Morphology, natural history and molecular identification of tadpoles of three endemic frog species of *Nectibatrachus* Boulenger, 1882 (Anura: Nectibatrachidae) from Central Western Ghats, India

Hebbar Priti, K.V. Gururaja and G. Ravikanth

*Ashoka Trust for Research in Ecology and the Environment (ATREE), Suri Sehgal Centre for Biodiversity and Conservation, Bengaluru, India; Manipal University, Manipal, India; Gubbi Labs LLP, R & D Center, WS-5, Entrepreneurship Center, SID, Indian Institute of Science Campus, Bengaluru, India

(Received 4 January 2015; accepted 23 March 2015; first published online 5 May 2015)

Western Ghats of India is known for its high amphibian diversity, but very little is known about their tadpoles. Here, for the first time, we describe tadpoles of three species of an endemic genus, *Nectibatrachus*, namely *N. kempholeyensis*, *N. jog* and *N. kumbara* using morphology and molecular techniques. Tadpoles were found in the streams and Myristica swamps of evergreen forests of central Western Ghats. They have a robust body, complete marginal papillae and lack keratodonts. The morphology indicates that these tadpoles are adapted to lotic habitats. In recent years, many of the streams these tadpoles inhabit have been diverted for agriculture and areca plantations. The descriptions of these tadpoles bear relevance for their conservation and can help in understanding amphibian larval ecology.

Keywords: amphibians; night frog; tadpole description; psammonic; Western Ghats

Introduction

The Western Ghats of India, along with Sri Lanka, is a global biodiversity hotspot (Myers et al. 2000) and has a high diversity of amphibians (Aravind and Gururaja 2012). In the last two decades, over 100 new species of frogs have been described from this region (Frost 2014). However descriptions of their larval counterparts are few. Tadpoles are an important stage in the amphibian life history and detailed studies of tadpoles can be useful in understanding phylogeny (Haas 2003), natural history (Thomas et al. 2005) and evolution (Roelants et al. 2011).

*Nectibatrachidae* represents one of the ancient frog families (Late Cretaceous in origin) in the Western Ghats with the genus *Nectibatrachus* endemic to the Western Ghats and *Lankanectes* endemic to Sri Lanka (Boeslauer et al. 2012). The genus *Nectibatrachus* was discovered by Boulenger in 1882 and recently Biju et al. (2011) revised its taxonomy. At present there are 28 species in *Nectibatrachus* (Gururaja et al. 2014). They are predominantly stream dwelling and found in torrent streams or in the leaf litter on the forest floor. The adults are brownish dorsally and are distinguished by rhomboid pupil, glandular wrinkled skin, notched tongue, subocular gland and pointed vomerine teeth (Biju et al. 2011).

*Corresponding author. Email: priti.gururaj@atree.org*
Tadpoles of the *Nyctibatrachus* genus are poorly studied with morphological descriptions available only for four species: *N. pygmaeus* (Annandale 1918, 1919); *N. sanctipalustris* (Rao 1923); *N. humayuni* (Bhaduri and Kripalani 1955); and *N. major* (Pillai 1978). With the recent taxonomic revision of the *Nyctibatrachus* genus and due to lack of molecular support, identities of these four species require validation. Molecular techniques using gene markers like 16S rRNA have been used for tadpole identification (Grosjean et al. 2005). In this paper we provide, for the first time, descriptions of tadpoles of three species of *Nyctibatrachus* (*N. kempholeyensis*, *N. jog* and *N. kumbara*) from the central Western Ghats using morphology and molecular techniques. We discuss the taxonomic gaps in the previous studies of *Nyctibatrachus* tadpoles and the natural history of the three species, and highlight the use of the 16S rRNA gene marker in tadpole identification from biodiversity rich regions like the Western Ghats.

**Material and methods**

**Study area**

The study was conducted in streams and *Myristica* swamps of evergreen forests in Sharavathi and Aghanashini river basins (14.2330°N, 74.6355°E to 14.4130°N, 74.8141°E). These two rivers are the major west-flowing rivers of the central Western Ghats and form a part of Aghanashini Lion Tailed Macaque Conservation Reserve. The *Myristica* swamps (lowland swampy forests) are unique ecosystems and are relict forests of Late Cretaceous origin (Doyle et al. 2004; Chandran et al. 2010) (Figure 1). They harbour many endemic flora and fauna (Kumara et al. 2008) and are highly threatened ecosystems. This region has a tropical climate and receives rainfall (3000–5000 mm) from south-west monsoons (Chandran et al. 2010). The mean temperature range is 18–29°C and the altitude is up to 600 m.

