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First record of *Gracixalus quangi* Rowley, Dau, Nguyen, Cao & Nguyen, 2011, from Hoa Binh Province, Vietnam, including the first documentation of advanced larval stages and an extended tadpole description

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***Abstract.*—We provide the first record of *Gracixalus quangi* from Hoa Binh Province, Vietnam, based on morphological and molecular evidence. The species was originally described from Nghe An Province and subsequently recorded from Son La and Thanh Hoa provinces in Vietnam. The first documentation of advanced larval stages up to Gosner stage 45 is provided from observations on the development of two egg clutches at the indoor amphibian facility of the Me Linh Station for Biodiversity. The clutches were collected in Hang Kia - Pa Co and Ngoc Son - Ngo Luong Nature Reserves in Hoa Binh. An extended description of a tadpole in stage 35 is presented including the morphology of the oral disc and the first complete larval staging table (comprising stages 14 to 45) for *G. quangi*. This is the first record of the species from a karst forest and we provide the first natural history data of *G. quangi* from such a habitat type.**

Keywords. Morphology, molecular biology, tadpole matching, new record, karst forest, Southeast Asia, development, tree frog

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Introduction

The Quang's Tree Frog, *Gracixalus quangi*, was recently described by Rowley et al. (2011) from the montane evergreen forest of Pu Hoat Nature Reserve in western Nghe An Province, Vietnam. The species has also been recorded from Copia Nature Reserve in Son La Province by Pham et al. (2012a) and from Xuan Lien Nature Reserve in Thanh Hoa Province by Pham et al. (2012b). The Quang's Tree Frog inhabits dense and relatively undisturbed montane rainforest between 600 and 1,300 m above sea level. Reproduction occurs from April to November when males aggregate at breeding sites. Eggs are deposited on vegetation overhanging shallow, muddy ponds, into which the larvae fall after hatching. *Gracixalus quangi* is listed as Vulnerable in the IUCN Red List of Threatened Species, being known only from few localities in Vietnam. Its habitat continues to decline both in quality and extent (IUCN SSC Amphibian Specialist Group 2015).

Recent herpetological surveys conducted in Hang Kia - Pa Co and Ngoc Son - Ngo Luong nature reserves allowed a range gap in the distribution of *G. quangi* to be filled by providing the first record of the species from Hoa Binh Province (Fig. 1), based on morphological and molecular evidence, and recorded the species for the first time from karst forest habitat.

In general, the importance of detailed morphological larval descriptions have been underestimated, and only limited information on morphology and natural

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Fig. 1. Adult *Gracixalus quangi* from Hoa Binh Province. *Photography by T. Ziegler.*

history is available for anuran larvae, compared to adult stages. However, the biphasic life cycle of anurans imposes dramatically different selective regimes on the aquatic larval stage, the metamorphic stage, and the terrestrial post-metamorphic stage (Haas and Das 2011). Knowledge about tadpole development is crucial for the proper determination of anuran larval stages in the field, e.g., for assessments of habitat, ecological adaptations, and population density, which all provide important information for potential conservation actions such as conservation breeding programs in the context of the global amphibian crisis.

Thus, detailed descriptions of developmental stages from egg until metamorphosis are important additions to larval descriptions, which are usually limited to one developmental stage that represents a very short period of the anuran life cycle. Rowley et al. (2011) observed larvae of *G. quangi* within clutches up to stage 24 of development (Gosner 1960) and provided some measurements and a photograph of a tadpole at stage 24. However, tooth rows were not yet obvious in that early developmental stage, so the oral disc was not described adequately. Rowley et al. (2015) published new information on the breeding habitats, eggs, embryos, and tadpoles of three *Gracixalus* spp. and a description of the tadpole of *G. quangi* up to stage 26. To provide a more complete description of larval development, including the advanced larval stages, two egg clutches were transferred from the newly recorded population in Hoa Binh Province to the indoor amphibian facility of the Me Linh Station for Biodiversity (Ziegler et al. 2016). Based on the larvae of *G. quangi* from Hoa Binh which subsequently hatched and further developed, we herein provide the first documentation of advanced larval stages together with an extended tadpole description, including the morphology of the oral disc.

Material and Methods

Field surveys. Field surveys were conducted in Hoa Binh Province in April 2014 (Fig. 2). Minimum and maximum air temperatures and humidity were measured daily with a thermo-hygrometer (TFA Dosmann/Wertheim Kat.

Fig. 2. Map of Vietnam showing previous distribution records (1: Pu Hoat Nature Reserve, Nghe An; 2: Copia Nature Reserve, Son La; 3: Xuan Lien Nature Reserve, Thanh Hoa) after Rowley et al. (2011), Nguyen et al. (2012), and Pham et al. (2012a, b), as well as the new findings from Hoa Binh Province (4: Hang Kia - Pa Co and 5: Ngoc Son - Ngo Luong nature reserves).

