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First record of the Vietnam Flying Frog, *Rhacophorus calcaneus* Smith, 1924, from Khanh Hoa Province, including the first molecular identification and morphological description of larval stages

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Abstract: We herein report the first record of the Vietnam Flying Frog, *Rhacophorus calcaneus* Smith, 1924, which is previously known only from Dak Lak and Lam Dong provinces, from Hon Ba Nature Reserve, Khanh Hoa Province, Vietnam. We further describe the larval morphology of the species for the first time, based on a tadpole collection from Hon Ba which was genetically matched with topotypic metamorphosed *Rhacophorus* and *R. calcaneus* from Dak Lak Province. Morphological characters of the tadpoles (Gosner stages 32, 36, 37 and 41) are as follows: Body and tail brown with numerous dark spots, ventral surface whitish. Body medium-sized, slightly depressed, elliptical in shape. Eyes of moderate size, located dorsolaterally. In lateral view, spiracle single, sinistral; lower and upper tail fin nearly of same size. Oral disc anteroventrally positioned, of trapezoidal shape and laterally emarginated, surrounded by papillae. Keratodont row formula (LTRF): 7(2-7)/3(1). Larvae are exotrophic, lentic; benthic; larval type after Orton (1953) is type IV.

Keywords: Rhacophoridae - *Rhacophorus calcaneus* - DNA barcoding - tadpole description - Vietnam - Tay Nguyen Plateau.

INTRODUCTION

The Vietnam Flying Frog, *Rhacophorus calcaneus* Smith, 1924, is endemic to the Annamite Mountains of southern Vietnam (type locality: Langbian peaks, see Smith, 1924). The taxonomic history of the *R. calcaneus* species group is complicated (Orlov *et al.*, 2012). *R. calcaneus* was previously extensively confused with the recently described *R. robertingeri* (Inger *et al.*, 1999), and another recently described species, *R. chuyangsinensis* (Orlov *et al.*, 2008), which was subsequently synonymized with *R. calcaneus* (Orlov *et al.*, 2012). According to Frost

(2017), the latter species is known with certainty only from Chu Yang Sin National Park, Krong Bong and Lak districts, Dak Lak Province, and from Bidoup - Nui Ba National Park, Lam Dong Province. Because of the complicated taxonomic history, not much is known about the natural history of *R. calcaneus* sensu stricto (Van Dijk & Nguyen, 2009). We herein report the species for the first time from Khanh Hoa Province. Based on tadpoles collected in Hon Ba Nature Reserve, which were genetically matched with topotypic metamorphosed frogs and *R. calcaneus* from Dak Lak Province (Nguyen

et al., 2014) and subsequently identified as *R. calcaneus* we herein further describe the larval morphology of the species for the first time.

MATERIAL AND METHODS

Material examined: Collected specimens were deposited in the herpetological collection of the Vietnam National Museum of Nature (VNMN), Vietnam Academy of Science and Technology in Hanoi. One adult male (VNMN 06317), one adult female (VNMN 0965), one juvenile (VNMN 0969) and six *Rhacophorus* tadpoles (VNMN 06318-06323) were collected in Hon Ba Nature Reserve, Khanh Hoa Province, Vietnam. Two adult males of *R. calcaneus* (VNMN 0608, 0610) from Chu Yang Sin National Park, Dak Lak Province, Vietnam (Nguyen *et al.*, 2014) served for morphological and molecular comparisons (Table 1).

Molecular analysis: For the molecular analysis we used the protocols of Kuraishi *et al.* (2013), modified by Nguyen *et al.* (2014), for DNA extraction, amplification, and sequencing. Fragments of the mitochondrial DNA gene 16S rRNA was amplified using the primers following Kuraishi *et al.* (2013). Chromas Pro software (Technelysium Pty Ltd., Tewantin, Australia) was used to edit the sequences, which were aligned using MAFFT version 7 (Kato & Standley, 2013) with default setting (FFT-NS-2 algorithm). We then checked the initial alignments by eye. Phylogenetic trees were constructed by using maximum likelihood (ML) and Bayesian

