Description of three Rhacophorus tadpoles (Lissamphibia: Anura: Rhacophoridae) from Sarawak, Malaysia (Borneo)

ALEXANDER HAAS¹,², STEFAN T. HERTWIG², WENKE KRINGS³, ENZO BRASKAMP⁴, J. MAXIMILIAN DEHLING³, PUI YONG MIN⁴, ANDRÉ JANKOWSKI¹, MANUEL SCHWEIZER² & INDRANEIL DAS⁴

¹ Zoologisches Museum Hamburg, Martin-Luther-King-Platz 3, 20146 Hamburg, Germany
² Naturhistorisches Museum der Burgergemeinde Bern, Bernastrasse 15, CH-3005 Bern, Switzerland
³ Institut für Integrierte Naturwissenschaften, Abteilung Biologie, Universität Koblenz-Landau, Universitätsstraße 1, 56070 Koblenz, Germany
⁴ Institute of Biodiversity and Environmental Conservation, Universiti Malaysia Sarawak, 94300 Kota Samarahan, Sarawak, Malaysia; idas@ibec.unimas.my

5 Corresponding author: E-mail: alexander.haas@uni-hamburg.de

Abstract

This communication reports the discovery of the hitherto unknown larval forms of Rhacophorus rufipes and R. penanorum, and re-describes the tadpole of R. dulitensis. Tadpoles of all three species were discovered at Gunung Mulu National Park, Sarawak (Borneo), Malaysia. The identity of the larvae was determined by DNA barcoding techniques using partial 16S rRNA mitochondrial gene sequences. Larval DNA sequences matched those of syntopic adults of respective species. Detailed descriptions of external morphology and colouration in life are provided along with ecological notes. The tadpole of R. rufipes and R. dulitensis can be classified as generalized, benthic-nectonic type, whereas tadpoles of R. penanorum show adaptations typical for a lotic, rheophilous lifestyle.

Key words: Rhacophorus rufipes, R. penanorum, R. dulitensis, tadpole description, larval morphology, oral disc, rheophilous tadpole, DNA barcoding

Introduction

The identification of anuran amphibian larval stages is essential for many purposes and research objectives, such as regional surveys, habitat inventories, studies on resource use, interspecific competition studies, and for conservation. For rapid assessments of study sites, searching for, and identifying larvae in the field is necessary to optimize amphibian species counts. This is particularly true in structurally complex and species rich tropical habitats. A large number of frogs still lack detailed descriptions of their larval forms, although there has been a recent increase in efforts in tadpole surveys and tadpole fauna inventories (for example, Anstis 2002; Chou & Lin 1997; Gawor et al. 2009; Leong & Chou 1999; Das & Haas 2011; Haas & Das 2011). The Sundaland is a biodiversity hotspot of global importance and the true number of frog species in the region remain unknown, and is probably underestimated (Inger 1999). The general lack of reliable information about larval forms is not expected to change in the near future as many new species of frogs continue to be discovered (AmphibiaWeb 2010) and because the majority of new species descriptions still focuses on the adult and very often neglect larval forms.

The tadpole fauna of the East Malaysian states of Sabah and Sarawak has been investigated since the mid-1960s in a series of publications by Robert F. Inger and collaborators (Inger 1966; Inger 1983; Inger 1985; Inger et al. 2006). Recently, Das & Haas (2005) and Haas & Das (2011) gave an overview on the literature that provides data on larval identities of Bornean amphibians. Haas & Das (2011) identified 32% of all East Malaysian (Borneo) anuran species to have unknown larval forms. Even where descriptions are available for tadpoles, these are often incomplete (such as lacking images) or only in an abbreviated form. Often differential diagnostics or identification keys are not provided, making unambiguous identification of larvae in the lab or in the field challenging.
A total of 17 species of Rhacophorus have been reported from Borneo, two of which were described in the past three years (Dehling 2008; Dehling & Grafe 2008). Tadpole descriptions are available for only 11 of them (Das & Haas 2005). In the present account, we describe the tadpoles of two additional species, R. rufipes Inger, 1966 and R. penanorum Dehling, 2008. In contrast to these two species, Rhacophorus dulitensis Boulenger, 1892 (Fig. 1) has long been known to science and its tadpole had been described by Inger (1966, 1985). Because of the technical limitations of the time, Inger did not provide photographic documentation along with his description. Furthermore, we found some discrepancies between our sample of R. dulitensis tadpoles and that in Inger’s description that justify comparison and discussion.

Rhacophorus penanorum (Fig. 2) is endemic to Gunung Mulu in northeastern Sarawak; it is currently known only from a single, small stream that forms the headwaters of Sungei Tapin on the southern flank of the mountain at ca. 1650 m a.s.l. (Dehling 2008). Little is known of its natural history and ecology. All specimens observed and collected so far were males that were encountered calling from leaves overhanging the stream at heights between 1.5 and 2 m above ground. It is a small tree frog (SVL [snout-vent length] of males 33–34 mm, females currently unknown) with a light green dorsal colouration.

Rhacophorus rufipes (Fig. 3) has a patchy distribution in northern Borneo. Originally described from central Sarawak, it was subsequently recorded from eastern Sabah and Brunei Darussalam (north-eastern Grafe & Keller 2008; Inger 1966; Inger et al. 2000). We herein report it from Gunung Mulu National Park in north-eastern Sarawak. All known localities were in primary forests at elevations from 100–300 m. R. rufipes breeds in small bodies of stagnant water. Eggs are deposited in foam nests attached to leaves, branches, or roots overhanging the surface of the water (pers. obs.). The SVL of males is in the range 33–38 mm, females 48–50 mm (Inger & Stuebing 1989). These frogs have a light to reddish brown dorsal colouration (Fig. 3). The webbing between the fingers and toes, the groin, and parts of the lateral surfaces of arms and legs show a conspicuous red colouration.

