

The tadpole of the Helmeted Toad, *Ingerophrynus galeatus* (Günther, 1864), from Vietnam (Anura: Bufonidae)

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Abstract. Based on identification through DNA barcoding we describe the tadpole morphology of the Helmeted Toad, *Ingerophrynus galeatus*. The description is based on twelve tadpoles that were collected in the karst forest of Phong Nha – Ke Bang National Park, Quang Binh Province, Truong Son, central Vietnam. The larvae, collected in slowly running forest streams or nearby ponds on gravel, are of generalized morphology of Orton's type 4, lentic: benthic with a keratodont formula of 2(2)/3.

Keywords. Vietnam, Anura, Bufonidae, *Ingerophrynus galeatus*, DNA barcoding, tadpole description, morphology.

Introduction

The Mountain or Helmeted Toad, *Ingerophrynus galeatus*, is known from Cambodia and Laos through Vietnam to southern China (Hainan) (Stuart, 1999; Ziegler, 2002; Nguyen et al., 2005, 2009). Both sexes bear a thick bony crest between the eye and the parotoid gland, but only adult females have the canthal crest conspicuously raised and arched (Fig. 1). The larger females, exceeding 89 mm in snout-vent length, bear as secondary sex character prominent, sharply pointed, conical tubercles on the sides (Inger et al., 1999). The barely known species is known to inhabit forests from 100-1,300 m above sea level, where it can be found in shallow water near streams and ponds (Ryboltofsky, 1997; Stuart, 1999; Ziegler, 2002). The Helmeted Toad is listed as rare in the Red Data Book of Vietnam (MOSTE 2000) and its first breeding in captivity was published by Ryboltofsky (1997). We herein describe the larval morphology for the first time, based on specimens recently collected from central Vietnam.

Material and Methods

The larvae were collected in slowly running limestone forest streams or nearby pools on gravel in the Phong Nha – Ke Bang National Park, Quang Binh Province, Truong Son, central Vietnam (Fig. 2), in June 2005 in the Cha Noi area (by TZ) and in July 2006 in the Khe Van area (Fig. 3), 17°29'.N, 106°17'.E (by RH). An adult specimen (AMNH A163648) was collected by Frost et al. (2006) in Quang Nam, Tre My, Tre Tep Commune, stream near Thon 2 Village, central Vietnam (15°09'.N, 108°02'.E) at an elevation of 920-1060 m.

Some tadpoles (n = 2) were fixed in 3% formalin, and later preserved in 70% ethanol, while others (n = 10) were fixed in 4%



Figure 1. Adult *Ingerophrynus galeatus* from Phong Nha – Ke Bang NP, Quang Binh Province, Vietnam, in amplexus (August 2006). Photograph: R. Hendrix.

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Table 1. Minimum-, maximum-, mean values, standard deviation, and number of the measured tadpoles (ZFMK 87000). All measurements are in millimeters.

SE	RN	NP	ED	BL	TAL	TL	IND	IOD	BW	TMW
1.13-1.40	0.58-0.80	0.52-0.60	0.22-0.42	3.60-4.20	4.93-6.13	8.67-10.33	0.65-0.74	1.17-1.41	2.41-2.87	0.42-0.55
1.24	0.68	0.56	0.36	3.82	5.31	9.13	0.70	1.30	2.63	0.48
± 0.07	± 0.06	± 0.03	± 0.07	± 0.18	± 0.33	± 0.47	± 0.03	± 0.07	± 0.12	± 0.04
(n = 10)	(n = 10)	(n = 10)	(n = 10)	(n = 10)	(n = 10)	(n = 10)	(n = 10)	(n = 10)	(n = 10)	(n = 10)
BH	TMH	UF	LF	HT	ODW	SS	NK			
1.61-1.94	0.63-0.71	0.57-0.70	0.45-0.63	1.50-1.85	1.19-1.47	2.48-3.07	44-48			
1.80	0.67	0.62	0.57	1.70	1.31	2.69	46			
± 0.09	± 0.03	± 0.04	± 0.06	± 0.12	± 0.07	± 0.16	± 1.23			
(n = 10)	(n = 10)	(n = 10)	(n = 10)	(n = 10)	(n = 10)	(n = 10)	(n = 8)			