inference (BI). Prior to ML and Bayesian analyses, the optimum substitution models for the 16S rRNA partition was selected by Kakusan 4 (Tanabe, 2011), based on the Akaike information criterion (AIC). We performed ML analyses with Treefinder version March 2011 (Jobb, 2011), while we estimated BI and Bayesian posterior probabilities (BPP) with MrBayes v.3.2.1 (Ronquist & Huelsenbeck, 2003). The best model selected for ML was the general time reversible model (GTR: Tavaré, 1986) with a gamma shape parameter (G: 0.255 in ML and 0.259 in BI). The BI summarized two independent runs of four Markov Chains for 10,000,000 generations. A tree was sampled every 100 generations and a consensus topology was calculated for 70,000 trees after discarding the first 30,001 trees (burn-in = 3,000,000). We checked parameter estimates and convergence using Tracer version 1.5 (Rambaut & Drummond, 2009). The strength of nodal support in the ML tree was analyzed using non-parametric bootstrapping (MLBS) with 1,000 replicates. We regarded tree nodes in the ML tree with bootstrap values of 75% or greater as sufficiently supported (Hillis & Bull, 1993), and nodes with a BPP of 95% or greater as significant in the BI analysis (Leaché & Reeder, 2002). Pairwise comparisons of uncorrected sequence divergences (p-distance) were calculated for 16S rRNA fragments. Resulting DNA sequences from this analysis were submitted to GenBank (Accession Numbers: LC375231 to LC375239; Table 1). For tadpole matching and specific identification, sequences of the 16S rRNA of the tadpoles from Hon Ba Nature Reserve, Khanh Hoa Province, were compared with

Table 1. Samples of Vietnamese *Rhacophorus* and other rhacophorid species used for DNA analysis in this study together with information on voucher, collection locality and GenBank accession numbers. Voucher abbreviations: KIZ = Kunming Institute of Zoology; KUHE = Graduate School of Human and Environmental Studies, Kyoto University; VNMN = Vietnam National Museum of Nature.

Sample No.	Species	Voucher	Locality	Accession No.	Reference
1.	<i>Rhacophorus calcaneus</i>	VNMN 4096	Vietnam, Dak Lak, Chu Yang Sin	LC010573	Nguyen <i>et al.</i> (2014)
2.	<i>R. calcaneus</i>	VNMN 4097	Vietnam, Dak Lak, Chu Yang Sin	LC010574	Nguyen <i>et al.</i> (2014)
3.	<i>R. calcaneus</i>	VNMN 6317	Vietnam, Khanh Hoa, Hon Ba	LC375239	This study
4.	<i>R. calcaneus</i>	VNMN 0965	Vietnam, Khanh Hoa, Hon Ba	LC375237	This study
5.	<i>R. calcaneus</i>	VNMN 0969	Vietnam, Khanh Hoa, Hon Ba	LC375238	This study
6.	Tadpole 01	VNMN 6318	Vietnam, Khanh Hoa, Hon Ba	LC375231	This study
7.	Tadpole 02	VNMN 6319	Vietnam, Khanh Hoa, Hon Ba	LC375232	This study
8.	Tadpole 03	VNMN 6320	Vietnam, Khanh Hoa, Hon Ba	LC375233	This study
9.	Tadpole 04	VNMN 6321	Vietnam, Khanh Hoa, Hon Ba	LC375234	This study
10.	Tadpole 05	VNMN 6322	Vietnam, Khanh Hoa, Hon Ba	LC375235	This study
11.	Tadpole 06	VNMN 6323	Vietnam, Khanh Hoa, Hon Ba	LC375236	This study
12.	<i>R. reinwardtii</i>	KIZ-Rao081205	Malaysia	JX219443	Li <i>et al.</i> (2012)
13.	<i>Kurixalus odontotarsus</i>	KIZ 201307012	China, Yunnan	AB933302	Nguyen <i>et al.</i> (2014)

these from topotypic adults and a topotypic juvenile together with one male and one female of *R. calcaneus* from Chu Yang Sin National Park, Dak Lak Province (Nguyen *et al.*, 2014). Morphological identification of adult frogs followed Smith (1924), Orlov *et al.* (2008, 2012) and Tran *et al.* (2011).

