

Molecular identification and description of the tadpole of the Annam Flying Frog, *Rhacophorus annamensis* SMITH, 1924 (Anura: Rhacophoridae)

RALF HENDRIX, STÉPHANE GROSJEAN, LE KHAC QUYET, MIGUEL VENCES,
VU NGOC THANH & THOMAS ZIEGLER

Abstract. Based on identification through DNA barcoding we describe the tadpole morphology of the Annam Flying Frog, *Rhacophorus annamensis*. The description is based on four exotrophic larvae of ORTON's type IV, lentic: benthic in developmental stage 41 that were collected in a karst forest stream at Phong Nha - Ke Bang National Park, Quang Binh Province, in central Vietnam. DNA sequences of the mitochondrial 16S rRNA gene obtained from the tadpoles had less than 0.6% sequence divergence to those of sympatric adult male frogs, making the identification unambiguous. The tadpoles, collected in a rock pool of the slowly moving stream, are of rather generalized morphology with a keratodont formula of 2:5+5/3.

Keywords. Rhacophoridae: *Rhacophorus annamensis*; DNA barcoding; tadpole description; morphology; Vietnam: Quang Binh Province, Phong Nha - Ke Bang National Park.

Introduction

Until recently, the Annam flying frog, *Rhacophorus annamensis* SMITH, 1924, was known only from the unique male holotype from "Daban, Phan Rang, S. Annam" in southern Vietnam (SMITH 1924, BOURRET 1942, INGER et al. 1999). The species meanwhile has been reported to occur in the southern and central provinces of Dak Lak, Gia Lai, Kon Tum, Lam Dong, Ninh Thuan, Thua Thien-Hue, and Quang Nam (INGER et al. 1999, ORLOV et al. 2002, NGUYEN et al. 2005, ORLOV 2005). The geographic range of the species is still inadequately known (see below), and likewise reports on its natural history (see ORLOV & HO 2000) are rare. RYBOLTOVSKY (1999a, b) pointed to the reproduction of the species in captivity, and these are to our knowledge the only papers referring to the tadpole of this species. However, the only information given by RYBOLTOVSKY (1999a, b) beside a single tadpole photograph is that the larvae are light grey in colour, have muscular tails and are about 1.5 cm long after

having left their foam nests, and that the hind legs already were visible after three months, with a total length of about 6 cm at this stage; the development in the terrarium lasted up to four months (RYBOLTOVSKY 1999b).

During recent field work in central Vietnam, *Rhacophorus annamensis* was recorded for the first time for Phong Nha - Ke Bang National Park (PNKB) in Quang Binh Province (ZIEGLER et al. 2005), representing the northernmost record known for the species (Figs. 1-2). DNA sequences of some of these adult specimens of *R. annamensis* closely matched those of a tadpole that had also been collected from PNKB nine years ago (see ZIEGLER & HERRMANN 2000: 50) which led to the classification of this larva as *R. annamensis*. Based on this material, we here provide a detailed description of the larval morphology of this tree frog species.

Material and methods

Tadpoles were fixed in 3% formalin and later

preserved in 70% ethanol (Figs. 3-5), and deposited in the herpetological collection of the Zoologisches Forschungsmuseum Alexander Koenig (ZFMK), Bonn, Germany under the collection number ZFMK 71342.

For a specific assignment of the four tadpoles of *Rhacophorus annamensis*, molecular data were obtained for one specimen (ZFMK 71342d, with partly removed tail muscle tissue) and compared with homologous DNA fragments of four syntopic adult males of *R. annamensis*.

The adult frogs from PNKB and its north-western border area in Quang Binh Province, Vietnam, were recently collected by LE KHAC QUYET and VU NGOC THANH and deposited in the scientific collections of Phong Nha - Ke Bang National Park, Quang Binh Province, Vietnam (PNKB LV1, specimen from PNKB), of the Vietnam National University Hanoi (VNUH LV2, specimen from north-west to PNKB), and of the Zoologisches Forschungsmuseum Alexander Koenig (ZFMK 82900, specimen from PNKB, and ZFMK 82918, specimen from north-west to PNKB). Adult frogs were compared with the holotype of *R. annamensis* from the Natural History Museum, London, and determination followed INGER et al. (1999); for measurements see Tab. 1 and for the listing of further characteristic features we refer to ZIEGLER et al. (2006).