Nr.30.5015). Of the twelve egg clutches observed near small ponds in the limestone forest one egg clutch was collected on 11 April 2014 from Hang Kia - Pa Co Nature Reserve and one clutch was collected on 17 April 2014 from Ngoc Son - Ngo Luong Nature Reserve.

For proper species identification of larvae, four syntopically occurring adult frogs (three males and one female) were collected in Hang Kia - Pa Co and Ngoc Son - Ngo Luong in April 2014, euthanized with ethylacetate, preserved in 70% ethanol, and deposited in the herpetological collection of the Institute of Ecology and Biological Resources (IEBR): One female IEBR 4329 (field number HB 2014.25) and one male IEBR 4330 (field number HB 2014.26) collected by T.Q. Nguyen, C.T. Pham, and H.N. Ngo on 12 April 2014 in Hang Kia - Pa Co Nature Reserve, Hoa Binh Province, at an elevation of 1,376 m a.s.l.; two adult males IEBR 4331, 4332 (field numbers HB 2014.73, 2014.74) collected by C.T. Pham, H.N. Ngo, and H.T. An on 18 April 2014 in Ngoc Son - Ngo Luong Nature Reserve, Hoa Binh Province, at an elevation of 605 m a.s.l.

Abbreviations: a.s.l. = above sea level; EN = distance from anterior corner of eye to the nostril; HL = head length; HW = head width; IND = internarial distance; IOD = interorbital distance; NS = distance from nostril to the tip of the snout; SVL = snout-vent length; UEW = maximum width of upper eyelid.

Husbandry conditions and rearing. To further investigate the development of the eggs and larvae, the two collected egg clutches were transferred to the indoor amphibian room at the Me Linh Station for Biodiversity.

The amphibian facilities at Me Linh were established to keep and breed threatened or poorly known species from Vietnam, both for research on husbandry, reproduction, and natural history and for building up *ex situ* breeding programs and captive assurance populations (see Ziegler et al. 2016). Room temperature was about 25–28 °C, and the humidity was 70–90%. Egg clutches were left on leaves and kept in covered petri dishes before hatching. After hatching, tadpoles were housed in plastic boxes measuring 36.5 × 26 × 13.5 cm (length × width × height); the water depth was about 3 cm, and air pumps provided for additional oxygen supply. Water was changed manually every day. Feeding occurred 4–5 times a day with fish flakes (SERA vipan). Water parameters were: pH 6.6, KH (carbonate hardness) 9, GH (general hardness) 4°, NO_2 0 mg/l, NO_3 0 mg/l.

Larval staging and description. Identification of larval stages followed Gosner (1960) as reproduced in McDiarmid and Altig (1999). The two egg clutches were photographed daily. Either daily or every few days after hatching, up to five tadpoles from each clutch were photographed in a small aquarium and the total length was measured (from the tip of the snout to end of the tail) with a caliper. The day when the first clutch was found is referred to as "day 1" in the Results section. One larva in an early developmental stage (IEBR 4333a, field number TZ 2014c) was preserved for genetic identification. Five additional tadpoles (IEBR 4333b, c, d, ZFMK 101032, 101033) in stages 35–38 were preserved for comparisons of their oral discs with that of IEBR 4333a and for subsequent morphological description. Preserved specimens were deposited in the herpetological collections of the Institute of Ecology and Biological Resources (IEBR) of the Vietnamese Academy of Science and Technology, Hanoi, Vietnam and the Zoologisches Forschungsmuseum Alexander Koenig, Bonn, Germany.

Measurements of preserved larvae were made with a sliding caliper. Terminology for morphometric data and abbreviations followed McDiarmid and Altig (1999), Altig (2007), and Haas and Das (2011). The Labial Tooth Row Formula (LTRF) with A (keratodont rows on upper labium) and P (keratodont rows on lower labium) was applied as in McDiarmid and Altig (1999), and the ecomorphological assignment of larval types followed Altig and Johnston (1989) and Orton (1953) as extended in McDiarmid and Altig (1999). Abbreviations are as follows: BH = body height; BL = body length (from snout to the point where the axis of the tail myotomes meets the body wall); BS = body end to center of spiracle; BW = maximum body width; ED = eye diameter; ES = eye-snout distance; IND = internarial distance (center to center); IOD = interorbital distance; LFH = lower fin height (at MTH); MTH = maximum tail height; NE = distance from center of naris to center of eye; ODW = oral disc width; SN = distance of naris (center) to snout; SS = distance of snout to center of spiracle; TAL = tail length (total length minus body length); TMH = tail muscle height at body-tail junction, where ventral line of musculature meets trunk contour; TMW = tail muscle width at the same level as TMW; TTL = total length (from the tip of the snout to end of the tail); UFH = upper fin height.