Morphological analysis: Terminology for morphometric data and abbreviations followed Altig & McDiarmid (1999) and Grosjean (2005). Tadpoles were staged according to Gosner (1960). The labial tooth row formula (LTRF) was determined according to Altig & McDiarmid (1999) and for general larval types see Orton (1953). Tadpoles were photographed alive in a cuvette, subsequently euthanized with ethyl acetate and preserved in 70% ethanol. Prior to preservation, a piece of the lower tail fin and tail musculature was taken from each tadpole and preserved in a 98% ethanol solution for further genetic analysis.

The measurements were taken with a dial calliper to the nearest 0.1 mm. Abbreviations are as follow: BH: maximum body height; BL: body length; BW: maximum body width; HT: maximum tail height; LF: maximum height of lower tail fin; IND: internarial distance (measured between centers of narial apertures); PP: interpupilar distance (measured between centers of pupils); RND: rostro-narial distance (measured between the tip of the snout and the center of the nostril); SS: distance from tip of snout to opening of spiracle; SU: distance from the tip of snout to insertion of upper tail fin; TL: total length; TAL: tail length; UF: maximum height of upper tail fin; VT: distance from vent to tip of tail; TMH: height of the tail musculature at base; TMW: width of tail musculature at base; FL: forelimb length; HLL: hindlimb length; SVL: Snout-vent length; ODW: oral disc width; ED: maximum diameter of eye (horizontal); LTRF: Labial Tooth Row Formula with A (number of rows on anterior labium) and P (number of rows on posterior labium); NPD: nario-pupilar distance (measured between the center of the nostril and the center of the pupilla).

RESULTS

Phylogenetic analyses: Aligned, combined sequences of 16S rRNA yielded a total of 457 characters. Of 457 nucleotide sites, 2 were variable within the in-group. The ML and Bayesian analyses produced topologies with $-\ln L = 1029.838$ and 1057.656 , respectively. Phylogenetic analyses employing ML and BI methods yielded identical topologies, and only the ML tree is presented (Fig. 1). The comparisons of the resulting 457 bp long fragment of the 16S rRNA between the tadpoles and the topotypic frogs from Hon Ba Nature Reserve, Khanh Hoa Province, Vietnam, and adults from Chu Yang Sin National Park, Dak Lak Province, Vietnam and, showed only a negligible single base pair difference (corresponding to 0.4%), and

thus an unambiguous specific assignment of the tadpoles both to the topotypic frogs and adult *R. calcaneus* from Dak Lak Province is guaranteed. Furthermore, the adults from Hon Ba Nature Reserve morphologically well agreed with the original description of *R. calcaneus* provided by Smith (1924), and with subsequent reviews (Orlov *et al.*, 2008; Tran *et al.*, 2011; Orlov *et al.*, 2012), as well as with adults from Chu Yang Sin National Park, Dak Lak Province, from where *R. calcaneus* already was proven to occur with certainty. Thus the identity of the tadpoles from Hon Ba Nature Reserve to represent *R. calcaneus* is sufficiently proven.

Morphological analysis: The collected tadpoles from Hon Ba Nature Reserve, Khanh Hoa Province, Vietnam were in the developmental stages 32, 36, 37, 41, and 42 according to Gosner (1960) (Tabs 2-3, Figs 2-3). Larvae are exotrophic, lentic: benthic; larva type IV after Orton (1953); for comparisons with larvae of other *Rhacophorus* species occurring in Vietnam (Tab. 4). In the following we provide a detailed description of a larva of *Rhacophorus calcaneus* at stage 32 (VNMN 06318).

Dorsal view: Body somewhat elliptically protracted, with a slightly pointed snout; widest portion being at midbody; maximum body width 0.68 times of body length (BW 11.48 mm; BL 16.86 mm). Nares small, rounded, positioned dorsolaterally in anterolaterally direction; naris closer to the pupil than to the tip of snout, rostro-narial distance 1.11 times of nario-pupilar distance (RND 3.2 mm; NPD 2.88 mm); internarial distance about 0.44 of interpupilar distance (IND 2.83 mm; PP 6.34 mm). Eyes of moderate size (ED 1.36 mm); eye diameter 0.12 times of maximum body width and 0.08 times of body length, positioned dorsolaterally, directed more laterally than anteriorly, slightly bulging. The tail musculature is of moderate size, tail muscle width 0.28 times of body width (TMW 3.19 mm; BW 11.48 mm).