Figure 1. (a) Sampling localities; (b) *Myristica* swamp forest, India.
Sampling
The sampling was carried out during June–September (monsoon season) and during January–April (post-monsoon season) in 2012–2013. Figure 1(a) provides sampling localities. Locations reference are Kathalekan: 74.74679°E, 14.27401°N; Baregudda: 74.75233°E, 14.27073°N; Banglikoppa: 74.81414°E, 14.2391°N; Baginapalajaddi: 74.73962°E, 14.41291°N; Hejanifalls: 74.75845°E, 14.27325°N; Mahime: 74.63551°E, 14.30256°N. The streams were sampled every 300 m from upstream to downstream. In the upstream areas, the water from the streams was diverted to areca plantations and paddy fields. The downstream area comprised evergreen forest and Myristica swamps. Tadpoles were collected with an aquarium dipnet (15 cm by 15 cm). They were euthanized with chlorobutanol solution and sorted into different series based on stage, body size and relative tail pattern and tail length. The tadpoles were photographed and then tail tips of about 10–15 mm were excised from two individuals from each species. The tadpoles were preserved in 10% formalin and tail tips were preserved in absolute ethanol. Specimens are deposited in the Bombay Natural History Society museum with voucher numbers (BNHS 5900–5907 for N. jog; BNHS 5908–5913 for N. kempholeyensis; BNHS 5914–5920 for N. kumbara).

Tadpole identification
Morphological characters were selected based on Altig and McDiarmid (1999), Grosjean (2005) and Schmidt et al. (2009). Morphological terminology follows that of Altig and McDiarmid (1999). Measurements (to closest 0.1 mm) were taken from tadpoles using a Leica MZ75 Stereo zoom microscope (Leica Microsystems Ltd., Heerbrugg, Switzerland) and Mitutoyo digital calliper (Mitutoyo, Japan). Morphometric abbreviations used in the descriptions are as follows: BL, body length (from tip of the snout to the trunk–tail junction); BH, body height; BW, body width; TaL, tail length; TL, total length (sum of BL and TaL); TMW, tail muscle width (at the beginning of the tail); TMH, tail muscle height (at the mid-length of the tail); MTH, maximum tail height (tail height at the mid-length of the tail including caudal fin and tail musculature); TMHM, height of tail muscle at the mid-length of the tail; DF, dorsal fin (height measured at the mid-length of the tail); VF, ventral fin (height measured at the mid-length of the tail); ODW, maximum oral disc width; ED, eye diameter; ESD, eye to spiracle distance; IOD, inter orbital distance (measured from the centre of each pupil); and IND, inter-narial distance (measured from the centres of the narial apertures). Developmental stages are based on Gosner (1960). Using PAST ver. 3.04 (Øyvind Hammer, Natural History Museum, University of Oslo, Norway; http://folk.uio.no/ohammer/past/), principal component analysis (PCA) for morphometric data with correlation matrix was carried out to reduce the number of dimensions required to describe the variation between species.