Genomic DNA was extracted from the liver of the adult *Rhacophorus annamensis* using Chelex-extraction. A fragment of the mitochondrial 16S rRNA gene as recommended for amphibian DNA barcoding by VENCES et al. (2005a, b) of each specimen was amplified by 35 cycles of PCR: initial denaturation, 120 sec. at 95 °C, 34 cycles of 30 sec. at 95 °C, 60 sec. at 55 °C and 60 sec. at 72 °C, annealing temperature 55 °C. Double-stranded DNA was sequenced on an ABI 3770 capillary sequencer. We obtained sequences of specimens ZFMK 82900, ZFMK 82918, VNUH LV2, and PNKB LV1, which have been deposited in Genbank under the accession numbers DQ665265 - DQ665268. These sequences

were compared with the DNA voucher sequence of the single tadpole (ZFMK 71342d) which had been obtained previously (Genbank accession number AF285229; for further information and methods of DNA-extraction see ZIEGLER 2002: chapter 3.2.6, and ZIEGLER & VENCES 2002).

Terminology of the morphological tadpole description follows ALTIG & MCDIARMID (1999a), the keratodont row formula is determined according to DUBOIS (1995). Terminology of the oral disk follows ALTIG (1970) and determination of the larval stages is set out according to GOSNER (1960). Morphometric data and abbreviations follow to a large extent those of GROSJEAN (2001), except the additional values of TMH (height of tail muscle at base), TMW (width of tail muscle at base), BL (body length), and TAL (tail length) which follow ALTIG & MCDIARMID (1999a); for details we refer to Tab. 2. Because the tadpole ZFMK 71342a has a somewhat dented snout on the right side, respective measurements are approximate values. All measurements were taken in millimetres (mm) with a digital calliper gauge.

Results and discussion

Four tadpoles of *Rhacophorus annamensis* in the developmental stage 41 according to GOSNER (1960) were collected in Phong Nha - Ke Bang National Park, Quang Binh Province, Vietnam by T. ZIEGLER during fieldwork in early September 1998 (field number TZ' 98/30). Tadpoles were collected at night (09:30) in a slowly flowing karst forest stream near a small path (surroundings of N 17°28', E 106°13') at an altitude of about 350-500 m above sea level (Fig. 6). The rock pool in which the larvae were found was partly shaded by vegetation; it had a maximum width of about 1 m and the maximum water depth measured ca. 15 cm. The shallow water was clear and the loamy to stony ground was covered with leaves. It did not rain and thus was relatively dry for the beginning rainy season;

ambient air temperature was 24.6 °C at the collecting site and relative air humidity was 78 %.

The tadpole DNA sequence showed less than 0.6 % sequence divergence compared to the adult sequences of ZFMK 82900 and VNUH LV2 (identities: 513/516 bp) and less than 0.4 % divergence compared to the adult sequences of ZFMK 82918 and PNKB LV1 (identities: 513/515 bp). A comparison with a further sequence of *R. annamensis*, obtained by WILKINSON et al. (2002) and available from Genbank (accession number AF458143) yielded similar results (identities: 492/495 bp of the sequence of ZFMK 82900 and VNUH LV2, respectively 492/494 bp of ZFMK 82918 and PNKB LV1). Considering that species of amphibians are often characterized by rather strong intraspecific genetic differentiation (up to 4 % in the 16S gene; VENCES et al. 2005a, b), the small differences observed between *R. annamensis* haplotypes are not surprising and the molecular tadpole identification is to be seen as very reliable.

General appearance of the tadpoles in preservative: The tadpoles are medium-sized (TL = 39.37– 41.69 mm). They are generalized extrophic tadpoles of ORTON's (1953) type IV, lentic: benthic (ALTIG & MCDIARMID, 1999b) without obvious specializations.