Lateral view: Body slightly depressed, body height 0.5 times of body length (BH 8.51 mm; BL 16.86 mm); spiracle single, sinistral, ventrolaterally positioned at midbody, oriented in posterodorsal direction and entirely attached to the body; opening of the spiracle oval. Distance from tip of snout to opening of spiracle 0.6 times of body length (SS 10.05 mm; BL 16.86 mm).

Tail tapered and long; body length 0.64 times of tail length (BL 16.86 mm; TAL 26.26 mm); tail musculature moderately developed; maximum height of tail musculature 0.67 times body height and 0.46 times maximum tail height (TMH 5.7 mm; BH 8.51 mm; HT 12.43 mm). Tail musculature from the proximal to its distal half in parallel, then gradually tapering, reaching the tip of the tail. Upper and lower tail fins almost equal in size (LF 4.15 mm; UF 4.16 mm), enlarged, maximum at the end of tail; tip of tail rounded; distance from tip of snout to insertion of upper tail fin 0.83 times body length (SU 13.99 mm; BL 16.86 mm); maximum height of upper

and lower tail fin 0.33 times of maximum tail height (UF 4.16 mm; LF 4.15 mm; HT 12.43 mm). Vent tube dextral, located directly at end of body between limbs; posterior part of vent tube coadunate with lower tail fin, moderate size, margin thick.
Lateral line organ present and well developed on body and along the apex of the caudal musculature.
Oral disc: Anteroventrally positioned, of nearly triangular shape in expanded state (see Fig. 2), and laterally emarginated; two short rows of papillae situated laterally

of the lower labium and margin of the mouth present, which is restricted by two side corners of the mouth; absence of papillae on margin of the upper labium; lower lip with medial gap; in preservative, tip of the papillae rounded, white; base of the papillae brown. Jaw sheaths black and convex; both upper and lower jaw sheaths serrated; upper jaw sheath semicircular and narrow, and stretched into a wide arch; lower jaw sheath V-shaped. Mouth part medium-sized (ODW 3.8 mm); oral disc width 0.33 times of maximum body width and 0.23