**Materials and methods**

Tadpoles of all three Rhacophorus species were from Gunung Mulu National Park, Sarawak, north-west Malaysia (Table 1). Tadpoles of R. rufipes were collected by AH, STH, and JMD on 20 March 2009, northwest of Camp 5, from a small pool in a patch of Kerangas forest approximately 50 m off the Kerangas Trail on top of a hill (N04.14840°, E114.88890°). Tadpoles were caught with a dip net (25 cm diameter). Tadpoles of R. penanorum were collected by STH on 31 March 2007 by dip net and by hand at the type locality of this species (N04.056017°, E114.858067°) in a small permanent stream headwater of Sungei Tapin (see also Dehling 2008; Dring 1983a; Dring 1983b). This stream is situated at a steep hillside in lower montane forest at an altitude of about 1,650 m a.s.l., approximately 200 m below the ridge-top and a basic shelter, referred to as Camp 4. Tadpoles of R. dulitensis were collected from a pond at Mentawai Ranger Station (N04.2389°, E114.87685°). Tadpoles could be observed in large numbers at night, occasionally forming congregations over muddy bottoms and vegetation of the ca. 3 x 20 m pond. A second sample was taken from a pond at the helipad of Gunung Mulu National Park Headquarters (N04.042333°, E114.813767°), where adults were calling from a tree in the pond (adult vouchers).

Living tadpoles were anesthetized (approx. 2% aqueous 1,1,1-trichloro-2-methyl-2-propanol) and photographed alive (Nikon D90, Canon EOS 5D-MkII, 105 mm and 180 mm macro lenses, double flashes) in a small glass tank (20 x 10 x 5 cm; width x height x depth). Specimens were euthanized in chloretone solution and preserved in either 4% neutral-buffered formalin or absolute ethanol. Adults from the respective locality were taken as voucher specimens (Table 1) and sampled for liver and muscle tissue, that were stored in absolute ethanol or buffer solution (RNALater® Ambion/Applied Biosystems). Tissues and larval samples examined for this study are summarized in Tables 1 and 2.

Tadpoles were matched to syntopic adults based on partial sequences of the mitochondrial 16S rRNA gene. Total genomic DNA was isolated from macerated muscle or liver tissue using peqGOLD tissue DNA Mini Kit (Peqlab) or DNeasy® Blood & Tissue Kit (Qiagen) following manufacturer’s manual. The following primers were used for PCR amplification: 16SC (forward) 5'- GTRGGCCTAAAAGCAGCCAC - 3', 16SD (reverse) 5'- CTC-CGGTCTGAACCTCAGATCAGTAG - 3' (Rafe Brown, pers. comm), and 16SA-L CGCCTGTTTATCAAAAACAT. To solve amplification problems with some samples, a new forward primer was designed, 16SCH TCAAHTAAGGCCAAGCCTTA, and combined with a modified reverse primer 16SB-H CCGGTCTGAACCTCAGATCAGTAG (Palumbi et al. 1991; Vences et al. 2005). The cycling conditions for amplification were: denatur-
MEGA 4 (Tamura 2008) to choose the best fitting model of sequence evolution. Minimum evolution analysis was performed using the Wizard® SV Gel and PCR Clean-UP System (Promega). To increase concentration of PCR product for sequencing, usually, two 25µl reactions were run for each sample and excised bands were put together for cleaning. Sequencing was done in both directions by Microsynth AG (Balgach, Switzerland), LGC Genomics (Berlin, Germany) and Macrogen Inc. (Seoul, South Korea) using the same primers as for amplification. Sequence preparation, editing and management was done with BioEdit 7.0.5.2 software (Hall 1999). Chromas lite 2.01 (Technelysium Pty. Ltd., www.technelysium.com) software was used for checking trace files from the sequencers.

Adults of *Rhacophorus penanorum* were diagnosed following the character set described in comparison to all other Bornean rhacophorids in Dehling (2008). Adult *R. dulitensis* were diagnosed with a combination of characters listed in Boulenger (1892) and Inger (1966), e.g.: SVL 40–45 mm; skin smooth above; pea green dorsally, with some white dots; purplish dots on head and back; purplish line from eye to eye around the snout and passing through nostrils; reddish-brown patch on each eyelid, flap above vent present, narrow dermal fringe passing along outer side of the forearm; features of hand webbing as in (Inger 1966); small cone on heal. The identification of adult *R. rufipes* followed Inger (1966): males < 40 mm SVL; web of hand reaching discs of all fingers, except for first; webbing orangish-red; lack of dermal appendages on limbs or at vent; large tympanum, at least 1.5 x width of disc of third finger; brown above with irregular dark spots; posterodorsal face of thigh pinkish-red; light line from eye to snout along canthus.

The larval DNA sequences of *Rhacophorus rufipes*, *R. penanorum* and *R. dulitensis* were compared with a data set of sympatric or related species in the Rhacophoridae (Table 1). Alignment analysis was performed using the online server of MAFFT (Katoh et al. 2002) accessible at http://align.bmr.kyushu-u.ac.jp/mafft/online/serverl. Alignments were calculated with the E-INS-i algorithm and standard parameters (manual). MAFFT was chosen for the alignment of ribosomal sequences, because MAFFT performs best in comparison with other algorithms based on maximizing sequence similarity (Morrison 2009). This optimality criterion for alignment is considered adequate in the case of DNA matching of closely related species. We tested the final alignment with jModelTest (Posada 2002) to choose the best fitting model of sequence evolution. Minimum evolution analysis was performed using MEGA 4 (Tamura et al. 2007). FigTree 1.2 (http://tree.bio.ed.ac.uk/software/figtree) and Inkscape (http://www.inkscape.org) were used for tree graphics (Fig. 4).

Colour images and scanning electron microscope (SEM) images were mildly retouched by removing dirt, adjusting contrast, and sharpness. Apple Aperture software was used for retouching and storage. The taxonomy of Frost (2011) is applied herein. We follow terms for tadpole descriptions from standard sources (e.g., Altig 2007; Anstis 2002; Grosjean 2005; McDiarmid & Altig 1999). Despite the known limitations of Gosner’s staging table (Gosner 1960; Gosner & Rossman 1960), when applied to rheophilous tadpoles (Nodzenski & Inger 1990), it is used here in the lack of a generally accepted alternative. Descriptions of colouration features were derived from on-screen representations (sRGB colour space) of digital images taken in the field of living specimens. We used publicly available colour lists (http://en.wikipedia.org/wiki/List_of_colours) for colour descriptions. Labial Tooth Row Formulas (LTRF) were applied as in Altig & McDiar mid (1999).

