance of 0.002–0.007 from both *F. limnocharis* and *F. multistriata*. The specimen with the highest divergence was found to be the specimen of *F. multistriata* (MW687118) from Manipur with an uncorrected *p*-distance of 0.017 used in the study by Decemson *et al*. (2021b) (Fig. 1, Sup. Table 1

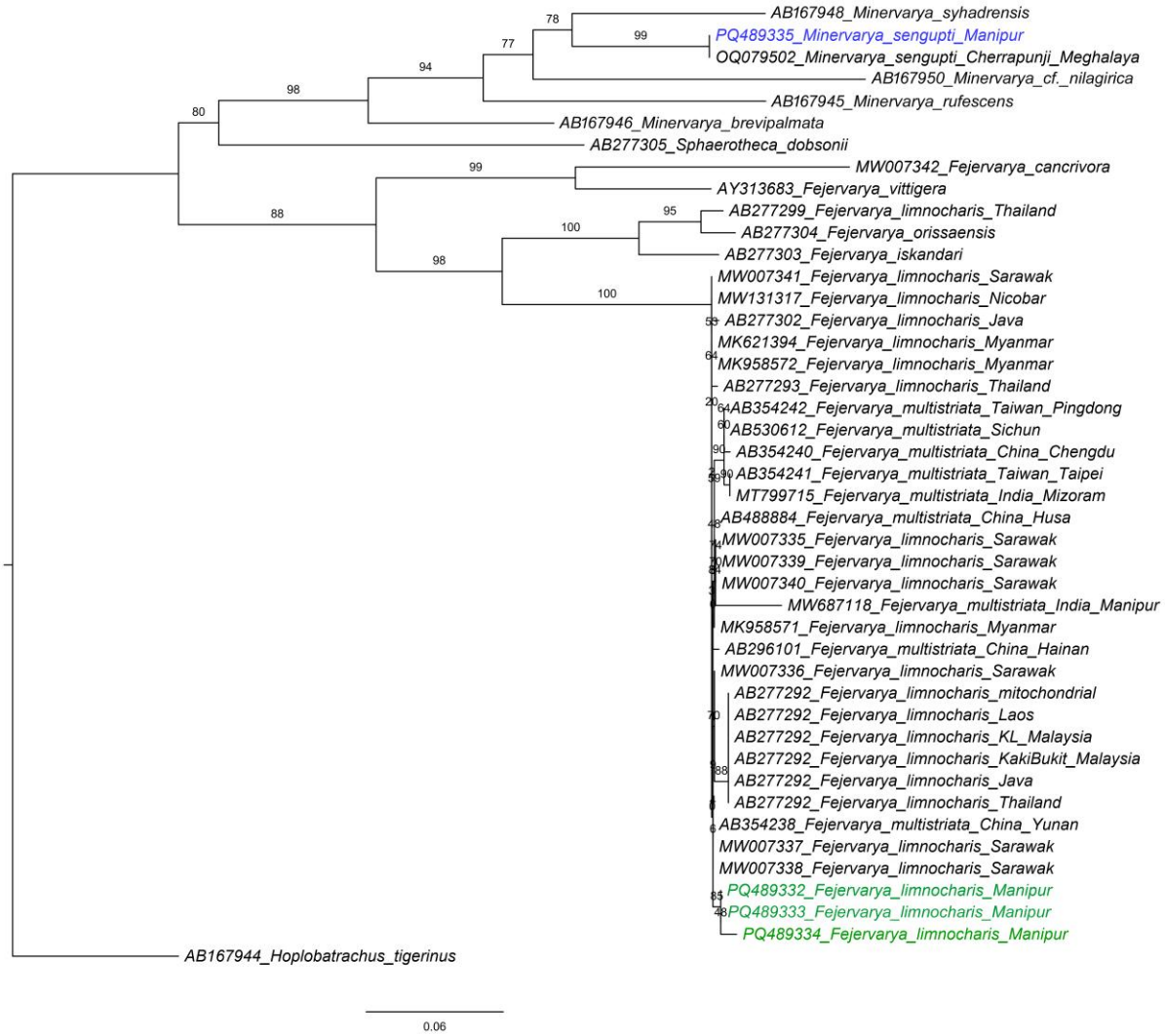

Figure 1. Maximum Likelihood tree (16s rRNA) showing members of *Fejervarya* and *Minervarya* species used in this study; the number preceding the species name is the GenBank accession number