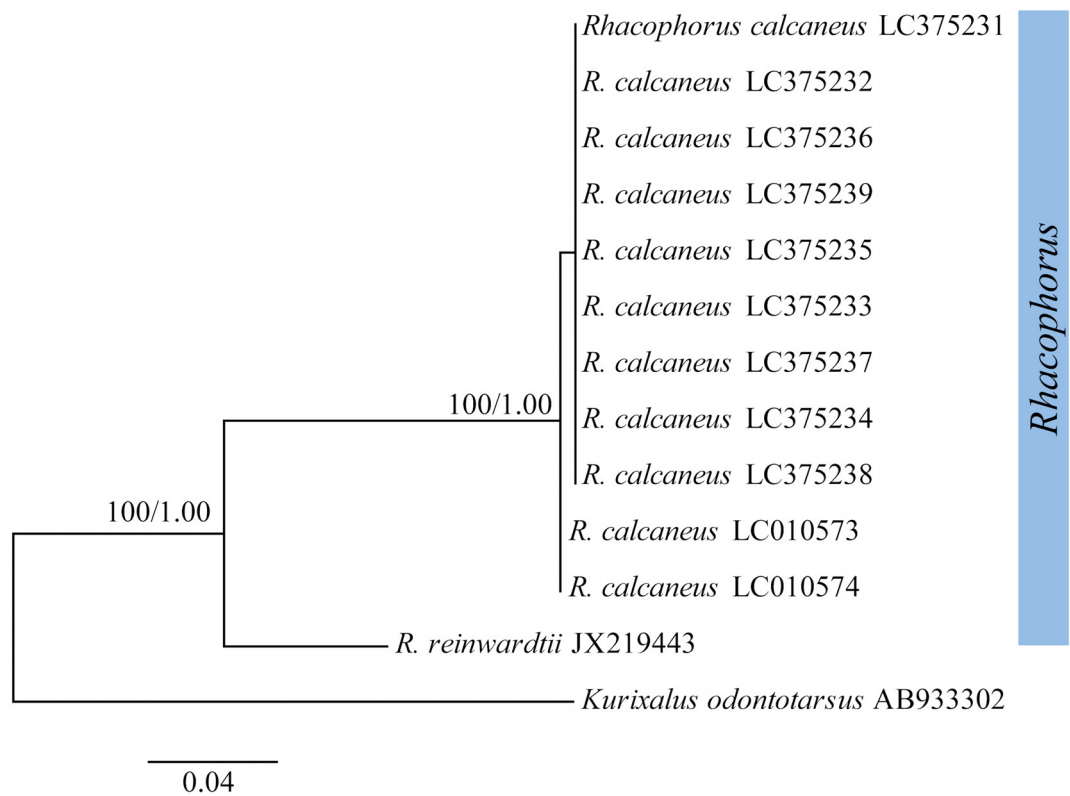


Fig. 1. Maximum-likelihood (ML) and Bayesian inference (BI) tree based on the partial 16S rRNA mitochondrial gene. Numbers above and under branches are ML bootstrap values and Bayesian posterior probabilities.

Table 2. Developmental stages, total length in mm and morphological characters of the six collected larvae of *Rhacophorus calcaneus* from Hon Ba Nature Reserve, Khanh Hoa Province; stage diagnostic characteristics according to Gosner (1960) are italicized.

Stage	n	TL (mm)	Morphological characters
32	1	42.79	<i>Indentation between toes 4 and 5</i>
36	1	47.37	<i>Toes 3-5 separated</i>
37	1	54.7	<i>Toes well developed and fully separated</i>
41	2	54.02	<i>Mouth parts atrophy</i> ; tongue well developed; <i>fore limbs visible under the skin</i> ; hind limbs without metatarsal tubercle, toe discs start to develop; <i>reduction of upper and lower tail fins</i> ;
		54.05	eye convex, eyelid distinct; <i>atrophy of vent tube</i> ; cloaca appears between hind limbs, close to tail base
42	1	52.64	<i>Keratodons and jaw sheaths disappeared</i> ; tongue completely developed; eye enlarged; <i>fore limbs with four fingers emerged</i> ; spiracle disappeared; <i>tail further atrophied</i>

Table 3. Measurements (in mm) of the six collected larvae of *Rhacophorus calcaneus* from Hon Ba Nature Reserve, Khanh Hoa Province; for abbreviations see Material & Methods.

	Stage	BH	BL	BW	HT	LF	IND	PP	NPD	RND	SS	SU
VNMN 6318	32	8.51	16.86	11.48	12.43	4.15	2.83	6.34	2.88	3.2	10.05	13.99
VNMN 6321	36	8.11	16.99	9.95	12.1	4.22	2.86	5.95	3	2.99	8.5	15.52
VNMN 6320	37	6.97	17.91	8.9	11.43	3.74	3.05	5.42	3.4	3.03	9.49	16.65
VNMN 6319	41	5.61	16.89	10.72	10.37	3.28	2.48	5.97	3.68	2.38	10.58	15.14
VNMN 6323	41	6.22	17.44	10.62	9.07	2.98	2.51	5.62	3.09	2.87	10.98	14.65
VNMN 6322	42	6.11	17.56	9.61	6.22	1.66	2.77	6.14	3.68	1.7	-	18.92

	Stage	TL	TAL	UF	VT	TMH	TMW	FL	HLL	SVL	ODW	ED
VNMN 6318	32	42.79	26.26	4.16	21.48	5.7	3.19	-	1.7	18.74	3.8	1.36
VNMN 6321	36	47.37	31.79	4.23	29.01	5.26	3.77	-	2.17	18.88	4.11	1.75
VNMN 6320	37	54.7	37.17	4.14	34.63	5.07	3.9	-	8.16	20.77	4.22	2.23
VNMN 6319	41	54.02	39.49	3.91	36.83	4.88	3.3	-	25.56	17.72	3.21	2.61
VNMN 6323	41	54.05	38.2	4.44	36.3	4.56	4.39	-	23.43	18.72	3.54	3.2
VNMN 6322	42	52.64	35.43	1.88	34.42	3.91	4.05	13.49	28.68	18.77	4.73	3.18

Table 4. Comparison of the larvae of *Rhacophorus calcaneus* with these of other *Rhacophorus* species occurring in Vietnam (after Hendrix *et al.*, 2007; Wildenhues *et al.*, 2010, 2011; Rowley *et al.*, 2010; Vassilieva *et al.*, 2013, 2016; Grosjean & Inthara, 2016).