Lateral, ventral, and dorsal view digital images were taken with a calibrated Keyence VHX-500 digital microscopic. The following standard tadpole body measurements (Altig 2007; Grosjean 2005) were taken: BL, body length from snout to the point where the axis of the tail myotomes contacts the body wall; BH, maximal body height at trunk; BW, maximal body width; ED, eye diameter; ES, eye snout distance; IND, internarial distance (centre to centre); IOD, interorbital distance (centre to centre); LFH, lower fin height at point of maximal tail height; MTH, maximal tail height (fins included); NE, centre of naris to centre of eye distance; ODW, oral disc width; SN, distance of naris (centre) to snout; SS, snout to centre of spiracle distance; TAL, tail length = TTL-BL; TL, total length; TMH, maximal tail muscle height at body-tail junction, where ventral line of musculature meets trunk contour; TMW, maximal tail muscle width; and UFH, upper fin height at point of maximal tail height.

Further details of our procedures and general recommendations for field and lab work have been given elsewhere (Das & Haas 2011; Haas & Das 2011). For standard codes of herpetological collections, we refer to Sabaj Pérez (2012).
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Results

Molecular genetics

The final alignment of the sequences comprised 827 bp. The test by jModelTest revealed the TIM2+I+G with gamma shape = 0.5180 as the best fitting sequence evolution model following the Akaike information criterion. This model is not available in MEGA4, therefore, we selected the most complex model offered in MEGA4: Tamura-Nei with different rates among sites (gamma shape = 0.5180) and used the pairwise deletion option of gaps. The resulting minimum evolution tree (Fig. 4) illustrates the matching of 16SrDNA sequences of larval and adult samples of *Rhacophorus dulitensis*, *R. rufipes*, and *R. penanorum*, respectively. Furthermore, the tree gives a preliminary assessment of relationships of the taxa examined. The mtDNA sequences of *R. rufipes* adult and tadpole from the same site at Gunung Mulu National Park were identical (genetic distance 0.0%). Among the *R. penanorum* individuals examined, all from the same locality (Headwater of Sungei Tapin; Table 1), a genetic distance of 0.25% was detected (equaling two substitutions). The larval sample of *R. dulitensis* differed from the adults by a genetic distance of 0.1%, that is equivalent to one transversion. The genetic distance between *R. penanorum* and its sister species *R. angulirostris* Ahl, 1927 ranged from 7.1 to 7.3%, (6.0 to 6.2% uncorrected distance). *R. rufipes* had a genetic difference of 5.0 to 5.6% (4.2 to 4.5% uncorrected distance) to *R. harrissoni* Inger & Haile, 1959, which was the sister taxon in our sample.

Larval morphology

*Rhacophorus dulitensis*

**Colour in life** (Stage 38, ZMH A13093). The background colour of the body and the tail is ochre or buff brown (Fig. 1). The pigmentation is diffuse and there are melanocytes on the dorsal and lateral body. In dorsal view there is a rhomboidal figure of melanocytes, starting anterior the eyes and reaching the posterior end of the head (Fig. 1B) where it transforms into a band posteriorly and terminating at the end of the trunk.

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FIGURE 2. *Rhacophorus penanorum*. A, Stage 31 tadpole in lateral view (ZMH A10168, 394Z). Note the black spots and oranges margins of tail fins. B, Same individual in dorsal view, showing colouration features such as the golden iridocyte field between eyes. C, Same specimen, ventral view. The cup-like oral sucker is attached to the glass of the aquarium. The ventral colouration pattern (opaque silvery abdominal pigmentation, medial vena abdominalis/streak visible, bright red gills visible through skin and partly covered by silvery iridocytes) has also been found in other Bornean rheophilous tadpoles within the genus and is not unique to *R. penanorum* (UNIMAS 8954). D, *R. penanorum* adult male, day colouration. E, *R. penanorum* adult male, nocturnal colour morph.

In lateral view, the pigmentation of the trunk slightly decreases from dorsal to ventral and from anterior to posterior. Congregations of larger melanocytes can be found in some specimens in the infraorbital region and may also form a diffuse band from the anteroventral corner of the orbit to the snout, passing ventral to the nares. The epidermal layers covering the abdominal cavity superficially and laterally bear spindle-shaped epidermal melanocytes; the deeper abdominal lining also possesses melanocytes. The dark brown colouration in the lateral trunk region originates from the additive effect of both layers. The lungs are discernible through the skin in dorsal and lateral views as an arched line of spherical shiny shapes dorsal to the gut coil (Fig. 1A; Stage 38, ZMH A13093). The spiracular tube is translucent, but clearly visible against the almost black abdominal wall (Fig. 1A). Few iridocytes can be present on the spiracular tube and the lateral trunk of some specimens. The hind limbs have a darker pigmentation on the dorsal side. The pigmentation of the muscular part of the tail and of the peripheral fin areas decreases from anterior to posterior, resulting in an almost transparent distal half of the tail. Dorsal and ventral fins are mostly unpigmented. Some specimens show 2–3 circles of assembled melanocytes on the anterior part of the tail, approximately along the horizontal septum of the tail musculature. Melanocytes in the tail are assembled to a line along the vena lateralis, a line at the base of the myosepts of the musculature.
FIGURE 3. *Rhacophorus rufipes*. A, Stage 31 tadpole in lateral view (ZMH A10167). B, Same individual in dorsal view. C, Same individual in ventral view. Note the virtually unpigmented, translucent skin (small patch of iridocytes overlaying anterior parts of gut coils). D, Adult male *R. rufipes* (UNIMAS MOS0028), same locality from where tadpoles were collected.
FIGURE 4. Minimum evolution tree of Bornean members of the genus Rhacophorus (Amphibia: Anura: Rhacophoridae) based on the total alignment comprising 827 bp of 16S rDNA sequences. See text for details on methodology. Scale bar, bp changes per branch length.

The eyes are black showing scattered golden pigmentation increasingly dense towards the iris and forming a golden ring around the pupil. Golden iridophores are present posteroverentral to the eye (region of the cheek). The lateral line system and the nares are of a beige colour. This colour, albeit faded sometimes, is retained in preserved specimens.

In ventral view, the skin of head, trunk, and tail is almost completely unpigmented and translucent. Scattered melanocytes may be present in the gular region and lateral edges of the buccal area. In the latter, some iridocytes can also be present. The red gill tufts and developing forelimbs are visible through the skin in ventral view. The gut coil is clearly visible in ventral view and the gut is dark pigmented (Fig. 1C). Iridocytes form a diffuse triangular shaped area that the anterior sector perimeter of the gut coil, right posterior to the heart region (Fig. 1C).