They are consistently dark pigmented from the snout to the tip of the tail including fins. Dorsal and dorsolateral pigmentation of the body is more dense than tail pigmentation. The body coloration varies from greyish brown to brown and the tail musculature coloration from light brown to reddish brown (see Fig. 3). The ventral and ventrolateral body sides are white to yellow and more or less pigmented. The intestine is visible through the body. For measurements see Tab. 2.

The description in dorsal view is based on a single tadpole (ZFMK 71342c, with protruded right forelimb). Body somewhat elliptically protracted with a slightly pointed snout (Fig. 4A) and with widest portion being at midbody (body width 0.68 times of body length). Eyes of moderate size (eye diameter 0.17 times of body length), positioned dorsolaterally, directed more laterally than anteriorly, bulging, not visible in ventral view. Nares small, rounded, slightly rimmed, positioned dorsolaterally in anterolaterally direction. Naris closer to snout than to pupil (rostronarial distance 0.45 times of narpupilar distance). Internarial distance about 0.49 times of interpupilar distance. Well developed nasolacrimal duct from the naris to the anterior corner of the eye.

Tab. 1. Measurements (in mm) of adult *Rhacophorus annamensis* males, including mean value and standard deviation (SD); abbreviations are as follows: SVL = snout vent length, HW = head width, IMW = inner mouth width, NST = distance from the nostril to tip of snout, ED = diameter of the eye, TD = diameter of the tympanum, EN = distance from the eye to the nostril, HL = length of the hand, FLIT = length of the foot including tarsus, KL = length of the head, TIB = length of the tibia, EST = distance from the eye to the snout-tip, LH = length of the hind limb, FL = length of the foot without tarsus, IMT = inner metatarsal tubercle.

	SVL	HW	IMW	NST	ED	TD	EN	HL	FLIT	KL	TIB	EST	LH	FL	IMT
ZFMK 82900	62.20	21.40	17.20	4.40	7.60	3.50	6.50	16.30	39.80	20.60	31.30	10.30	99.10	25.80	2.50
ZFMK 82918	59.10	19.30	16.40	4.60	7.30	3.20	6.40	15.20	37.80	20.40	29.90	10.50	91.70	23.70	2.60
VNUH LV2	54.10	18.30	17.00	4.00	6.90	3.00	6.00	14.10	35.20	17.60	28.70	8.50	89.70	21.80	2.10
PNKB LV1	59.20	18.70	17.00	4.00	6.90	3.50	6.20	14.50	37.70	20.10	30.40	10.70	93.80	23.40	2.10
mean	58.65	19.43	16.48	4.23	7.18	3.30	6.28	15.03	37.63	19.68	30.08	10.00	93.58	23.68	2.33
SD	±3.36	±1.38	±0.58	±0.34	±0.34	±0.24	±0.22	±0.96	±1.9	±1.40	±1.08	±1.01	±4.05	±1.64	±0.26



Fig. 1. Adult male *Rhacophorus annamensis* (ZFMK 82900) from Phong Nha - Ke Bang National Park (May/June 2004). Photo: LE KHAC QUYET.

In lateral view, body slightly depressed (body height 0.82 times of body width), snout slightly rounded (Fig. 4B). Spiracle sinistral, ventrolaterally positioned at midbody (distance from snout tip to opening of spiracle 0.66 times of body length), conical, oriented in posterodorsally direction and entirely attached to the body. Opening of the spiracle oval, located on level between origin of hind limbs and longitudinal axis. Vent tube completely reduced at this stage. Myotomes of the tail musculature of moderate development (height of tail musculature at base 0.54 times of maximum body height, and 0.57 times of maximum tail height). Tail musculature from the proximal to its distal half parallel, then gradually tapering, reaching the tip of the tail. Myotomes appear not to be attached from the distal half to the tip. Tail fin moderate, rounded at the end. Highest point of the upper fin at the last third of the tail length (maximum height of upper tail fin 0.32 times of maximum tail height). Lower fin smaller than dorsal fin (maximum height of lower tail fin 0.27 times of maximum tail height). Lateral line organ existent and well developed on body and along the apex of the caudal musculature.