Molecular identification of tadpoles
Tadpoles were identified molecularly using the mitochondrial gene 16S rRNA. A 570 bp region was amplified using primers from Palumbi et al. (2002). The alcohol preserved tail tissues of tadpoles and thigh muscle of adults were used for DNA extraction. The PCR amplification for the mitochondrial gene was performed in 15 µl
reactions containing 2 µl each of 10× PCR buffer (Sigma-Aldrich, Bengaluru, India), deoxyribonucleotide triphosphates (1 mM), 2 µl of each primer (5 pmol µl$^{-1}$) and 0.2 µl of Taq DNA polymerase (5 units µl$^{-1}$) (Sigma-Aldrich), 5.8 µl of dH$_2$O and 1 µl of template DNA (> 50 ng). The amplified products were sent for purification and sequencing to Amnion Sequencing Services (Bengaluru, India). The obtained sequences were edited using Chromas (Version 2.4, Copyright 1998–2012; Technelysium Pvt Ltd, South Brisbane, Australia). For adults, the edited sequences of 16S rRNA of each sample were used to identify the species by comparing with known sequences retrieved from NCBI database by using BLAST (http://blast.ncbi.nlm.nih.gov/). The identification was accepted as confirmed when there was 99–100% identity between the query sequence and adult sequences in the NCBI database. For calculating the genetic distance, the 16S rRNA sequences of the tadpoles and the adults were first aligned using MAFFT algorithm (Katoh et al. 2002) and manually corrected in MEGA 5.10 software (Tamura et al. 2011). Genetic distances between the species were estimated using the Kimura 2-parameter model in MEGA 5.10 software. For understanding the clustering pattern between the tadpoles and adults, a neighbour-joining (NJ) tree was created in MEGA 5.10 software using Lankanectes corrugatus as an outgroup. The sequences have been deposited in GenBank (accession numbers: KF935242, KP317815–KP317820, KP893671–KP893672; refer to Appendix I for individual accession numbers of tadpoles and adults).

Results
Natural history
The tadpoles and adults of N. kumbara, N. jog and N. kempholeyensis often co-occur in the streams and were seen throughout the study period suggesting that the three species could be continuous breeders. Generally, the tadpoles are found near the edges of the stream where the water is shallow and slow flowing. The substrate is usually made of sand, small gravel and leaves. The tadpoles are diurnal and we did not observe any differences in day and night activities. They were seen scraping leaf debris and when approached hid in sandy substrate or under leaf debris. We did not observe schooling or cannibalistic behaviour among the tadpoles.

Along with tadpoles of Nyctibatrachus, tadpoles of the Indian golden-backed frog (Hylarana indica) were seen in the streams particularly during post-monsoon season. Micrixalus kottigeharensis, an endemic dancing frog of the Western Ghats, was also found breeding in the streams (Gururaja 2010) post-monsoon. But we did not observe its tadpoles in the streams.

Molecular identification
The neighbour-joining tree for the 16S rRNA gene shows 100% identity between tadpoles and their adults (Figure 2). Genetic distance comparisons show that there is no intraspecific divergence between tadpoles and adults of N. kempholeyensis and N. kumbara. For N. jog, the intraspecific divergence between tadpoles and adults is 0.21%. There is a high interspecific divergence between the three species (9–14%) (Table 1).
Figure 2. Neighbour-joining tree of tadpoles and adults of three species of *Nyctibatrachus* and an outgroup (*Lankanectes corrugatus*) based on mitochondrial 16S rRNA. Numbers at the branches indicate bootstrap values.

Table 1. Genetic distance (%) calculated using Kimura 2-parameter model based on 16S rRNA sequences of *Nyctibatrachus* adult and tadpole samples.

|   | 1   | 2    | 3    | 4    | 5    | 6    | 7    | 8    | 9    |
|---|-----|------|------|------|------|------|------|------|------|
| 2 | 18.47 |      |      |      |      |      |      |      |      |
| 3 | 18.47 | 0.00 |      |      |      |      |      |      |      |
| 4 | 18.47 | 0.00 | 0.00 |      |      |      |      |      |      |
| 5 | 19.90 | 9.20 | 9.20 | 9.20 |      |      |      |      |      |
| 6 | 19.90 | 9.20 | 9.20 | 9.20 | 0.00 |      |      |      |      |
| 7 | 19.90 | 9.20 | 9.20 | 9.20 | 0.00 | 0.00 |      |      |      |
| 8 | 18.15 | 14.14| 14.14| 14.14| 13.71| 13.71| 13.71|      |      |
| 9 | 18.15 | 14.14| 14.14| 14.14| 13.71| 13.71| 13.71| 0.00 |      |
| 10| 18.50 | 13.82| 13.82| 13.82| 13.40| 13.40| 13.40| 0.21 | 0.21 |

(1) *Lankanectes corrugatus* (outgroup); (2) *N. kempholeyensis* tadpole 1; (3) *N. kempholeyensis* tadpole 2; (4) *N. kempholeyensis* adult; (5) *N. kumbara* tadpole 1; (6) *N. kumbara* tadpole 2; (7) *N. kumbara* adult; (8) *N. jog* tadpole 1; (9) *N. jog* tadpole 2; (10) *N. jog* adult.