External morphological features, shared by Stages 38–39 (ZMH A 10840). The body shape is depressed dorsoventrally, tapering to the snout. In dorsal view, the body contour is ovoid. The oral disc is subterminal in position and cannot be seen in dorsal view. The snout profile is rounded in lateral view with a characteristic elevation dorsal the nares and a bulge just dorsal to the oral disc (Fig. 1A). The tail fin rises abruptly at the trunk-tail transition so that the beginning of the tail is clearly marked in lateral view (Fig. 1A). The tail contributes 60% of the total length, where earlier larval stages had relatively longer tails than late larval stages in our sample.

The dorsal fin originates in the same level as the tail muscles, at the trunk-tail junction. The ventral fin connects broadly to the trunk. The height of the tail increases gradually, achieving its maximal extent at the level of mid-tail. The dorsal and ventral fin each make up for on third of the maximal tail height (MTH). Posteriorly the tail tapers gradually and terminates in a narrow tip. The muscular part of the tail is moderately high (50% of body depth). The width of the tail’s base equals 27–35% of the maximal trunk width.

The eyes are positioned dorsolaterally. They are placed at 20–26% of the distance between the anterior tip of the snout and the trunk-tail junction. The nares are oriented anterolaterally and are closer to the snout than to the eyes. The internarial distance (IND) is 40–52% of the interorbital distance (IOD). The spiracle is sinistral. The spiracular tube is directed posteriorly and dorsally (approx. 27° in lateral view, Fig. 1), and opens laterally. Its medial
part is fused to the abdominal wall (but forms a shallow rim); the lateral circumference of the orifice is free. The opening of the spiraculum is positioned at 60–66% of the head-trunk length. The anal siphon is dextral.

The oral disc is subterminal and can only be seen from the ventral aspect of body. The width of the oral disc is 41–54% of the maximal width of the trunk (Fig. 1C). In lateral view and at rest, the lower lip sticks out ventrally at about 90° (Fig. 1a). Papillation is present along the margin of the lower lip and lateral parts of the upper lip (Fig. 5a). The marginal papillae are arranged mostly bi-serially; papillae are short and rounded.

The labial ridges bear uniserial rows of spoon-shaped keratodonts showing fine incisions along their edges (Fig. 5). The Labial Tooth Row Formula (LTRF) is 5(2–5)/3 to 6(2–6)/3. In the upper lip only keratodont row A-1 is continuous; five divided rows follow caudally. Three undivided keratodont rows are present on the lower lip. The size of the keratodonts decreases in the upper lip from anterior to posterior rows. In the lower lip the size decreases from posterior to anterior. The beaks are well-keratinized, black, and have sharp serrations (Fig. 5B). The upper beak is broadly arched in ventral view, whereas the lower jaw sheath is V-shaped.

**FIGURE 5.** SEM images of *Rhacophorus dulitensis* (ZMH A13094; A, C, D: 481–3902; B: 481–3802) in ventral views. A, Overview of oral disc in ventral view; the anterior lip is free of papillae, except for the lateral parts. Note the presence of a 6th tooth row on only one side of the oral disc. B, Close up of the right (of body) lateral mouth angle showing the serrated jaw sheath of the upper jaw (right in image); C, Keratodonts of row A2. D, Keratodonts of row P3.

**Variation.** In a total of 43 specimens (ZMH A10838, A10837, A13093, A13094; Stages 27 to 41), counts of the number of keratodont rows on the lower lip (P1–P3) were stable, whereas the keratodont row numbers on the upper lip varied: most specimens (47%) had 5(2–5)/3. Another 28% had an additional proximal interrupted row (A6), thus, 6(2–6)/3. Nine specimens (21%) had A6 only on the right or left side of the oral disc. Two specimens had only three (both sides; Stage 33) or four/five anterior rows (left/right; Stage 37). There was no clear correlation between stage and LTRF. Minor differences in pigment patterns were discerned in the samples: the dark band from the eye to the snout was inconspicuous in some (Fig. 1A), but more pronounced in other specimen, and the circular assemblages of melanocytes at the base of the tail can be present (Fig. 1A) or absent. The density of iridocytes varies individually.
**Ecological notes.** We found *Rhacophorus dulitensis* larvae in bodies of standing water. Tadpoles were collected from sun-exposed ponds (50–150 m², depth < 0.5 m), with muddy substrate. Adult *R. dulitensis* were encountered at the edges of these ponds. At night, the tadpoles were seen to form schools and to feed at the bottom as well as from surfaces of leaves of macrophytes. Apart from adult *R. dulitensis*, frogs of the following species were encountered at these ponds: *Rhacophorus appendiculatus* (Günther, 1858), *R. pardalis* Günther, 1858, *Kaloula baleata* (Müller, 1836), *Fejervarya limnocharis* (Gravenhorst 1829), *Polypedates leucomystax* (Boie, 1829), and *Hylarana raniceps* (Peters, 1871).

**FIGURE 6.** SEM image of *Rhacophorus penanorum* (ZMH A10169). A, Overview of oral disc in ventral view; anterior keratodont rows (A1–4) and posterior keratodont rows (P1-6) visible, note that seventh keratodont row on lower lip (P7) is present only as a small medial stretch of keratodonts and not visible from this perspective; LTRF 4(4)/7. B, Close up of the jaw sheaths; orientation as in A; showing details of conspicuously blunt serration on both upper (above) and lower jaws (below). C, Keratodonts of P3–P6 in lateral part of oral disc have possess serration of free margin, however, clefts are shallow. D, On keratodonts of P1–P3 serration is present only on newly formed keratodonts (right), whereas older ones seem to lack serration possibly due to wear.

*Rhacophorus penanorum*

**Colour in life** (Stage 31; ZMH A10168; Fig. 2). The background colour is pale amber with dusting of dark brown melanocytes on the trunk (less dense on the snout) and along the muscular portion of the tail.

Between the eyes, the specimens examined had a patch of pale golden pigment cells covering the braincase and otic region. Other accumulations of pale golden iridocytes are located on the cheek region and upper abdominal region. At the cheek and the posteroventral head region, the internal gills are externally visible as red structures. Two sharply defined round, black spots are present in specimen ZMH A10168 on the dorsal tail fin, and pale spots on the lower fin. The muscular part of the tail is finely mottled with black melanocytes (Fig. 2A); the margins of
the muscular portion are slightly pronounced by these melanocytes. The edge of the upper fin and the edge of the posterior lower fin are tinted with carrot orange. In the posterior part of the tail, the fin bases are dusted with melanocytes. Myosepta are discernible.