Abbreviations as follow: BH = maximum body height; BL = maximum body length; BW = maximum body width; ED = eye diameter; IND = internarial distance; IOD = interorbital distance; LF = maximum height of lower fin; HT = maximum tail height; NK = number of keratodonts per 0.25 mm; NP = nuro-pupilar distance; ODW = oral disc width; RN = rostro-narial distance; SE = distance from snout to eye; SS = distance from the tip of snout to the opening of spiracle; TAL = tail length; TL = total length; TMH = tail muscle height at proximal basis; TMW = maximum of the tail musculature at basis; UF = maximum height of upper fin; n = number of individuals.

formalin and subsequently preserved in a mixture of 4% formalin and 70% ethanol (1:1; see Grillitsch, 1984; Grosjean, 2001). All larvae were deposited in the herpetological collection of the Zoologisches Forschungsmuseum Alexander Koenig (ZFMK), Bonn, Germany, under the collection numbers ZFMK 87000 (Khe Van sample, n = 10), and ZFMK 87586 (Cha Noi sample, n = 2).

To reveal the taxonomic identity of the tadpoles, molecular data were obtained from a single individual from the Khe Van sample, Quang Binh Province (GenBank: EU366289) and compared with an homologous mitochondrial 16S ribosomal RNA gene fragment of an adult specimen from Quang Nam Province (Genbank accession number DQ283376; Frost *et al.*, 2006). Genomic DNA was extracted using a modified standard protocol for Chelex-Extractions (Walsh *et al.*, 1991). Obtained mitochondrial 16S ribosomal RNA gene fragment was amplified by primers 16Sar-L and 16Sbr-H of Palumbi *et al.* (1991). PCR product was purified by a modified QiaQuick-Purification-Protocol (QiaQuick Purification Kit, Qiagen), and sequenced using an automatic ABI capillary sequencer. Genetic uncorrected pairwise distances of edited sequences were calculated by the adjustment of pairwise alignment (optimal global alignment) in BioEdit version 7.0.9.0 (Hall, 1999).

Terminology for morphometric data and abbreviations follow McDiarmid & Altig (1999) and Grosjean (2005) as shown in table 1. Keratodont row formulae (KRF) follow the terminology of McDiarmid & Altig (1999). Developmental stages were determined according to Gosner (1960). Measurements were taken using a stereo microscope with a measuring device of 100 bars/cm converted into millimetres.

Identification

The DNA voucher individual originated from the same water body as the before mentioned tadpoles from

the Khe Van area (ZFMK 87000, n = 10). In this water body, no other bufonid species than *I. galeatus* were observed. The DNA voucher specimen was destroyed for DNA extraction, but fully agreed morphologically

**Figure 2.** Map showing the location (large dot) of Phong Nha - Ke Bang NP within Vietnam.

with the syntopically collected larvae, as well as with the described and drawn specimen (ZFMK 87586a) in the diagnostic characters (length of keratodont rows of lower labium: $P2 > P1 = P3$, and average density of 46 keratodonts per 0.25 mm). The mitochondrial 16S rRNA gene sequence of the tadpole comprised 544 base pairs and showed 0.74% (540 of 544 bps) sequence divergence with the sequence of the adult specimen from Quang Nam Province, which is located three provinces farther to the South. This result supports that our collected tadpoles from Quang Binh Province belong to the same species as the *I. galeatus* voucher specimen.

Results

The tadpoles of *Ingerophrynus galeatus* (ZFMK 87000 series) collected in July 2006 were in the developmental stage 27 according to Gosner (1960). Tadpoles were collected at afternoon (15:00 h) on 19 July in a separated gravel pool right next to a slowly running karst forest stream at an altitude of about 130 m above sea level. The pool was partly shaded by large trees; it had a maximum width of 1.2 m and the maximum water depth measured 6 cm. The shallow water was clear and the sandy ground was covered with decaying leaves. The larvae were discovered resting nearly motionless on the bottom of

the pool, where they fed upon the periphyton of the leaves surfaces. Ambient air temperature was 29°C at the collecting site and relative air humidity was 77%; the water temperature was 27.7°C, with a pH value of 6.6. Although the mating calls of adult male *I. galeatus* were regularly heard throughout July along forested streams after sunset, and a pair in amplexus was observed in early August, the above mentioned larvae were the only ones observed and collected in the year 2006.