The specimen ADBUA0100 (PQ489335) formed a sister clade to *M. sengupti* from Cherrapunji, Meghalaya (~20 km south of the type locality at Mawphlang, Meghalaya). The specimen from Manipur differed from that of Cherrapunji by an uncorrected *p*-distance of 0.0 (Sup. Table 2). Based on the above assessment, we hereby report two new records of amphibians from Manipur, *M. sengupti*, and *F. limnocharis*.

Fejervarya limnocharis
(Figs. 2, 3; Sup. Table 1)

Tadpole morphology. The dorsal view of the tadpole shows an oval-shaped body widening in the center. The ventral side is translucent with four and a half circular intestinal coils faintly visible through the body wall in some tadpoles. Tadpoles are small-sized, snout blunt but rounded in both dorsal and ventral views. SVL is approximately 40% of the total length (SVL:TL = 0.40±0.02) and it is maintained from Gosner stage 26 to 40. Body is the widest in the middle and the body width is 57% of the SVL (BW:SVL = 0.57±0.07), 136% of the body height (BW:BH = 1.36±0.13). Eyes are large and bulging (ED:SVL = 0.13±0.01) positioned and directed dorso-laterally Eye diameter is more than one-third of the interorbital distance (ED:IOD = 0.37±0.03). The nostril opening is oblong and positioned dorsolaterally; nostrils are closer to the eye than the snout tip (NS:EN = 1.04±0.14). Nostrils are moderately spaced and the internarial distance is equal to interorbital distance (IND:IOD = 0.50±0.12) but gets wider in the Gosner stage 32 to 40. Spiracle opening is oval and sinistral. Vent tube is medial, long, positioned supra-marginally, and not attached to the ventral fin. Tail is long (TAL:TL = 0.62±0.02), tip is attenuated and there is strong tail musculature. The upper part of the tail fin begins where the tail meets the body, the lower fin starts at the ventral terminal end of the body. The upper tail fin margin is slightly convex, the lower tail fin is almost obliterated and straight. The dorsal tail fin is speckled with small light and dark markings, the ventral tail fin is uniformly coloured without contrasting markings. Glands on the body surface are absent.

Oral morphology. The oral disc is non-protruding, located anteroventrally, and emarginated. It is composed of anterior (upper) labium and posterior (lower) labium. The oral disc is much wider than long (ODL: ODW = 0.34±0.03). The keratodonts (keratinized labial denticles) are arranged in a single row per ridge. The upper lip is slightly longer than the lower lip. Each jaw sheath had serrated edges along its entire length. The marginal papillae exhibit a large dorsal gap at the upper part of the labium and a modest ventral gap at the lower labium. The marginal and submarginal papillae have rounded tips. The opened mouth cavity measured 1.32±0.4 mm and 0.58±0.24 mm in both vertical and horizontal dimensions. The beak is thin; its anterior half is narrowly arch-shaped. The Upper jaw sheath is thick, broad, readily apparent, and entirely black. The lower jaw sheath is V-shaped and serrated. A magnified view of V-shaped lower jaw sheath is pointed. LTRF 2(2)/3. Order of length of denticle row is A2>A1>P1>P2>P3.

Colouration. In life, tadpoles are black, wholly pigmented but slightly translucent at the ventral side. The tail tip is dark.

Habitat. Tadpoles occurred in slow-flowing streams with a maximum depth of approximately 60 cm in a rocky and sandy substratum covered mostly with leaf litter. These tadpoles are faintly visible in the stream as they blend with the colours of the rock and leaf litter (Fig. 6).