**Tadpole descriptions**

*Nyctibatrachus jog* Biju, Van Bocxlaer, Mahony, Dinesh, Radhakrishnan, Zachariah, Giri and Bossuyt, 2011; Figure 3a–d, Voucher numbers: BNHS 5900–5907.

The tadpoles were collected from a fast-flowing stream. The site consisted of large boulders, small gravel, sand and leaf litter. Canopy cover > 90%. The average width of the stream was 90 ± 0.9 cm (n = 4) and average water depth was 8.0 ± 0.25 cm (n = 4). Adults were seen in the stream, on boulders and on the bark of trees.
Tadpole morphology

Based on stage 25 of a single specimen (BNHS 5900, Table 2). Body oval, longer than wide (BL 11.7 mm, BW 7.2 mm, TL 33.8 mm). In lateral view, body depressed. BH 70% of BW. Eyes positioned dorsally, directed dorsolaterally. Nares closer to eyes than snout. IND 56% of IOD. Intestinal spiral coiled and visible from dorsal, lateral and ventral view. Body length 53% of tail length. Dorsal fin originates after the trunk–tail junction. Dorsal fin

Figure 3. Tadpole of *N. jog*, BNHS 5900. (a) Dorsal view; (b) ventral view; (c) lateral view; (d) mouth part (not to scale).

Table 2. Morphometric measurement of tadpoles of *N. jog*, *N. kempholeyensis* and *N. kumbara*. Measurements in mm.

| Species          | Voucher # | N. jog  | N. kempholeyensis | N. kumbara |
|------------------|-----------|---------|-------------------|------------|
|                  | BNHS 5900 | BNHS 5908 | BNHS 5914       |            |
| Stage            | 25        | 25      | 25                |            |
| BL               | 11.7      | 6.4     | 20.2              |            |
| BH               | 5.1       | 3.2     | 8.6               |            |
| BW               | 7.2       | 4.0     | 13.3              |            |
| TL               | 33.8      | 18.4    | 56.9              |            |
| TAL              | 22.1      | 12      | 36.7              |            |
| ED               | 1.1       | 0.5     | 1.8               |            |
| IOD              | 3.6       | 1.8     | 6.0               |            |
| IND              | 2         | 1.3     | 3.1               |            |
| ESD              | 3.6       | 2.6     | 5.9               |            |
| TMH              | 4.1       | 2.4     | 7.8               |            |
| TMW              | 2.8       | 1.3     | 5.5               |            |
| MTH              | 5.4       | 2.7     | 10.1              |            |
| DF               | 1.5       | 0.8     | 3.1               |            |
| TMHM             | 2.7       | 1.4     | 4.8               |            |
| VF               | 1.2       | 0.5     | 2.2               |            |
| ODW              | 2         | 0.8     | 3.3               |            |
higher than ventral fin. Spiracle sinistral (ESD 3.6 mm), directed laterally. Vent tube dextral. Oral disc small, anteroventral, not emarginated and surrounded by papillae. ODW 17% of BL and 39% of BH. Papillae complete with rounded tip. Keratodonts absent. Jaws-sheath keratinized, finely serrated and saw-toothed. (Figure 3a–d).

Morphological variation among individuals (N = 8, stage 25) given in Table 3.

**Table 3. Morphometric variations in tadpoles (stage 25) of N. jog (n = 8), N. kempholeyensis (n = 6) and N. kumbara (n = 7). Measurements in mm.**