The sclera of the eye is black with scattered golden iridocytes. The iris is densely covered with pale golden iridocytes, visible as a solid golden ring towards the pupil.

The oral disc and sub-buccal region are mostly unpigmented and translucent (Fig. 2). The gills are visible in ventral view as bright red structures. There is a patch of iridocytes ventral to each of the gill baskets. The abdomen and area ventral to the heart are silvery and opaque, the \textit{vena abdominalis} is visible as a midline streak. The gut coils are not visible.

The \textit{vena caudalis ventralis} and \textit{vena caudalis lateralis} are visible in ventral and lateral views, respectively. The ventral side of the muscular part of the tail lacks pigmentation.

The colour in preservation is lighter than in life. All silvery or golden iridocytes become invisible in preservation, thus, rendering the ventral skin translucent; the gut coils become clearly visible and the eyes turn homogeneously black (no golden dust of cells). The carrot orange colour along the tail fin margin disappears in preservation, but conspicuous spots on the tail prevail in preservation.

\textbf{External morphological features} (Stages 26–28, $n=9$, ZMH A 10168). The examined specimens share the following morphological features. \textit{Rhacophorus penanorum} larvae are of rheophilous, sucker-mouthed type, with streamlined bodies and strongly muscular, elongated tails. They are mid-size tadpoles; the maximum total length in the sample examined was 34.2 mm (Table 2); the tail accounts for 64\% (median of sample in Table 2) of the total length. In dorsal view, the body contour is pear shaped, the head broader than the trunk. There is a slight constriction of the contour behind the level of the gill region (Fig. 2B). The body is widest posterior to the eyes and dorsally depressed. The snout is greatly expanded, bearing a large sucker (oral disc) ventrally. The snout profile is convex and the tip of the snout moderately rounded in lateral view (Fig. 2A). The eyes are located dorsolaterally, set medially from the body contour in dorsal view.

The external nares are at approximately equidistant to the eyes and to the snout and lightly elevated (Fig. 2A). The nares are elliptical and face anterolaterally. The rims of the nares are smooth, lacking projections. The spiracle is sinistral, extended into a short tube, the spiracular orifice is free. The spiracle is low on the flank in lateral view (below horizontal mid-trunk line) when the tadpole is attached to the substrate (Fig. 2C). The spiracular siphon is directed at a flat angle (almost horizontally) posterodorsally.

The oral disc is ventral and, in adhesion state, is wider than the snout (Fig. 2). A multi-serial row of marginal papillae is present on the lower lip of the oral disc; the papillation along the margin of the upper (anterior) lip has a broad medial gap (Fig. 2C, 6). The oral disc margins lacks lateral indentations between upper and lower lip sections. Marginal, distal papillae are fairly long (length $>2x$ diameter), blunt and adjoining. There is no clear differentiation between marginal and submarginal papillae. Proximal papillae, however, are gradually reduced in length, down to a knob-like shape.

The labial ridges bear uniserial keratodont rows. The Labial Tooth Row Formula (LTRF) ranges from 4(4)/6 to 4(4)/7 in the sample examined. If present, row seven on lower lip (P7) is discontinuous; irregular gaps may also be present in row A1 (Fig. 6). Rows A3 and P1 show a slight indentations medially, however, without a clear gap between keratodont-bearing ridges and medial keratodonts. Despite the indentation, we count these as undivided rows in the absence of a clear gap. Upper and lower lip keratodont rows are long, span most of the oral disc, and almost meet in the lateral parts of the oral disc (Fig. 2C, 6A). Single keratodonts in the distal rows of upper and lower lip (A1, P4–7), respectively, are substantially smaller than keratodonts in the rows adjacent to the mouth (A2–4, P1–3; Fig. 2C, 6A). The latter have fine serration that seems to wear off in feeding action (Fig. 6D). The edge of the upper beak is of sigmoid shape (Fig. 6B), the lower jaw shallow U-shaped. The jaws are strongly developed and keratinized to approximately 80\% of the jaw height, and undivided. The edges of the jaw sheaths are coarsely serrated; the serration is blunt (Fig. 6B).

The tail musculature is strong and almost as high as the trunk (in lateral view) at the trunk-tail junction and it reduces its height gradually distally. The dorsal tail fin expands at the trunk-tail junction. The dorsal fin is higher than the ventral fin. The tail reaches its maximum height at about mid-tail position. The edges of the fins are shallowly convex in lateral view. The tail fins taper gradually to a moderately rounded tip (Fig. 2). The anal siphon is dextral.
### TABLE 2. Body measurements of *Rhacophorus dultilinus*, *R. penanorum*, and *R. rufipes* larvae in mm. ZMH, Zoological Museum Hamburg; ***, not measured due to damage. Stages according to Gosner (1960), however see Nodzenski & Inger (1990) for limitations of applying this staging table to rheophilous tadpoles, BL, head-body length from snout to the point where the axis of the tail myotomes contacts the body wall; BH, maximal body height at trunk; BW, maximal body width; ED, eye diameter; ES, eye snout distance; IND, intermaxilar distance (center to center); IOD, interocular distance (center to center); LHF, lower fin height at point of maximal tail height; MTH, maximal tail height (fins included); NE, center of naris to center of eye distance; ODW, oral disc width; SN, distance of naris (center) to snout; SS, snout to center of spiracle distance; TAL, tail length=TTL-BL; TTL, total length; TMH, maximal tail muscle height at body-tail junction, where ventral line of musculature meets trunk contour; TMW, maximal tail muscle width; UFH, upper fin height at point of maximal tail height.