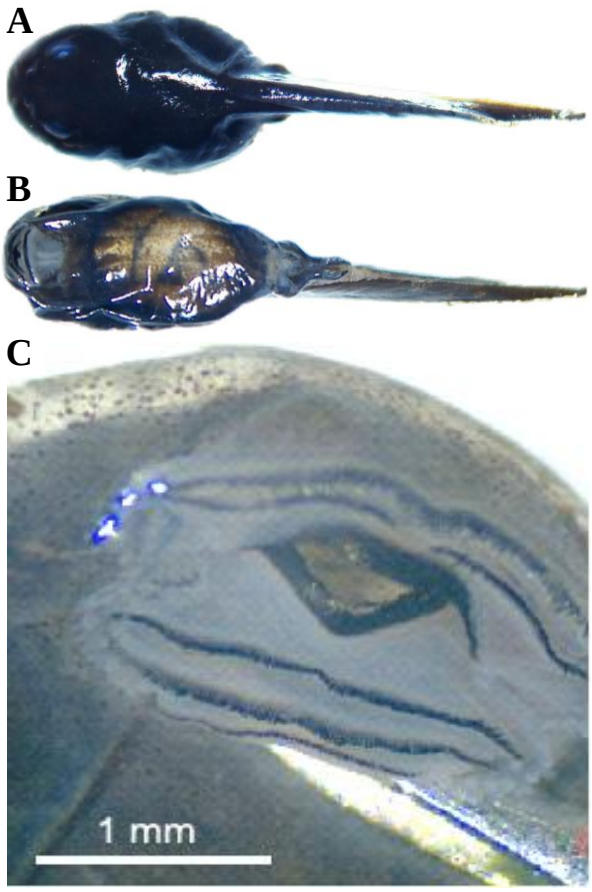

Figure 2. *Fejervarya limnocharis* tadpole (Gosner stage 36): **(A)** dorsal and **(B)** Ventral view of the body; **(C)** oral disc