Oral disk anteroventrally positioned (about 0.4 times of body width), of triangular shape in relaxed state, of nearly trapezoidal shape in expanded state (see Fig. 5), and laterally emarginated. Oral disk framed by finger shaped papillae of moderate size except for a large medial gap of the upper la-

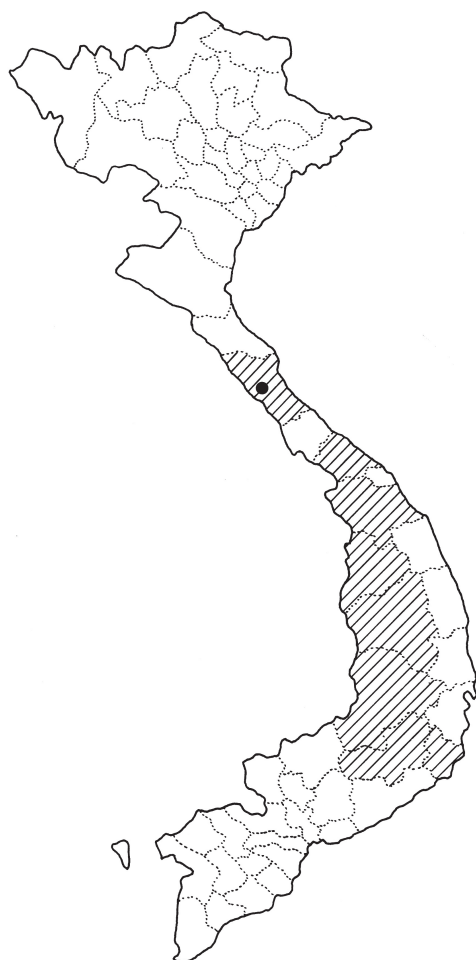


Fig. 2. Vietnamese provinces, from which *Rhacophorus annamensis* has already been reported (INGER et al. 1999, ORLOV et al. 2002, NGUYEN et al. 2005, ORLOV 2005, ZIEGLER et al. 2005, 2006). From North to South: Quang Binh, Thua Thien-Hue, Quang Nam, Kon Tum, Gia Lai, Dak Lak, Lam Dong, and Ninh Thuan provinces; the dot marks the locality within the Phong Nha - Ke Bang National Park where the tadpoles (ZFMK 71342) were collected. Drawing: T. ZIEGLER.

bium which is as long as the first keratodont row. Few small submarginal papillae situated laterally of the upper labium, two at each end of keratodont rows A2 – A4; three further at each end of the rows A5 – A7 in somewhat

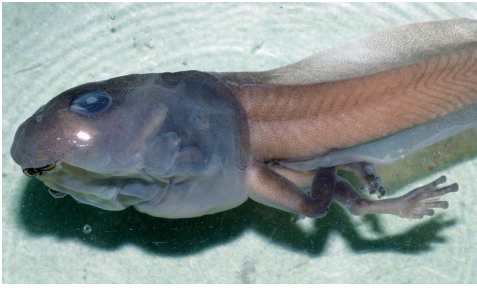


Fig. 3. Lateral aspect of the preserved tadpole of *Rhacophorus annamensis* (ZFMK 71342c) from Phong Nha - Ke Bang. Photo: T. ZIEGLER.

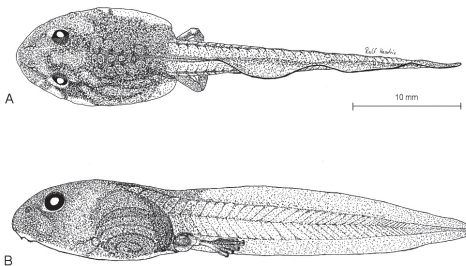


Fig. 4. Dorsal (A) and lateral (B) aspects of the preserved tadpole of *Rhacophorus annamensis* (ZFMK 71342c) from Phong Nha - Ke Bang; scale bar = 10 mm. Drawings: R. HENDRIX.