	LTRF	tail tip	BL	TL
<i>R. calcaneus</i> (stage 32, n=1)	7(2-7)/3(1)	rounded	16.99	47.37
<i>R. annamensis</i> (stage 41, n=4)	7(3-7)/3	rounded	13.22±0.17	41.22±1.48
<i>R. helenae</i> (stage 37, n=3)	5(2-5)/3	rounded	15.17±0.55	40.63±1.96
<i>R. maximus</i> (stage 35, n=1)	5(2-5)/3(1)	-	13.1	39.3
<i>R. kio</i> (stage 36, n=6)	5(2-5)/3	rounded	20.0±1.42	53.9±3.06
<i>R. orlovi</i> (stage 40, n=1)	4(2-4)/3(1)	rounded	8.3	24.45
<i>R. rhodopus</i> (stage 36, n=8)	6(2-5)/3(1)	-	18.4±0.82	45.7±3.77
<i>R. vampyrus</i> (stage 36, n=1)	upper labium reduced, upper jaw sheath with a few huge, widely spaced hook-shaped serrations, lower jaw sheath absent	pointed	9.9	40.3

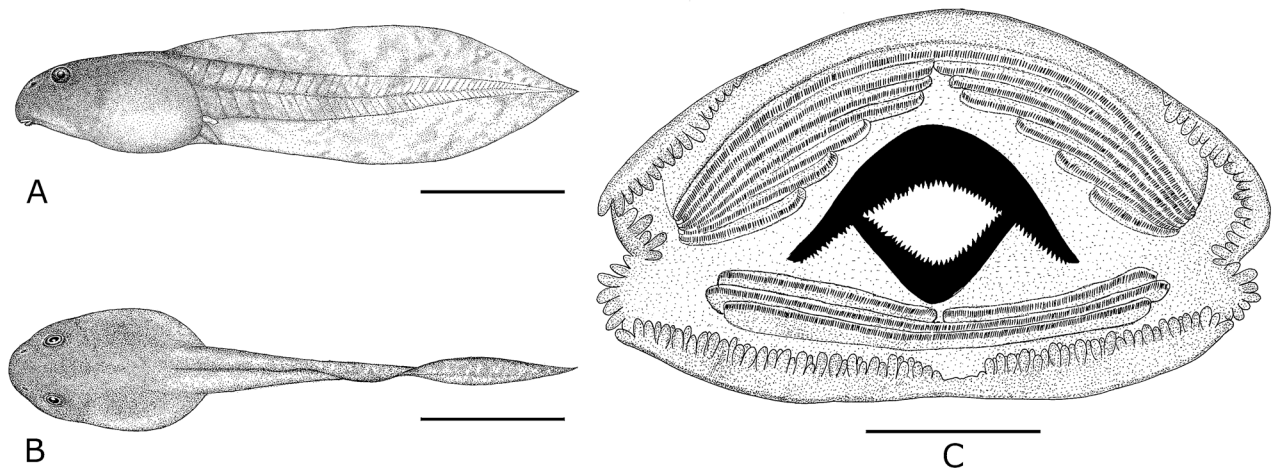


Fig. 2. Drawings of the preserved tadpole (VNMN 6318) of *Rhacophorus calcaneus* from Hon Ba Nature Reserve in Gosner Stage 32: lateral view (A), dorsal view (B) (scale bar = 1 cm); oral apparatus (C) (scale bar = 0.5 mm).