<table>
<thead>
<tr>
<th>Species</th>
<th>Stage</th>
<th>n</th>
<th>BL</th>
<th>BH</th>
<th>ED</th>
<th>ES</th>
<th>IND</th>
<th>IOD</th>
<th>NE</th>
<th>ODW</th>
<th>SN</th>
<th>SS</th>
<th>TAL</th>
<th>TMH</th>
<th>TMW</th>
<th>TTL</th>
<th>UFH</th>
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<tr>
<td><em>dultilinus</em></td>
<td>2</td>
<td>26</td>
<td>5.42 ± 0.31</td>
<td>2.64 ± 0.23</td>
<td>3.19 ± 0.42</td>
<td>0.91 ± 0.10</td>
<td>1.69 ± 0.18</td>
<td>1.02 ± 0.43</td>
<td>2.40 ± 0.50</td>
<td>0.49 ± 0.63</td>
<td>2.40 ± 0.36</td>
<td>1.35 ± 0.50</td>
<td>2.00 ± 0.97</td>
<td>7.59 ± 0.23</td>
<td>1.13 ± 0.18</td>
<td>1.43 ± 0.18</td>
<td>1.09 ± 0.28</td>
</tr>
<tr>
<td><em>penanorum</em></td>
<td>2</td>
<td>26</td>
<td>6.37 ± 0.24</td>
<td>2.94 ± 0.42</td>
<td>3.66 ± 0.66</td>
<td>0.10 ± 0.13</td>
<td>1.94 ± 0.35</td>
<td>1.23 ± 0.29</td>
<td>2.78 ± 0.45</td>
<td>0.56 ± 0.10</td>
<td>2.93 ± 0.75</td>
<td>3.18 ± 0.18</td>
<td>1.91 ± 0.28</td>
<td>8.08 ± 0.13</td>
<td>3.30 ± 0.51</td>
<td>13.72 ± 0.24</td>
<td>13.82 ± 0.22</td>
</tr>
<tr>
<td><em>rufipes</em></td>
<td>2</td>
<td>26</td>
<td>8.75 ± 0.44</td>
<td>4.21 ± 0.64</td>
<td>4.96 ± 0.64</td>
<td>0.94 ± 0.16</td>
<td>2.60 ± 0.25</td>
<td>1.54 ± 0.98</td>
<td>3.60 ± 0.85</td>
<td>0.81 ± 0.09</td>
<td>4.11 ± 0.59</td>
<td>1.61 ± 0.98</td>
<td>2.21 ± 0.31</td>
<td>0.93 ± 0.13</td>
<td>5.21 ± 0.57</td>
<td>12.70 ± 1.25</td>
<td>2.62 ± 0.52</td>
</tr>
</tbody>
</table>

**Note:** ZMH A10839: 13 RHACOPHORID TADPOLES FROM SARAWAK.
Variation. Little can be said about the natural variation in morphological features in *Rhacophorus penanorum* tadpoles. The most peripheral lower lip row seem more incomplete in early larval stages than in more advanced tadpoles, and the seventh row on the lower lip was absent in the smallest individual examined. From the size and stages represented in our sample, we conclude that the dot pattern on the tail fins starts with two dots on the upper fin in early stages and increases to three dots on the upper and one dot on the lower fin in more advanced stages. Because *R. penanorum* larvae are highly specialized rheophiles, their hindlimb development may be altered heterochronically in comparison to other species (Nodzneski & Inger 1990), thus, rendering predictions about maximal size difficult, based on individuals examined and their Gosner stages.

Ecological notes. The tadpoles were found in shallow stony pools with moderate current, which are connected by steep, narrow channels on bedrock. Within the pools, *R. penanorum* tadpoles are syntopic with larvae of *Leptobrachium* sp., *Limnonectes kuhlii* (Tschudi, 1838), and *Xenophrys dringi* (Inger, Stuebing & Tan, 1995). Further, the tadpole community of this stream comprises *Leptobrachella brevicrus* Dring, 1983 and *Leptolalax dringi* Dubois, 1987 larvae, both of which burrow in the gravel of the pool substrate. *Ansonia* sp. tadpoles were present in sections with faster current in the connecting channels or below water-chutes. Adults of *R. penanorum* were found in relatively low vegetation (1.5 to 3 m) along the streams. Adult frogs of the following species were detected in the immediate vicinity: *Ansonia hanitschi* Inger, 1960, *A. torrentis* Dring, 1983, *Leptobrachella brevicrus*, *Leptobrachium* sp., *Leptolalax dringi*, *Limnonectes kuhlii*, *Meristogenys amoropalamus* Matsu, 1986, *M. kinabaluensis* (Inger, 1966), *Philautus mjobergi* Smith, 1925, *Philautus everetti* (Boulenger, 1894), *Staurois tuberilinguis* Boulenger, 1918, and *Xenophrys dringi*.

*Rhacophorus rufipes*

Colour in life (Stage 31; ZMH A10167; Fig. 3). The basic colouration of the body dorsum and tail is grey. The pigmentation is diffuse, and there are no sharply defined spots or blotches. The grey background colour is modified with a slight tint of olive on the head and trunk dorsally. There is a superficial layer of small, spindle-shaped epidermal melanocytes and deeper body layers with stellate or irregular polygonal melanocytes. The overall colouration stems from the additive interaction of both layers. For example, around the eyes there are only epidermal melanocytes, whereas the cheeks bear a deeper layer (Fig. 3). In dorsal and lateral views, the colouration is darkest along the abdominal cavity. The red branchial structures and developing forelimbs are partially visible through the skin in lateral view. There are only a few iridocytes that appear in light blue or gold; they are located in small scattered patches between the eye and the spiracle, along the spiracular tube, on the lower cheek, and on the snout close to the oral disc. The spiracular tube is mostly translucent, but clearly visible against the almost black abdominal wall.

The pigmentation of the trunk extends seamlessly onto the muscular part of the tail, but with a more brownish hue. The peripheral areas of the tail fins are without pigmentation and are clear. However, the dorsal tail fin bears pigmentation along its entire base. The ventral fin is almost entirely clear, except for melanocytes in the distal third of the tail, along the neighbouring muscular tail portion. The *vena caudalis lateralis* is visible as a red line in living specimens due to the erythrocytes in it, but it is not particularly lined with melanocytes.

The ventral body surface is mostly unpigmented and translucent, except for areas below the cheek, where scattered melanocytes in deeper body layers reach the venter. The translucency of the ventral skin is combined with slight iridescence. The gut coil is clearly visible in ventral view. The gut is dark pigmented. The gills and the heart are visible through the clear ventral skin in red. They contrast with the pale sub-buccal region anteriorly and the dark gut coils posteriorly.

The background iris colour is black with dense scattered golden and coppery pigment cells. Around the pupil, the golden pigmentation fuses into a closed ring, which lines the pupil. The skin of the oral sucker is without pigmentation. The colour in preservation shows the same markings as living specimens, however, melanocytes become paler and iridocytes (any silver or golden cells) disappear.