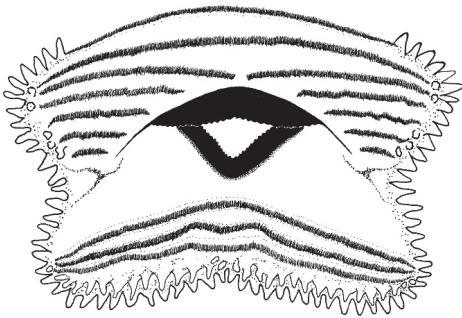


Fig. 5. Oral disk of the tadpole of *Rhacophorus annamensis* (ZFMK 71342c). Drawing: R. HENDRIX.

distance to the upper submarginal papillae. Posterior border of the lower labium slightly emarginated with one additional row of sub-



Fig. 6. Habitat of *Rhacophorus annamensis* in Phong Nha - Ke Bang National Park at daytime (September 1998) where the larvae were collected. Photo: T. ZIEGLER.

marginal papillae over the length of lower keratodont row. Keratodont rows A1 and A2 continuous, A3 divided, A4 – A7 entirely separated by the upper jaw sheath. Keratodont rows P1 – P3 of the lower labium undivided. Keratodont row formula (KRF): 2:5+5/3. Jaw sheaths black, and slightly serrated. Upper jaw sheath narrow, and stretched into a wide arch. Lower jaw sheath V-shaped.

Ten species of rhacophorid frogs are currently known to occur in PNKB (ZIEGLER et al. 2004, OHLER & DELORME 2006; systematics according to FROST et al. 2006): *Chiro-mantis vittatus* (BOULENGER, 1887), *Kurix-alus verrucosus* (BOULENGER, 1893), *Poly-pedates megacephalus* HALLOWELL, 1861, *P. mutus* (SMITH, 1940), *Rhacophorus annamensis* SMITH, 1924, *R. bipunctatus* AHL, 1927, *R. dennysi* BLANFORD, 1881, *R. kio* OHLER & DELORME, 2006, *R. orlovi* ZIEGLER & KÖHLER, 2001, and *Theloderma asperum* (BOULENGER, 1886). Furthermore, ORLOV & HO (2005) list their new species *Philautus truongsoneensis* as occurring in Phong Nha – Ke Bang National Park, but this record was based on a photograph only. ZIEGLER et al. (2006) report of a single female *Philautus* specimen from Minh Hoa district in Quang Binh Province adjacent to Phong Nha - Ke Bang National Park, that was provisionally (because male sexual characters could not be taken into consideration) allocated to *Philautus* cf. *jinxuensis* HU, 1978.

Tadpoles of the genus *Rhacophorus* are morphologically uniform (INGER 1966, 1985, CHOU & LIN 1997, GROSJEAN 2004). However, some species of *Rhacophorus* possess an uninterrupted row of marginal papillae on the lower labium whereas others show a short medial gap. The larva of *R. annamensis* belongs to the first group. It also possesses two uninterrupted rows of keratodonts on the upper labium, a feature shared only by *R. aurantiventris* from Taiwan (CHOU & LIN 1997), and *R. pardalis* from Borneo, Indonesia (INGER 1966, 1985) and Negros Island, Philippine (ALCALA, 1962). The high number (seven) of keratodont rows of the upper labium in *R. annamensis* is also striking. In most other *Rhacophorus* species these otherwise occur in six rows (*Rhacophorus* sp. [GROSJEAN 2004], *R. bipunctatus* [BOURRET 1942, GROSJEAN unpublished data], *R. dulitensis* [INGER 1966, 1985], *R. margaritifera* [VAN KAMPEN 1909, 1923 under the name *R. javanus*], *R. nigropalmatus* [VAN KAMPEN 1923, INGER 1966] and *R. prominans* [BERRY 1972]) or four or

five rows (CHOU & LIN 1997, ALTIG & McDIARMID 1999b: 333, GROSJEAN 2004). The high number of keratodont rows observed on the upper labium of *R. annamensis* is shared by the tadpoles of *R. monticola* (VAN KAMPEN 1923) and *R. nigropalmatus* (BERRY 1972), and maybe by other species of the *nigropalmatus* group (including *R. nigropalmatus*, *R. prominans*, *R. kio* and *R. reinwardtii*) (GRANDISON 1972).