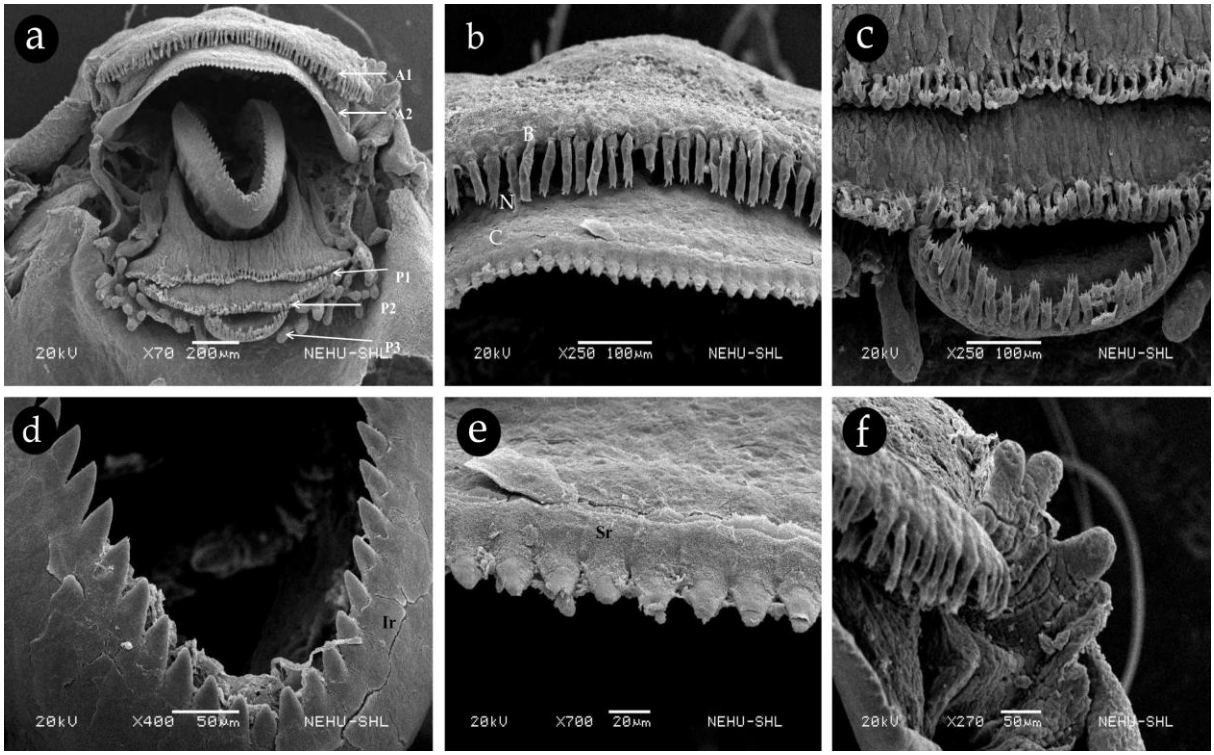

Figure 3. SEM micrograph of the oral disc of *Fejervarya limnocharis* tadpole showing the keratinized beak and labial tooth rows: **(a)** A1, A2 = 1st, 2nd anterior tooth rows; P1–P3 = 1st–3rd posterior tooth rows; **(b)** upper labial teeth with B = base, N = neck & C = cusp; **(c)** lower labial teeth; **(d)** serration of the infrarostrodont; **(e)** serration of the suprarostrodont; **(f)** marginal papillae

Minervarya sengupti
(Figs. 4, 5; Sup. Table 1)

***Tadpole morphology*.** Tadpoles are medium sized with a depressed body and rounded snout; body elliptical and globular in dorsal view. SVL 40 % of the total body length (SVL:TL = 0.04±0.02). Body width 52% of the SVL (BW:SVL = 0.52±0.01), and 114% of the body height (BW:BH = 1.14±0.08). Large eyes (ED:SVL = 0.12±0.01), one-third of the interorbital distance (ED:IOD = 0.33±0.02), placed dorsally. Nostrils slightly elevated and rounded, closer to the snout tip than the eye (NS:EN = 0.62±0.06). Internarial distance is less than one-third of the interorbital distance (IND: IOD = 0.29±0.03); internarial distance and interorbital distance 10% (IND:SVL = 0.10±0.005) and 35% (IOD:SVL = 0.35±0.02) of the SVL, respectively. Spiracle is sinistral, short, and is attached to the body wall along its entire length, located below the medial line of the lateral side. The vent tube is medial. Tail length is 64% of the total length (TAL:TL = 0.64±0.05), tail fin is moderately high (MTH:TAL = 0.27±0.06). The upper part of the tail fin begins where the tail meets the body, the lower fin starts at the ventral end of the body. Glands are absent on the outer surface of the body.

Oral morphology. The mouth is anteroventrally located. The oral apparatus is non-protruding and the oral disc is emarginated, and elongated horizontally. The oral disc is wider than long (ODL: ODW = 0.67±0.08). The upper jaw sheath and the lower jaw sheath are of intermediate length where the upper lip is projected over the lower lip. The marginal papillae are thick, with rounded tips, interrupted broadly on both upper and lower labia medially; a single row of marginal papillae on lateral commissures is present. The moderately keratinized jaw sheaths are serrated, the upper jaw sheath is wide and arc-shaped while the lower jaw sheath is V-shaped with an arched outline. The mode of arrangement of keratodonts is uniserial. LTRF 2(2)/3. Order of length of denticle row is A1>A2>P1>P2>P3.

Coloration. In life, greyish-brown hue, with a translucent ventral abdomen revealing coils of the gut. The fins are transparent, marked with several moderate-size spots and a few blotches.

Habitat. *Minervarya sengupti* occurred amongst aquatic vegetation in shallow standing water bodies (Fig. 6) with muddy substratum near rivers, for instance, tire tracks created during rainfall.