The tadpole description is based on a single specimen in lot ZFMK 87586a, while the ratios are based on measured mean values of $n = 10$ larvae (ZFMK 87000). Body and tail basis of tadpoles in life is dark grey to dark brown pigmented. Upper fin becomes more transparent to the end of the tail while the lower fin is entirely translucent. Ventral side of the body is transparent and scarcely pigmented, compared to dorsal side. Intestinal coils visible from lateral and ventral view.

Descriptions of preserved specimens correspond extensively to the living tadpoles except for a transparent margin of the body (Figs. 4-5).

Body in dorsal view (Fig. 4b) oval; nearly three-fourth as long as tail length (body length 0.72 times of tail length) and widest portion being at midbody (body width 0.79 times of body length). Snout slightly pointed. Eyes, in relation to body length, rather small (eye diameter 0.09 times of the body length); more dorsally than dorsolaterally positioned and laterally directed. Bulging of the eyes not visible from ventral view. Interorbital distance half of the body width (IOD 0.49 times of BW). Nares rounded, slightly rimmed and anterolaterally positioned; upper border somewhat prominent versus the lower border. Naris nearer to the eye than to the snout (rostronarial distance 0.55 times of rostro-pupilar distance). Tail musculature at basis, small (tail muscle width at basis 0.18 times of body width).

Body in lateral view (Fig. 4a) compressed (body height 0.68 times of body width); snout slightly pointed. Spiracle sinistral, tubular, and ventrolaterally positioned. Opening of the spiracle oval, applied somewhat below the longitudinal axis; equidistant to the eye and to the end of the body, and oriented in posterior direction. Inner wall of the opening entirely attached to the body. Caudal musculature at basis of the tail weakly developed; less than half of total height (tail muscle height at basis 0.39 times of maximum tail height). Tail musculature slightly rises from proximal to its distal last one-third, then gradually tapering to the end, not reaching the tip of tail. Maximum height in the middle of the tail. Upper and lower fin of nearly identical height (lower fin 0.92



Figure 3. Habitat of *Ingerophrynus galeatus* within Phong Nha – Ke Bang NP, Quang Binh Province, Vietnam (Khe Van area, July 2006). Photograph: R. Hendrix.

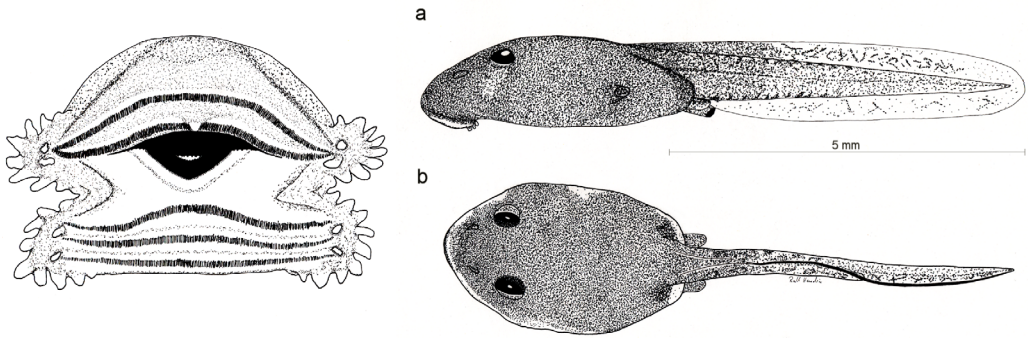


Figure 4. Left: Oral disk of the tadpole of *Ingerophrynus galeatus* (ZFMK 87586) from Cha Noi, Phong Nha – Ke Bang NP, Quang Binh Province, Vietnam, collected by T. Ziegler; right: lateral (a) and dorsal (b) aspects of the preserved tadpole (scale bar = 5 mm). Drawing: R. Hendrix.

times of upper fin); convex at the middle of the tail. Tip of tail broadly rounded. Anal tube strongly elongated and dorsomedially adnated to the lower fin.