| Characters | N. jog Mean ± SD | Range | N. kempholeyensis Mean ± SD | Range | N. kumbara Mean ± SD | Range |
|------------|------------------|-------|-----------------------------|-------|----------------------|-------|
| BL         | 11.2 ± 0.6       | (10.4–12.1) | 6.2 ± 0.5                  | (5.2–6.8) | 16.6 ± 1.9            | (13.8–20.2) |
| BH         | 5.1 ± 0.2        | (4.7–5.3) | 2.6 ± 0.4                   | (2.1–3.2) | 7.2 ± 1.1            | (5.7–8.7) |
| BW         | 6.8 ± 0.7        | (5.7–7.6) | 3.5 ± 0.4                   | (3.0–4.0) | 10.5 ± 2.0           | (7.9–13.3) |
| TL         | 31.6 ± 2.6       | (28.4–36.2) | 18.5 ± 1.8                 | (16.4–20.6) | 47.0 ± 5.8           | (39.0–56.9) |
| TAL        | 20.5 ± 2.0       | (18.0–24.1) | 12.3 ± 1.4                 | (10.6–13.9) | 30.4 ± 4.1           | (24.5–36.7) |
| ED         | 0.9 ± 0.1        | (0.8–1.1) | 0.5 ± 0.1                   | (0.4–0.6) | 1.4 ± 0.3            | (0.9–1.8) |
| IOD        | 3.3 ± 0.3        | (3.0–3.7) | 1.8 ± 0.2                   | (1.5–1.9) | 4.9 ± 0.7            | (3.7–6.0) |
| IND        | 2.0 ± 0.2        | (1.8–2.3) | 1.2 ± 0.1                   | (1.0–1.4) | 2.7 ± 0.3            | (2.1–3.1) |
| ESD        | 3.6 ± 0.3        | (3.3–4.2) | 2.3 ± 0.3                   | (2.0–2.6) | 5.2 ± 0.5            | (4.4–5.9) |
| TMH        | 3.8 ± 0.3        | (3.3–4.1) | 2.3 ± 0.2                   | (2.0–2.6) | 5.9 ± 1.1            | (4.6–7.8) |
| TMW        | 2.6 ± 0.4        | (2.1–3.2) | 1.3 ± 0.1                   | (1.1–1.4) | 4.3 ± 0.7            | (3.1–5.5) |
| MTH        | 5.1 ± 0.6        | (4.5–6.3) | 2.7 ± 0.2                   | (2.4–3.0) | 7.9 ± 1.4            | (5.9–10.1) |
| DF         | 1.5 ± 0.2        | (1.4–2.0) | 0.8 ± 0.1                   | (0.7–0.9) | 2.4 ± 0.5            | (1.6–3.1) |
| TMHM       | 2.5 ± 0.4        | (2.1–3.2) | 1.3 ± 0.2                   | (1.1–1.5) | 3.9 ± 0.6            | (3.2–4.9) |
| VF         | 1.1 ± 0.3        | (0.7–1.6) | 0.6 ± 0.1                   | (0.5–0.6) | 1.5 ± 0.4            | (0.9–2.2) |
| ODW        | 1.9 ± 0.2        | (1.5–2.0) | 1.0 ± 0.1                   | (0.8–1.1) | 2.7 ± 0.5            | (2.0–3.3) |

**Colouration**

In life, individual brown in colour. Eyes black in colour. In preservative, individual light brown in colour. Colour of the tail lighter than the body. Tail musculature strong with irregular dark spots. The anterior part of ventral fin translucent and devoid of spots and the posterior part made up of large irregular spots. The area surrounding the oral disc pale white in colour.

*Nyctibatrachus kempholeyensis* (Rao, 1937); Figure 4a–d, Voucher numbers: BNHS 5908–5913.

The tadpoles were collected from a slow flowing stream in an evergreen forest. The site of collection had high percentage of sand and leaf litter. The average width of the stream was 30 ± 0.5 cm (n = 4), water depth was 4.7 ± 0.61 cm (n = 4) and canopy cover > 80%. Adults were seen near the edges of the stream.

**Tadpole morphology**

Based on stage 25 of a single specimen (BNHS 5908; Table 2). Body shape oval, longer than wide (BL 6.4 mm, BW 4 mm, TL 18.4 mm). In lateral view, body...
depressed. BH 80% of BW. Eyes positioned dorsally, directed dorsolaterally. Nares closer to eyes than snout. IOD larger than IND (IND 72% of IOD). Intestinal spiral visible in dorsal, lateral and ventral views. Tail musculature well developed, broad at the base and tapers to form pointed tip. Body length 53% of tail length. Dorsal fin originates at the base of the caudal musculature. Dorsal and ventral fin show few irregular brown spots in alternating pattern. Dorsal fin higher than ventral fin. Spiracle visible, sinistral (ESD 2.6 mm). Vent dextral. Oral disc small, anteroventral, not emarginated and surrounded by papillae. ODW 13% of BL and 25% of BH. Papillae complete with rounded tip. Keratodonts absent. Jaws-sheath keratinized, finely serrated and saw-toothed. The lateral process of upper jaw extends well beyond lower jaw. Variation among individuals (N = 6, stage 25) given in Table 3.