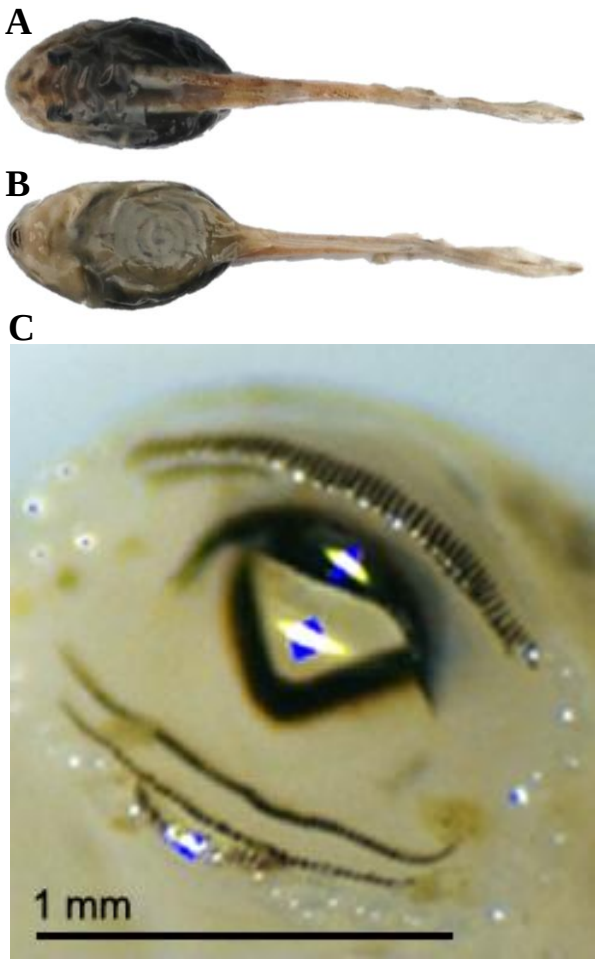

Figure 4. *Minervarya sengupti* tadpole (Gosner stage 30): **(A)** dorsal and **(B)** Ventral view of the body; **(C)** oral disc

Discussion

The *Fejervarya limnocharis* from Manipur is well nested within the *F. limnocharis* clade from different localities used in the study including the one from Java, the type locality. The sample from Mizoram differs from all the other *F. limnocharis* we examine by an uncorrected *p*-distance of 0.002–0.007 which also includes *F. multistriata* from China, Taiwan, and Thailand. The specimens from Mizoram (MT799715) and Manipur (MW687188), which were recorded as *F. multistriata*, differ from our specimen from Manipur by an uncorrected *p*-distance of 0.007 and 0.017, respectively. Only one specimen labelled as *F. limnocharis* from Thailand differs by a *p*-distance of 0.121, which should constitute a different species. The genetic divergence (*p*-distance) among *F. limnocharis* specimens from Manipur ranges from 0 to 0.004, with a predominant value of 0.002 when compared to populations from Myanmar, Thailand, Malaysia, and China. This suggests that the population in southeast Asia forms a continuous range with the Indian population. This minimal genetic variability also suggests that the genetic makeup of *F. limnocharis* remains relatively consistent and exhibits limited influence from geographic separation across its distribution range. The specimen of *M. sengupti* from Manipur was seen to be conspecific with *M. sengupti* from Cherrapunji, Meghalaya (~20 Km south of the type locality at Mawphlang, Meghalaya) not differing by any genetic distance (uncorrected *p*-distance = 0).

The morphology of the tadpole of *F. limnocharis* matches the description for the tadpole of this species from Singapore (Leong & Chou 1999), peninsular Malaysia (Leong 2005) and Sarawak (Malaysia; Min 2007). However, in the present study, the nostril is found to be placed almost equidistant from the eye and snout tip, a little closer to the eye in contrast to the findings of earlier workers (Leong & Chou 1999, Leong 2005, Min 2007), who observed the nostril closer to the snout tip than the eye. Leong & Chou (1999) and Leong (2005) recorded the vent tube as dextral, whereas we observed the vent tube as medial, which conforms with the findings of Min 2007. The tadpoles of Manipur morphology differ significantly from the *F. limnocharis* tadpoles from Nicobar described by Chandramouli *et al*. (2020) in the following parameters SVL:BW = 1.78 (vs 2.4), BW: BH = 1.38 (vs 1.07), ED:SL = 0.32 (vs 0.66), IOD:IND = 2.09 (vs 1.42), vent tube-medial (vs. dextral) LTRF = 2(2)/3 (vs 3(2)/1(1).