Oral disc anteroventrally positioned, of nearly trapezoidal shape in relaxed state, laterally strongly emarginated and framed by small finger-shaped papillae which encompass the rows of fan-shaped keratodonts (Fig. 4). Anterior labium with large medial gap almost as long as the first keratodont row. Posterior labium with medial gap two-third as long as keratodont row P3. One to two submarginal papillae situated at the end of the keratodont rows of the upper and lower labium. Keratodont rows A1, P1, P2 and P3 continuous; A2 slightly interrupted above the upper jaw sheath. Keratodont row formula: 2(2)/3. Length of keratodont rows $A1 > A2$; $P2 > P1 = P3$ with an average number of 46 keratodonts per 0.25 mm. Jaw sheaths dark brown to black at the edges, shallow, and faintly serrated. Posterior jaw sheath V-shaped, anterior jaw sheath M-shaped and stretched widely into a light arc.

Discussion

In general, the morphology and ecology of bufonid tadpoles are poorly known (Inger, 1985). Bufonid tadpoles often show simply undivided and serrated beaks, marginal papillae at each side of the oral disk, an oval body shape, and a dark coloured skin (Pope, 1931; Inger, 1985). Furthermore, many species show a nearly identical keratodont row formula. The upper labium usually bears 1-2 (rarely three) mostly continuous keratodont rows and three continuous keratodont rows on the lower labium (KRF: 2(2)/3), see Manthey & Grossmann (1997), and Altig & McDiarmid (1999: 302).

Exceptions for South East Asian bufonids are *Pelophryne* larvae (see Manthey & Grossmann, 1997), which only bear few keratinized teeth in a single row on the upper labium (*P. brevipes*), whereas species of *Ansonia* and *Leptophryne* additionally show a continuous row of marginal papillae on the lower labium (Inger, 1985). Furthermore, *Ansonia* larvae are characterized by the presence of a large suctorial ventral disk (Inger, 1985; Manthey & Grossmann, 1997). Inger (1985) compared tadpoles of *Phrynoidis aspera* and *Ingerophrynus divergens* from Sarawak, Borneo and found out that larvae of both species are easily distinguishable based on their webbing in older developmental stages but also based on the length of the posteroventral liver lobe, that shows through the ventral skin from stage 25 onwards and which is in relation to the body length larger in *P. aspera* than in *I. divergens*.

Within the family Bufonidae, six species are known to occur in Vietnam: *Bufo cryptotypanicus* Liu & Hu, 1962; *B. pageoti* Bourret, 1937; *Duttaphrynus melanostictus* (Schneider, 1799), *Ingerophrynus galeatus* (Günther, 1864), *I. macrotis* (Boulenger, 1887), and *Phrynoidis aspera* (Gravenhorst, 1829) (Nguyen *et al.*, 2009). However, descriptions of the larval morphology are only available for *Duttaphrynus melanostictus* (e.g. Flower, 1896; Annandale, 1918; Van Kampen, 1923; Pope, 1931; Bourret, 1942; Manthey & Grossmann 1997; Leong & Chou, 1999), *Ingerophrynus macrotis* (Inthara *et al.*, 2005), and *Phrynoidis aspera* (e.g. Van Kampen, 1910, 1923; Smith, 1930; Bourret, 1942; Berry, 1972; Inger, 1985; Manthey & Grossmann, 1997).