**Colouration**

In life, individual brown in colour. Eyes black in colour. The colour of the preserved individual light brown. Dark pigmentation posterior to eye. Few small spots on dorsal part of the tail. Laterally, tail muscle axis consists of a stripe of dark pigmentation (extending from anterior to mid portion of tail musculature). Ventrally, tail pale white in colour with very few dark spots distributed at the posterior end. Pupil white in colour. The area surrounding the oral disc pale white in colour.

*Nyctibatrachus kumbara* Gururaja, Dinesh, Priti and Ravikanth, 2014; Figure 5a–d, Voucher numbers: BNHS 5914–5920.

The tadpoles were collected from a slow-flowing stream. The substrate at the site of collection had small gravel, sand and leaf litter with intermittent big boulders. The
width of the stream was 30 ± 0.5 cm \((n = 4)\) and average water depth 4.0 ± 0.5 cm \((n = 4)\), shallower at the edges and deeper in the middle. Adults are sympatric with \(N. kempfaleyensis\).

**Tadpole morphology**

Based on stage 25 of a single specimen (BNHS 5914; **Table 2**). Body shape oval, longer than wide (BL 20.2 mm, BW 13.3 mm, TL 56.9 mm). In lateral view body depressed. Eyes large (ED 1.8 mm), positioned dorsally, directed dorsolaterally. Nares closer to eyes than snout. IND 52% of IOD. Intestinal spiral visible in dorsal, lateral and ventral view. Tail musculature well developed. Body length 55% of tail length. Dorsal fin higher than ventral fin. Dorsal fin originates posterior to the trunk–tail junction. Spiracle sinistral (ESD 5.9 mm). Vent tube dextral. Oral disc small, anteroventral, not emarginated and surrounded by papillae. ODW 16% of BL and 38% of BH. Papillae complete with rounded tip. Keratodonts absent. Jaws-sheath keratinized, finely serrated and saw-toothed. Upper jaw-sheath longer than the lower jaw-sheath. Upper jaw-sheath resembles inverted U in shape, lower jaw-sheath V-shaped. Variation among individuals \((N = 7, \text{ stage 25})\) given in **Table 3**.

**Colouration**

In life, individual light brown in colour. Eyes black in colour. In preservative individual grey in colour. Tail colour lighter than body and bears dark irregular spots distributed more on the tail muscle axis. In lateral view both fins show irregular spots but irregular spots distributed more on dorsal fin than ventral fin. Pupil white in colour. The area surrounding the oral disc pale white in colour.

Figure 5. Tadpole of \(N. kumbara\), BNHS 5914. (a) Dorsal view; (b) ventral view; (c) lateral view; (d) mouth part (not to scale).
Tadpole comparison

The tadpoles of *N. kempholeyensis* are smallest and those of *N. kumbara* the largest among the three species. The tadpoles of *N. kempholeyensis* can be distinguished from *N. kumbara* and *N. jog*, by smaller size (BL, TL and TaL), by tail pattern (presence of dark pigmented stripe along the sides of mid tail) and dark pigmentation behind eyes. Tadpoles of *N. kumbara* and *N. jog* have a dark horizontal stripe dorsally at the trunk–tail junction, while *N. kempholeyensis* do not have this horizontal stripe. *N. kumbara* differs from *N. jog* in size (BL, TL and TaL). Morphological variations of these tadpoles are given in Table 3. Principal component analysis of morphometric data, after transforming to log₁₀, revealed clear grouping of these tadpoles (Figure 6). Principal component 1 and 2 explained 96.7% and 1.1% variance respectively.