External morphological features (Stages 33–34, n=4, ZMH A 10167). A medium sized tadpole (TTL 24.79 mm at Stage 34; Table 2), with long tail (55–60% of total length). The body shape is depressed and ovoid in lateral view, tapering to the snout. In dorsal view, the body contour is slightly inverse pear shaped. The body is widest at the gill region (posterior to eyes) in dorsal view. Between the gill part of the body and the trunk there is a shallow constriction of the body contour. The body is moderately depressed dorsoventrally.
The tail shape is moderately arched in lateral view, tapering in the posterior two thirds with straight contour lines into narrow, pointed tip; a flagellum is not formed. The muscular part of the tail is moderately high (49–54% of body depth). The dorsal fin starts at the trunk-tail junction, the ventral fin connects broadly to the trunk. The maximum height of the tail (fins included) is 37–43% of the tail length.

The eyes are positioned dorsally, at clear distance from the body contour in dorsal view. The nares are closer to the snout than to the eyes and are spaced moderately wide (IND = 62–69% IOD). The nares are round. The rims of the nares are elevated posteriorly and flat anteriorly. The spiracle is sinistral. The spiracular tube opens posterolaterally and is below the longitudinal body axis in lateral view (Fig. 3). The medial part of the spiracular orifice is attached to the abdominal wall. Gut coils are concentric. The anal siphon is dextral.

**FIGURE 7.** SEM image of *Rhacophorus rufipes* (ZMH A10167) in ventral view. A–C taken with the same orientation of the specimen. A, Overview of oral disc in ventral view; the anterior lip is free of papillae, except for the lateral parts. B, Close up of the jaw sheaths; orientation as in A; showing details of sharp serration on both upper (above) and lower jaws (below). C, Keratodonts of P1. D, Keratodonts of A1.

The oral disc is subterminal (Fig. 7A). The marginal papillation of the oral disc is present on the lower lip and lateral parts. There is a broad medial gap in the papillae row of the dorsal lip. Marginal papillae are arranged bilaterally on the lower lip. The lateral part of the upper lip bears a uniserial row of marginal papillae plus a short row of submarginal papillae. The oral disc margins possess lateral indentations between upper and lower lips. Papillae are short (length ≤ 2x diameter), blunt and adjoining (Fig. 7A). The labial ridges bear uniserial keratodont rows. The Labial Tooth Row Formula (LTRF) is 4(2–3)/3 or 5(2–4)/3. Generally, distal keratodont rows are long and extend far laterally on both the upper and lower lip, however, the keratodont rows of the divided series become shorter towards the mouth. Row A5, if present, is very short and bears only a few keratodonts (one specimen, Stage 34). The keratodonts are spoon-shaped with fine incisions along their edges (Fig. 7C–D). The beaks are well-keratinized and have sharp serrations. The upper beak is almost straight in ventral view, whereas the lower jaw sheath is V-shaped (Fig. 7A–B). They are keratinized to about half of the total jaw height.
Variation. The most proximal, fifth keratodont row of the upper lip was present only in the most advanced specimen (Stage 34).

Ecological notes. We encountered *Rhacophorus rufipes* adults in Kerangas forests (Bornean heath forests) on numerous occasions. Tadpoles were collected from a pool of water created by an uprooted tree, which was partially covered by a fallen tree trunk. Tadpoles of *R. rufipes* were sighted hovering at the surface of the pool by night. The water was tea-coloured and peaty; its depth was ca. 50 cm, the pool area ca. 1 m². The bottom of the pool was covered with leaf litter and a thick layer of humic debris. Adults of *R. rufipes*, some in amplexus, were spotted in the vicinity of the pool. Other species encountered at the site with *R. rufipes* included: *Calluella flava* (Kiew, 1984), *Kalophrynus cf. heterochirus* Boulegger, 1900, *Limnonectes malesianus* (Kiew, 1984), *Megophrys nasuta* (Schlegel, 1858), *Metaphrynella sundana* (Peters, 1867), *Microhyla nepenthicola* Das & Haas, 2010, *Pelophryne cf. guentheri* (Boulegger, 1882), *Philautus kerangae* Dring, 1987, and *Polypedates colletti* (Boulegger, 1890).

Discussion

*Rhacophorus penanorum* was described recently from Gunung Mulu by Dehling (2008). Although the adults of this species are morphologically similar to *R. angulirostris*, they differ in a number of colouration features, some morphological characters, and call parameters. It remained unclear to what extend both species are related. Our genetic analysis recovered the two species as closest known relatives (sister taxa) within the sample of Bornean rhacophorids examined herein. They showed a difference of 7.1–7.3 % in their partial sequences of the 16S rDNA gene. *R. angulirostris* and *R. penanorum* must, thus, be considered allopatric sister taxa with a disjunct distribution in higher elevations in northern Borneo.

Because closely related frogs tend to have similar tadpoles, it could be expected that the tadpoles of *Rhacophorus penanorum* resemble those of *R. angulirostris* (see Malkmus et al. 2002). We identified tadpoles of both species by genetic matching with the respective adult. Indeed, tadpoles of both species are streamlined and rheophilous, possessing oral discs modified to form suckers that are adaptive for clinging on to hard substratum, such as rocks, and strong tails to maneuver in the current. Superimposed line drawings of two specimens, *R. angulirostris* (Stage 37) and *R. penanorum* (Stage 31), tentatively suggest larval proportions (Fig. 8). When scaled to a baseline set by the centre of the eye and the end of the body landmarks, snout and tail were relatively longer in *R. penanorum* than in *R. angulirostris*. Larger series are needed to corroborate this preliminary evidence in the future. A general difference between the two species may be preference for faster waters in larval *R. penanorum* compared to those of *R. angulirostris*.