Based on the descriptions provided by Van Kampen (1923), Bourret (1942), and Berry (1972) for *Phrynoidis*

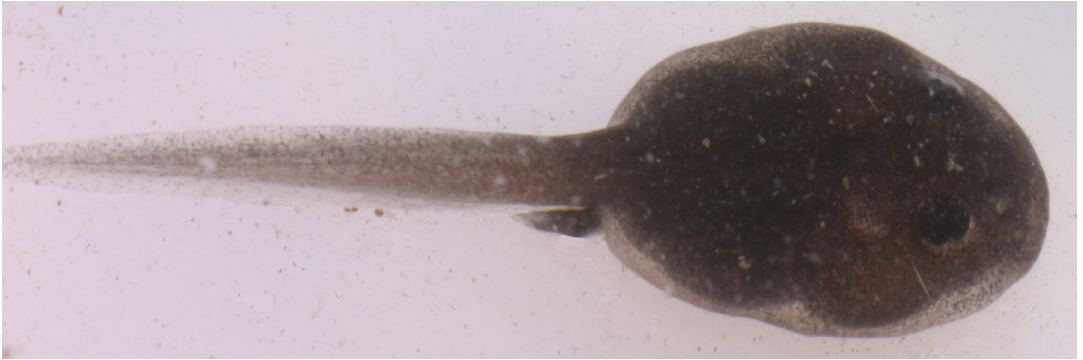


Figure 5. Photograph of the tadpole of *Ingerophrynus galeatus* (ZFMK 87586) from Cha Noi, Phong Nha – Ke Bang NP, Quang Binh Province, Vietnam, in life. Photograph: T. Ziegler.

aspera, larvae of *I. galeatus* are distinguishable from the afore-mentioned species in the absence of a large sucker-like posterior labium, which is completely emarginated by small papillae, and a set of undivided keratodont rows in the anterior labium (KRF in *P. aspera*: 2/3). To distinguish between the larvae of *I. macrotis* and *I. galeatus* becomes more difficult, due to the undetermined information given by Inthara et al. (2005) for the mouth part structure of *I. macrotis* from Thailand. The authors only provide descriptive information about the keratodont formula (KRF: 2(2)/3) and provide photographs as well as a drawing of the tadpole and the oral disk, respectively. The drawing shows one marginal row and two submarginal rows of small papillae along the lower labium of *I. macrotis*, which are not visible on the photograph. A similar situation can be found for the drawing of the oral disk of *D. melanostictus* from Thailand compared to the according photograph provided by Inthara et al. (2005) that also shows two marginal rows of papillae in the lower labium. However, this feature has neither been mentioned in previous descriptions for this tadpole species (see Flower, 1896; Annandale, 1918; Van Kampen, 1923; Pope, 1931; Bourret, 1942; Manthey & Grossmann 1997; Leong & Chou, 1999) nor was it observed in *D. melanostictus* larvae from Phong Nha – Ke Bang National Park (Hendrix, 2007). Consequently, we are not sure whether the existence of three rows of papillae in *I. macrotis* is a true distinguishing character or due to an error in the description provided by Inthara et al. (2005).

We observed a relative similarity in external morphology and coloration in syntopic tadpoles of *Duttaphrynus melanostictus* and *Ingerophrynus galeatus* from Phong Nha – Ke Bang National Park. Both species have equal keratodont row formulae

(2(2)/3) with an average density of 46 keratodonts per 0.25 mm in *I. galeatus* (n = 10 examined specimens) versus 48 keratodonts per 0.25 mm in *D. melanostictus* (Hendrix, 2007), which does not allow for a proper identification. However, a distinction between both species is at least possible in the samples at hand based on the length of keratodont rows on the posterior labium. In *I. galeatus* keratodont row P2 is somewhat longer than P1 and P3, whereas latter rows are of equal length. In contrast, the length of keratodont rows in *D. melanostictus* continuously decreases from P1 to P3. Furthermore, total lengths of *I. galeatus* larvae as well as the relation of body lengths to tail lengths are lower when compared with *D. melanostictus* [TL in *I. galeatus* (stage 27, n = 10) 8.5–10.3 mm vs. 11.4–12.1 mm in *D. melanostictus* (stage 27–28, n = 2), and mean value of relation of BL to TAL 0.72 vs. 0.90 (see Hendrix, 2007)].

According to Ryboltovsky (1997), *I. galeatus* larvae start swimming on the third day, and begin feeding on the sixth day. Tadpoles may reach 2.5 cm length within 30 days and develop rear limbs from day 35 onwards. After 45 days, metamorphosis was completed and the juvenile toads left the water with sizes of about 5 mm.

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