The present study suggests further in-depth study is required of the so-called *F. limnocharis* tadpoles of Nicobar. We also compared the description of *F. multistriata* tadpoles recorded from Mizoram, India (Siammawii *et al*. 2022) with the descriptions from Nicobar. We found both descriptions are more or less similar except for a minor difference in LTRF [2(2)/3 vs 2(2)/3(1)]. Khan & Mufti (1994) opined that the oral discs of tadpoles are relatively similar across different genera. The LTRF of *F. limnocharis* appeared to be 2(2)/3 as reported in the present study and by earlier workers (Leong & Chou 1999, Leong 2005, Inthara *et al*. 2005, Min 2007). The LTRF within the same species may vary a little as was reported from *F. kawamurai* [2(2)/3 or 2(2)/3(1)] by Djong *et al*. (2011).

The present study found that *M. sengupti* had twice the ODL:ODW ratio of *F. limnocharis*. Compared to *M. sengupti*, *F. limnocharis* has a more pointed infrarostrodont, although both species have a wide suprarostrodont.

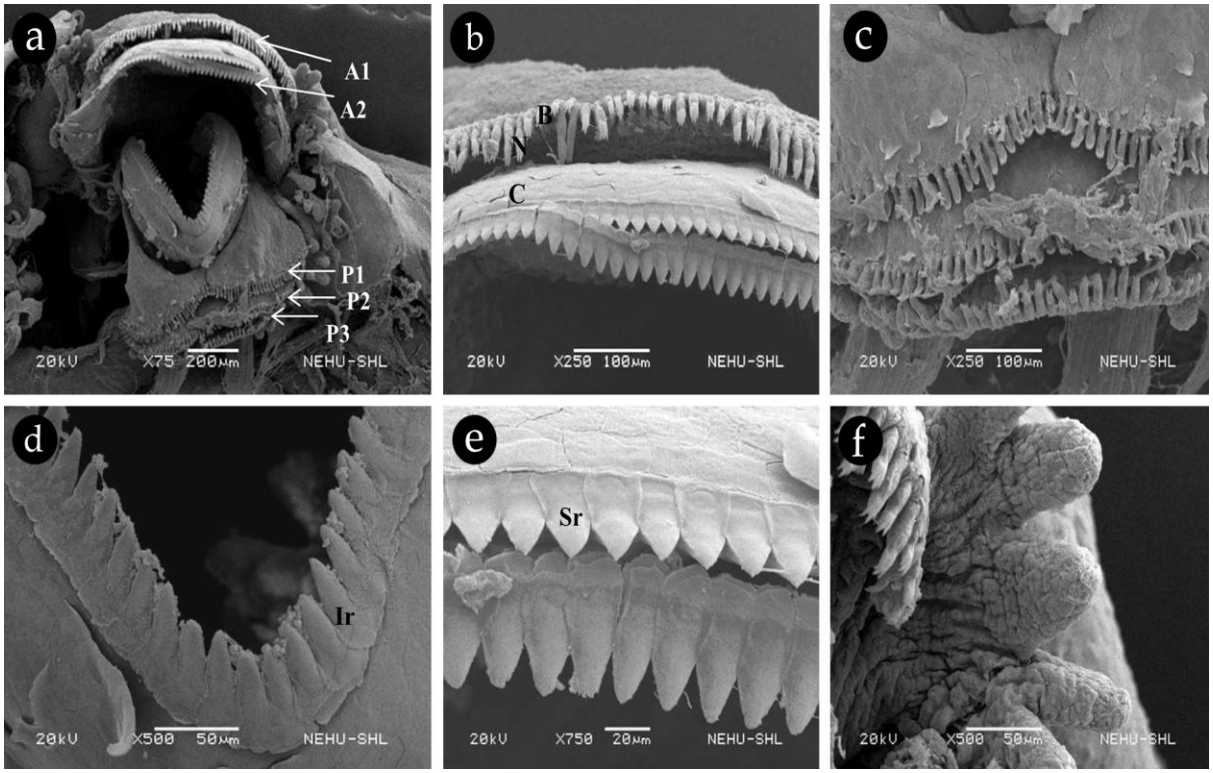

Figure 5. SEM micrograph of the oral disc of *Minervarya sengupti* tadpole showing the keratinized beak and labial tooth rows: **(a)** A1, A2 = 1st, 2nd anterior tooth rows; P1–P3=1st–3rd posterior tooth rows; **(b)** upper labial teeth with B = base, N = neck & C = cusp; **(c)** lower labial teeth; **(d)** serration of the infrarostrodont; **(e)** serration of the suprarostrodont; **(f)** marginal papillae