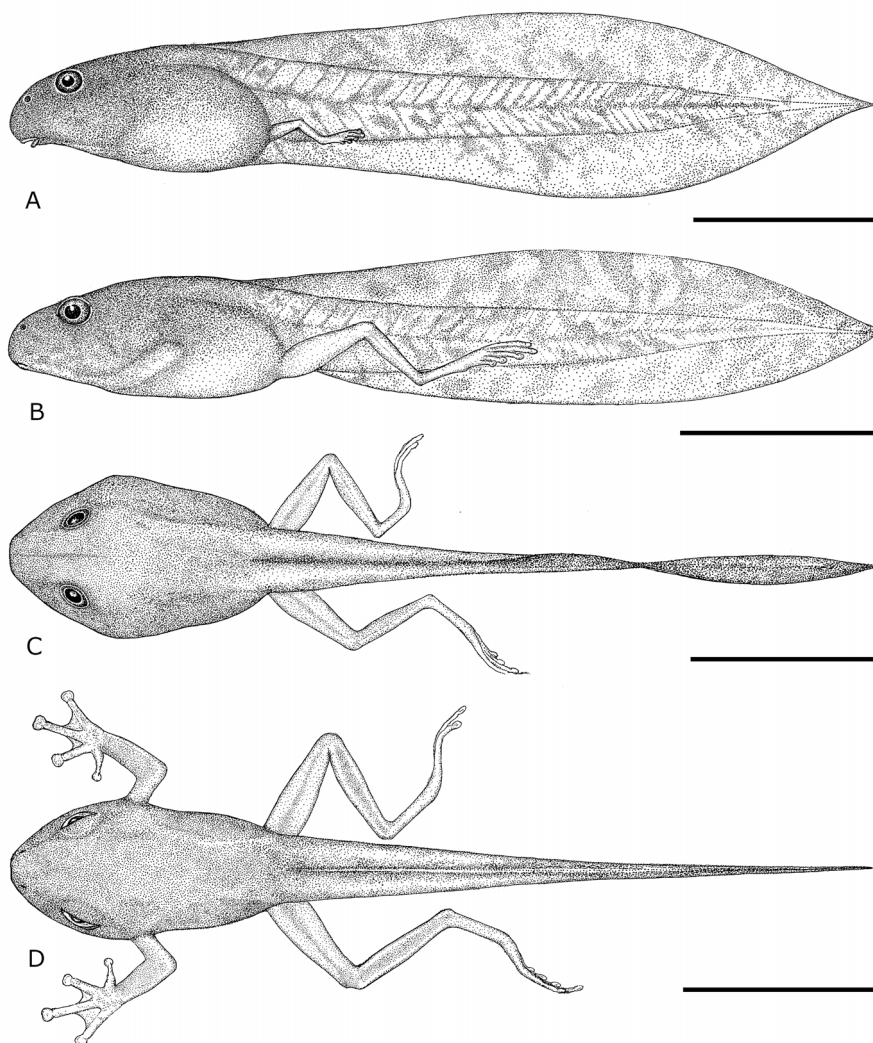


Fig. 3. Drawings of the preserved tadpoles of *Rhacophorus calcaneus* from Hon Ba Nature Reserve in advanced Gosner stages. (A) Stage 36. (B) Stage 37. (C) Stage 41 and (D) Stage 42 (scale bar = 1 cm).

times of body length (BW 11.48 mm; BL 16.86 mm). Keratodont row formula (LTRF): 7(2-7)/3(1). Upper and lower labium with black keratodont rows; keratodonts positioned at margin; lateral keratodont rows absent; keratodont rows with numerous small black keratodonts. The upper labium with seven keratodont rows, A1 positioned at margin, continuous and in curved shape, A2 - A7 divided and separated by upper jaw sheath. The lower labium with three parallel keratodont rows, P1 divided, P2-P3 continuous.

Coloration: Dorsal surface of head and body brown, with several small dark dots; the tail brown, with numerous small dark dots, edge and end of the tail light yellowish brown. Ventral surface whitish; vent tube region opaque-white; abdominal fins slightly lighter than tail; limbs white.

In general, the collected tadpoles of *R. calcaneus* in Gosner stages 32-37 can be diagnosed as follows: medium-sized; oral disk anteroventrally positioned; LTRF: 7(2-7)/3(1); upper jaw sheath semicircular and narrow; spiracle sinistral, single, ventrolaterally positioned at midbody; vent tube open, round, dextral and located directly at end of body between limbs; tail tip round; body and tail brown with numerous small dark dots.

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