![FIGURE 8. Line drawing superimposition of *Rhacophorus penanorum* (ZMH A10168; Stage 31; full line) and *R. angulistrois* (ZMH A 13098; Stage 37; dashed line), drawn from photographs in dorsal (left) and lateral (right) views. Drawings were scaled (normalized) to equal distances between centre of eye and end of body (vertical lines). The superimposition may suggest that the snout and the tail are relatively longer in *R. penanorum* than in the otherwise similar tadpole of *R. angulistrois*. However, the superimposition was based on only one specimen per species for which adequate photographs of living tadpoles had been available and future data must corroborate this preliminary evidence.](image-url)}

Malkmus *et al.* (2002) described the LTRF of *R. angulistrois* as 4(3–4)/4–5, however, their Fig. 200 seems to shows a continuous, gap-free row A3. Bearing in mind the limited knowledge on the variation of tadpole features in both species, we can tentatively conclude that the tadpole of *R. penanorum* has at least one additional row of keratodons on the lower lip compared with *R. angulistrois*. Furthermore, in the samples examined, *R. penanorum* differs from *R. angulistrois* in the more intense carrot orange tinge at the dorsal fin margin and tail tip. More information on variation is needed to corroborate the nature of the observed interspecific differences in these sister-taxa

Malkmus *et al.* (2002) described the LTRF of *R. angulistrois* as 4(3–4)/4–5, however, their Fig. 200 seems to shows a continuous, gap-free row A3. Bearing in mind the limited knowledge on the variation of tadpole features in both species, we can tentatively conclude that the tadpole of *R. penanorum* has at least one additional row of keratodons on the lower lip compared with *R. angulistrois*. Furthermore, in the samples examined, *R. penanorum* differs from *R. angulistrois* in the more intense carrot orange tinge at the dorsal fin margin and tail tip. More information on variation is needed to corroborate the nature of the observed interspecific differences in these sister-taxa.
tadpoles. The conspicuous ventral colouration in life with the opaque silvery abdominal part, medial streak embedding the *vena abdominalis*, and the bright red gills visible through the transparent skin (Fig. 3C), is not unique to *R. penanorum*. It is also present in *R. angulirostris* (Malkmus et al. 2002: Fig. 200), *R. gauni*, (pers. obs.) and possibly other related species. Our limited sample did not allow any conclusions about the intraspecific variability or the ecological plasticity of these tadpoles.


Among the rhacophorid tadpoles from Borneo that have been identified unequivocally and that have cup-like mouths, *Rhacophorus penanorum* and *R. angulirostris* are the only known species with distinct black, round spots on the tail fins. All known Bornean rhacophorid larvae have at least four keratodont rows on upper and three keratodont rows on the lower lip, respectively (Inger 1985; Leong & Chou 1999; Leong 2004; Malkmus et al. 2002). This minimum count of rows is found, for example, in *R. appendiculatus* (see Leong 2004; pers. obs.), *R. kajau* (Dring 1983b), and *R. gauni* (Malkmus et al. 2002, pers. obs; but see Inger 1985, who listed eight upper keratodont rows for *R. gauni*). Some species have increased the number or upper lip keratodont rows, such as *R. harrisoni* (5), *R. nigropalmatus* (6–7), and *R. pardalis* (6–7) (see Inger 1985; Malkmus et al. 2002; pers. obs.); *R. cyanopunctatus* is remarkable in having up to nine keratodont rows on the upper lip (LTRF 9(5–9)/3(1); Leong 2004). In contrast, species in the clade combining *R. gauni, R. penanorum* and *R. angulirostris* (Fig. 4; tadpole of *R. belalongensis* unknown) have either the minimal number (*R. gauni*, pers. obs.) or increased the number of keratodont rows on the lower lip.

The tadpole of *Rhacophorus rufipes* belongs to a lentic-benthic to lentic-nectonic tadpole type (Altig & Johnston 1989) by its body proportions, fin shape, oral disc features, and behaviour. In general body shape, the tadpoles of *R. rufipes* resemble those of its relatives (Fig. 4) *R. harrisoni* and *R. pardalis*. However, *R. rufipes* differs from *R. harrisoni* in: less dark pigmentation (vs. very dark pigmented), 4 upper lip keratodont rows (vs. 5), less deeper body, and use of standing water habitat (vs. treeholes). It can be distinguished from *R. pardalis* most easily by the lower number of keratodont rows in the upper lip (6–7 in *R. pardalis*), colouration (*R. pardalis* being usually beige to yellow in ground colour), and lack of black cheek and head roof markings (usually present in *R. pardalis*) (Inger 1985).

Tadpoles of *Rhacophorus dulitensis* are lentic-benthic, mostly observed at the bottom of ponds and among aquatic vegetation. *R. dulitensis* tadpoles differ from *R. pardalis* (that may occur in the same pond) by their smaller size and presence of the narial bump in the snout profile (Fig. 1A), and absence of black cheek markings; however, we found the cheek markings to be absent in some *R. pardalis* populations. In the samples we collected, all *R. dulitensis* specimens had a peculiar bump in the profile of the snout in lateral view; this character in combination with moderate size, iris colour and body colouration makes the tadpole easy to recognize in the field. However, Inger’s (1985) description of the larva or *R. dulitensis* departs from ours in several ways. He gave the head-body length as 13.2–16.3 mm (Stages 28 to 36) as compared to 5.4–10.4 mm (Stages 26–39) in our sample; the maximal total length was 42.7 mm (Stage 35) in Inger’s account and 25.9 mm (Stage 38) in ours; and LTRF 6(2–6)/3(1) (Inger 1986) versus 5(2–5)/3 to 6(2–6)/3 in our sample. His notes on colour in life do not fit our specimens in any of the character states: “pale grayish brown with a black vertical wavy line in the middle of the tail beyond which the tail is darker; underside of head-body whitish.” (Inger 1985:75). Although little is known on variation in any of the Bornean tadpoles, these differences in size and colouration appear substantial to us and may indicate two different taxa. Neither our, nor Inger’s sample were from the type locality, Gunung Dulit, Miri Division, Sarawak (N03.33°, E114.15°). Inger (1985:75) identified his specimens according to character states at Stage 42: "...webbing of feet and hands as in *dulitensis* and the diagnostic transverse supra-anal fold." The latter, however, has also been known as a character state of *R. reinwardtii* (sensu Inger, 1966), and the difference in webbing between *R.*
*R. dulitensis* and *R. reinwardtii* are restricted to the third finger and may not be fully diagnostic in Stage 42 metamorphs. Inger's (1985) description in size, colour, and LTRF fits specimens that we collected at Tawau Hills National Park and identified genetically as *R. reinwardtii* (Haas & Hertwig, unpublished data). All evidence from larval and adult genetic sequences considered, we maintain that our genetic evidence was unambiguous and the larval form presented herein is the tadpole of *R. dulitensis*.

A preliminary determination key for rhacophorid tadpoles from Borneo was given by Inger (1985), however, it will be in need for an update as more previously unknown larval forms become described.

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