The tadpole of *Rhacophorus annamensis* can be distinguished from the other *Rhacophorus* tadpoles from PNKB whose tadpoles are known, by its keratodont formula (2:5+5/3 versus 1:4+4/1+1:2 in *R. dennysi* from China [POPE 1931]; 1:4+4/3 in *R. kio* from Thailand [GROSJEAN unpublished data]; 1:5+5/1+1:2 in *R. bipunctatus* from Thailand [GROSJEAN unpublished data]).

The uncertainties surrounding some of the above mentioned records exemplify that further research concerning rhacophorid taxonomy as well as ecology is still required. For example, the reproductive ecology and

Tab. 2. Measurements (in mm) of *Rhacophorus annamensis* larvae, including mean value and standard deviation (SD); abbreviations after GROSJEAN (2001) and ALTIG & McDIARMID (1999a): **BH** = maximum body height, **BL** = body length, **BW** = maximum body width, **ED** = maximum diameter of eye, **HT** = maximum tail height, **LF** = maximum height of the lower tail fin, **NN** = internarial distance, **NP** = narpupilar distance, **ODW** = oral disk width, **PP** = interpupilar distance, **RN** = rostral distance, **SS** = distance from the tip of snout to opening of spiracle, **SU** = distance from the tip of snout to insertion of upper tail fin, **TL** = total length, **UF** = maximum height of the upper tail fin, **TAL** = tail length, **TMH** = height of the tail musculature at base, **TMW** = width of the tail musculature at base.

	BH	BL	BW	ED	HT	LF	NN	NP	ODW
ZFMK 71342a	7.62	13.08	9.04	2.21	7.15	2.11	2.11	2.44	3.81
ZFMK 71342b	6.87	13.13	9.06	2.11	7.22	2.21	2.08	3.16	3.90
ZFMK 71342c	7.64	13.47	9.28	2.12	7.33	1.97	2.24	2.83	3.88
ZFMK 71342d	6.66	13.18	8.09	1.94	6.71	1.75	2.23	2.57	3.41
mean	7.20	13.22	8.87	2.10	7.10	2.01	2.17	2.75	3.75
SD	±0.51	±0.17	±0.53	±0.11	±0.27	±0.20	±0.08	±0.32	±0.23
	PP	RN	SS	SU	TL	UF	TAL	TMH	TMW
ZFMK 71342a	5.57	1.08	7.73	11.63	41.14	2.52	28.06	3.96	3.54
ZFMK 71342b	5.78	1.39	8.49	12.03	39.78	2.52	26.65	4.19	3.66
ZFMK 71342c	5.73	1.27	8.87	12.43	43.27	2.35	29.80	4.17	3.86
ZFMK 71342d	5.43	1.12	8.76	11.86	40.68	2.07	27.50	4.03	2.90
mean	5.63	1.29	8.46	11.99	41.22	2.37	28.00	4.09	3.49
SD	±0.16	±0.23	±0.51	±0.34	±1.48	±0.21	±1.33	±0.11	±0.41

larval morphology of *Rhacophorus orlovi* is still unknown since its scientific description six years ago (ZIEGLER & KÖHLER 2001). The recent larval description of *Kurixalus verucosus* from central Vietnam (ZIEGLER & VENCES 2002) and the results of the present study show that molecular analyses can help to shed light upon the actual systematic relationships of frogs and affiliated tadpoles. DNA sequences, if deposited in public databases, provide unambiguous means to assign the larvae to adult stages, even after taxonomic redefinitions and rearrangements. They therefore provide substantial contributions to future works on larval morphology and ecology in amphibians. Facing the global amphibian extinction crisis, standardized collection of DNA sequences in approaches of DNA barcoding (HEBERT et al. 2003, SAVOLAINEN et al. 2005), and in conjunction with thorough morphological descriptive analyses, can be the key to more reliable knowledge on the distribution areas, geographical variation, reproductive diversity and larval ecology of these organisms.