Both species have keratinized and well-developed jaw sheaths. According to Raj *et al*. (2023), *M.* cf. *agricola* has jaw sheaths that are entirely serrated with tiny, uniformly sized serrations, a U-shaped infrarostrodont with a concave middle, and a broad, convex suprarostrodont with an arched outline. *Minervarya sengupti* is described by Purkayastha & Matsui (2012) from Mawphlang (Meghalaya, northeastern India) and later reported in Shillong by Saikia *et al*. 2021. Raj *et al*. (2023) described the tadpoles of *M. sengupti* collected from the locality from where it was reported to occur by Purkayastha & Matsui (2012). Our specimens of *M. sengupti* differ from those from Meghalaya in the position of the nostril. We observed that nostrils were closer to the snout vs nostrils closer to the eye and closer to each other in relation to inter-orbital distance compared to the Meghalaya population (Raj *et al*. 2023). Otherwise, the morphological parameters of the Manipur population are similar to the Meghalaya population. The present study genetically confirmed the presence of *F. limnocharis* in mainland India. One of the sequences (*AB277302*) is from Java, Indonesia, the type locality of *F. limnocharis*. All the other *F. limnocharis* used in the tree along with *F. multistriata* from Taipei, Taiwan; Sichuan, Chengdu, China and Mizoram, India seen to form a single cluster including topotypic material indicating that all these species are conspecific and should be treated as *F. limnocharis*. Hallowell (1861) described *Rana multistriata* based on two specimens but did not mention where the types were stored. Later Peters (1863) without any clarification noted a single specimen as a Holotype (ZMB 3255, see Frost 2024). Wiegmann (1834) described the species as very closely related to *F. limnocharis* and can only be distinguished from it by the presence of a blunt longitudinal keel on the forehead (between the eye sockets).

Kotaki *et al*. (2010) proposed that *F. multistriata* could potentially be conspecific with *F. limnocharis*, based on molecular phylogenetic analyses. This hypothesis is supported by significant genetic overlap between the populations of these two nominal species (Fig. 1; Sup. Table 1) and by morphological similarities between the tadpoles of these two species. Since we do not have genetic data for *F. multistriata* from its type locality in Hong Kong, China (see Hallowell, 1861) it will be difficult to conclude

the validity of the nomen but we propose that the specimens from Northeast India reported as *F. multistriata* by earlier studies (Siammawii *et al*. 2022) should be treated as *F. limnocharis* henceforth.

Figure 6. The stream, from which tadpoles of *Fejervarya limnocharis* and *Minervarya sengupti* were collected, Kangchup Hills, Manipur, India

The findings of this study contribute significantly to the understanding of amphibian diversity in Northeastern India, particularly concerning the genera *Fejervarya* and *Minervarya*. This region, characterized by its complex topography and diverse habitats, serves as a crucial confluence zone for these genera, supporting a rich assemblage of species. The documentation of *F. limnocharis* and *M. sengupti* in Manipur adds valuable data to the biogeographical distribution of these frogs and highlights the taxonomic complexities within these groups. The presence of *F. limnocharis* and *M. sengupti* in Manipur underscores the role of Northeastern India as a biodiversity hotspot. This region, which is situated at the junction of the Indian and Indo-Chinese biogeographic zones is characterized by complex topography and diverse habitats, providing unique ecological niches that support a rich amphibian biodiversity. Our findings point to a need for a detailed study of the *Fejervarya* and *Minervarya* species complex of northeast India.

Author contributions

All the authors contributed equally.

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Research permits

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Supplemental data

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