Discussion

The earliest descriptions of tadpoles of *Nyctibatrachus* were those of *N. pygmaeus* (Annandale 1918, 1919), *N. sanctipalustris* (Rao 1923), *N. humayuni* (Bhaduri and Kripalani 1955) and *N. major* (Pillai 1978). Apart from those of *N. humayuni*, tadpole identifications and descriptions are ambiguous due to discrepancies in the original descriptions and due to taxonomic revision. Compiled morphometric data of *N. major*, *N. humayuni* and *N. sanctipalustris* are given in Table 4. Annandale (1918) described the tadpole of *N. pygmaeus* collected by Capt. R.B.S. Sewell, I.M. S, in a small lake in the Nilgiris in June 1912. Later in 1919, Annandale stated that the description provided as *N. pygmaeus* was of *Ixalus variabilis* and true tadpoles of *Nyctibatrachus* did not have keratodents, and closely resembled ‘*Rana semipalmata*’ (*Indirana semipalmata*) described earlier in his 1918 paper. Rao (1923), while describing tadpoles of ‘*Nyctibatrachus sancti-palustris*’ (*Nyctibatrachus sanctipalustris*), examined one of the specimens of *N. pygmaeus* and considered it to be a tadpole of *N. major* and not *N. pygmaeus*. Later, Bhaduri and Kripalani (1955) and Pillai

Figure 6. Principal component analysis of morphometric data of tadpoles of *N. jog*, *N. kempholeyensis* and *N. kumbara.*
1978), referring to the original description of Annandale (1918), considered tadpoles described under ‘Rana leptodactyla’ (Indirana leptodactyla; refer to Annandale 1918, figure 2a, b, plate I) and ‘Rana semipalmata’ (Indirana semipalmata; Annandale 1918, figure 3a, b, plate I) to be Nyctibatrachus and disregarded the description of N. pygmaeus tadpoles.

Bhaduri and Kripalani (1955), when describing tadpoles of N. humayuni collected from Matheran, North Western Ghats (refer to Bhaduri and Kripalani 1955, figures 5 and 6), reported that the oral structure of N. sanctipalustris did not resemble that of N. humayuni. According to them, N. humayuni tadpoles were devoid of keratodons while tadpoles of N. sanctipalustris had keratodons. Based on our study, we agree with Bhaduri and Kripalani that keratodons are absent and therefore consider the tadpole description of N. sanctipalustris as ambiguous. The description of the tadpole of N. major by Pillai (1978) is of a Nyctibatrachus species, however the identity remains questionable due to the distribution range of N. major. According to Pillai (1978), the tadpole was collected from a small stream in Kurichi Reserve Forest, Chedleth, Wayanad, Kerala in October 1976 (refer to Pillai 1978, figure 1). Based on a recent review of Nyctibatrachus genus and due to clade-level endemism (Biju et al. 2011; Bocxlaer et al. 2012), N. major is a southern Western Ghats species (between 8 and 9°N latitude; south of a 32 km wide Palghat gap that acted as a natural barrier in contiguous Western Ghats) while the tadpole described by Pillai (1978) is from 11.7–12°N latitude. Therefore the specific identity is debatable although the genus is likely correct. Apart from these tadpole descriptions, Biju et al. (2011) recently provided descriptions of developmental stages of N. aliceae but did not provide a complete description of the tadpole. Of the existing four tadpole descriptions of Nyctibatrachus, only N. humayuni appears appropriate and unambiguous with respect to taxonomy and distribution. With the addition of three species from the current study, the total number of tadpole descriptions for Nyctibatrachus stands at four species; 24 are yet to be described.

Table 4. Compiled morphometric data of N. major, N. humayuni and N. sanctipalustris from Pillai (1978), Bhaduri and Kripalani (1955) and Rao (1923) respectively.

| Species     | N. major* | N. humayuni | N. sanctipalustris |
|-------------|-----------|-------------|--------------------|
| Stage*      | 41        | 41          | 41                 |
| BL          | 15        | 16          | 18                 |
| BW          | 10        | 10          | 11                 |
| TaL         | 22        | 32          | 34                 |
| TL          | 37        | 48          | 52                 |
| MTH         | 8         | 7           | 9                  |

*Questionable status of identity and tadpole stage.
the streams, molecular techniques in addition to morphology helped to identify the species. The low intraspecific distance values and high interspecific distances (9–14%) among these congeneric species (Table 1) indicate that 16S rRNA could distinguish the tadpoles of three congeneric species.