Acknowledgements

We thank Professor VO QUY and Professor Dr. TRUONG QUANG HOC (Centre for Natural Resources and Environmental Studies, Vietnam National University, Hanoi), the People's Committee of Quang Binh, and the Phong Nha - Ke Bang National Park directorate (NGUYEN TAN HIEP, CAO XUAN CHINH, and LUU MINH THANH) as well as DINH HUY TRI (Science Research Centre, Phong Nha - Ke Bang National Park) for their continuous assistance and encouragement as well as for issuing respective permits. The National Park staff helped again to make fieldwork successful. We are further grateful to Dr. JÖRN KÖHLER (Hessisches Landesmuseum Darmstadt) and to Drs. BARRY CLARKE and COLIN MCCARTHY (The Natural History Museum, BMNH, London) for enabling us to examine the holotype of *Rhacophorus annamensis*. We are also indebted to Professor Dr. DIETHARD TAUTZ and Dr. MEIKE THOMAS (University of Cologne, Institute of Genetics) for their support with DNA sequencing. We further thank

Dr. RAOUL BAIN (Centre for Biodiversity and Conservation, American Museum of Natural History, New York) for valuably commenting on an earlier version of the manuscript. Field work of LE KHAC QUYET was supported by Fauna & Flora International (FFI) - Vietnam Programme, Hanoi. Field work of T. ZIEGLER was funded in large by the Volkswagen Foundation (project no. I/72 843), by a grant of the "Graduiertenförderung" (GrFG NW, no. 1 26 10) in combination with a grant of the German Academic Exchange Service (DAAD, no. 213/327/501/7), as well as recent research by the Zoological Garden Cologne, the Kölner Kulturstiftung der Kreissparkasse Köln and BIOPAT (www.biopat.de). Current tadpole research in the Phong Nha - Ke Bang region is supported as well by the Alexander-Koenig-Gesellschaft (AKG), the Alexander Koenig Stiftung (AKS), the Deutsche Gesellschaft für Herpetologie und Terrarienkunde (DGHT), and by the European Union of Aquarium Curators (E.U.A.C.).

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Manuscript received: 8 June 2006

Authors' addresses: RALF HENDRIX, Zoologisches Forschungsmuseum Alexander Koenig, Adenauerallee 160, D-53113 Bonn, Germany and AG Zoologischer Garten Köln, Riehler Straße 173, D-50735 Köln, Germany, E-Mail: r.hendrix@uni-bonn.de; STÉPHANE GROSJEAN, Muséum National d'Histoire Naturelle, Département de Systématique et Evolution, UMS 602, Taxinomie-Collection – Reptiles & Amphibiens, Case Postale N° 30, 25 rue Cuvier, F-75005 Paris, Frankreich, E-Mail: sgrosjea@mnhn.fr; LE KHAC QUYET, Fauna & Flora International – Vietnam Programme, IPO Box 78, 340 Nghi Tam, Hanoi, Vietnam, E-Mail: quyet.khac.le@ffi.org.vn; MIGUEL VENCES, Division of Evolutionary Biology, Zoological Institute, Technical University of Braunschweig, Spielmannstraße 8, D-38106 Braunschweig, Germany, E-Mail: m.vences@tu-bs.de; VU NGOC THANH, Centre for Natural Resources and Environmental Studies (CRES), Vietnam National University, Hanoi, University of Science, Faculty of Biology, Department of Vertebrate Zoology, Zoological Museum, 334 Nguyen Trai Street, Thanh Xuan, Hanoi, Vietnam, E-Mail: vnthanh@fpt.vn; THOMAS ZIEGLER, AG Zoologischer Garten Köln, Riehler Straße 173, D-50735 Köln, Germany, E-Mail: tziegler@zoo-koeln.de.