The genus *Nyctibatrachus*, along with its sister genus *Lankanectes* from Sri Lanka, forms an ancient lineage of frogs from Western Ghats–Sri Lanka biodiversity hotspot (Roelants et al. 2004). According to Frost et al. (2006) there are considerable differences in morphological characters of adults of *Nyctibatrachus* and *Lankanectes* but based on molecular data the two genera have been put together as one family. There are significant differences in oral structure of tadpoles as well. The tadpole of *Lankanectes corrugatus* has keratodonts (Ukuwela and Bandara 2009) while tadpoles of *Nyctibatrachus*, as evident from this study, lack keratodonts (Figure 7). The loss of keratodonts in *Nyctibatrachus* species seems to be a unique trait. Description of the tadpoles of the remaining 24 *Nyctibatrachus* species and phylogenetic analysis of frogs from this genus with inclusion of larval characters could help in further understanding the evolution of larval traits.

The stream-dependent tadpoles of Nyctibatrachidae are mostly distributed in the second and third order streams of Sharavathi and Aghanashini river basins where the habitat is mainly evergreen forests and *Myristica* swamps. These swamps were once found throughout the watercourses of the Western Ghats but now have a patchy distribution due to anthropogenic activities (Chandran and Mesta 2001). Many of the streams in the study area have been diverted for agricultural purposes. Such changes could disturb the habitat of these tadpoles and affect both the tadpoles and adults. Ecological and genetic studies on effects of habitat alteration on tadpole populations would help in elucidating their impacts on larval ecology and population distribution and could be useful in

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**Figure 7.** Oral apparatus of *Lankanectes corrugatus* tadpole. Scale bar is 1 mm. Image courtesy of Kanishka D.B. Ukuwela.
making decisions on conservation and management of the amphibians of central Western Ghats.

Acknowledgements

The authors would like to thank Karnataka Forest department for permission (No. C1/WL/CR-6/2012–13). We thank Dr Aravind Madhyastha for the help in initial discussions and for reviewing the manuscript. Our thanks go also to Dr Rahul Khot, Curator, Bombay Natural History Society for help in accessing museum collections; and to Dr Priyadarsanan D.R. and Ms Seena K. for providing the Leica Microscope and camera. PH and KVG are grateful to Ashok Hegde for logistical support in the field. We thank the two anonymous reviewers for the critical comments on the manuscript.

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

PH is thankful to Madras Crocodile Bank Trust (MCBT) for the funding (MoU dated June 2012).

Geolocation information

N. jog (point): 74.74679°E, 14.27401°N; 74.75233°E, 14.27073°N; 74.75845°E, 14.27325°N
N. kempholeyensis (point): 74.74679°E, 14.27401°N; 74.75233 °E, 14.27073°N; 74.81414°E, 14.23291°N
N. kumbara (point): 74.74679°E, 14.27401°N; 74.75233°E, 14.27073°N; 74.73962°E, 14.41291°N; 74.63551°E, 14.30256°N

ORCID

K.V. Gururaja http://orcid.org/0000-0001-6907-9907

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**Appendix I.** GenBank accession numbers for the 16S rRNA of Lankanectes corrugatus and adult and tadpoles of Nyctibatrachus species used in the present work.

| Species                          | GenBank Accession # | Reference                        |
|----------------------------------|---------------------|----------------------------------|
| *Lankanectes corrugatus*         | AY880445            | Delorme et al. 2004              |
| *N. jog* tadpole 1               | KP317819            | Present work                     |
| *N. jog* tadpole 2               | KP317820            | Present work                     |
| *N. jog* adult                   | KP893672            | Present work                     |
| *N. kempholeyensis* tadpole 1    | KP317815            | Present work                     |
| *N. kempholeyensis* tadpole 2    | KP893671            | Present work                     |
| *N. kempholeyensis* adult        | KP317816            | Present work                     |
| *N. kumbara* tadpole 1           | KP317817            | Present work                     |
| *N. kumbara* tadpole 2           | KP317818            | Present work                     |
| *N. kumbara* adult               | KF935242            | Gururaja et al. 2014             |