Molecular analysis. Total genomic DNA was extracted from a tissue sample of larva IEBR 4333a using a commercially available DNeasy Tissue Kit following manufacturer's instructions (QIAGEN Inc., Valencia, California, USA). A fragment of 16S gene was amplified with the primer pair 16Sar + 16Sbr (Palumbi et. al. 1991). The standard PCR conditions for 16S were 95 °C for 5 min, 40 cycles of [95 °C for 30 sec., 50 °C for 45 sec, 72 °C for 60 sec] and 72 °C for 6 min. All PCR products were visualized on a gel before purification. Successful amplification was purified to eliminate PCR components with a GeneJETTM PCR Purification kit (Fermentas, Canada). The purified PCR product was sent to 1st Base (Malaysia) for sequencing. The sequence was compared to those available for other species with a BLAST search of GenBank.

Results

Morphology of adults. Morphological characters of the four preserved adults from Hoa Binh Province are similar to those in the original description of *Gracixalus quangi* provided by Rowley et al. (2011): Males smaller than females (SVL 22.8–24.0 mm male, 29.2 mm female); head longer than wide (HL 8.7–10.2 mm, HW 8.3–9.2 mm male, HL 11.0 mm, HW 10.2 female); snout pointed in dorsal view and in profile, projecting beyond margin of the lower jaw; canthus rostralis distinct, loreal region slightly concave; nostril oval, closer to tip of snout than eye (NS 1.6–1.7 mm, EN 1.8–1.9 mm male, NS 1.8 mm, EN 2.4 mm female); interorbital distance wider than internarial distance and upper eyelid (IOD 3.3–3.6 mm, IND 2.5–2.8 mm, UEW 2.3–2.4 mm male, IOD 3.9 mm, IND 3.4 mm, UEW 3.2 mm female); tympanum distinct, rounded, about 40% eye diameter; vomerine teeth absent; tongue notched posteriorly; external subgular vocal sac.

Forelimbs moderately robust; tips of fingers enlarged into round discs with circummarginal grooves; relative length of fingers I<II<IV<III; fingers free of webbing; males with nuptial pad on finger I.

Fig. 3. Adult couple of *Gracixalus quangi* from Hoa Binh Province (male above, female below). *Photo credit C.T. Pham.*

Hindlimbs: tips of toes enlarged into round dics with circummarginal grooves; relative length of toes I<II<III<V<IV; discs of toes slightly smaller than those of fingers; webbing formula I1-11/2II1/2-2III1/2-2IV2-0V; inner metatarsal tubercle present; outer metatarsal tubercle absent.

Dorsal surface of head, body, thigh, and shank with small tubercles; largest and most concentrated on eyelids; supratympanic fold present; throat and chest smooth; ventral surface of thighs and belly coarsely granular.

Coloration in life (Fig. 3): Dorsal surface olive-green, with brighter pale green on dorsal surface of upper arms; brown interorbital bar covering eyelids and faint darker X across back; black supratympanic line underneath supratympanic fold, extending from eye to axilla; line of large, black spots from supratympanic line to flanks; ventral surface of throat, chest and belly opaque white with translucent pale green margins.

Molecular analysis. According to the BLAST search of GenBank, the 556 bps sequence of the amplified DNA fragment was 99% similar to that of the *Gracixalus quangi* holotype (GenBank accession number JN862537) and thus the tadpole was clearly identified as conspecific. However, the new sequence differed from the type sequence in five positions.

Natural history. Adult frogs and 12 egg clutches were found in the early rainy season in four breeding sites of *G. quangi* in Hoa Binh Province, near two small ponds (each ~1 m^2 in area) in the limestone forests of Hang Kia - Pa Co and Ngoc Son - Ngo Luong nature reserves (Figs. 4, 5). Clutches were small and consisted of eggs in prominently clear jelly deposited in a clump at the tip of green leaves between 0.3 to 0.7 m above the water surface of the small ponds. In Hang Kia - Pa Co five egg clutches containing 8 to 14 eggs each were found at an elevation of 1,376 m a.s.l., and in Ngoc Son - Ngo Luong seven egg clutches containing 6 to 18 eggs each were found at an elevation of 650 m a.s.l. The mean clutch size for the 12 clutches was 11 eggs (minimum 6, maximum 18). The habitat was located in the secondary karst forest of medium and small hardwoods mixed with shrubs and vines. The surrounding vegetation consisted of ferns and other plants, less than one meter tall. Air temperature was 19.9–28.0 °C and relative humidity was 69–90%.

Developmental stages. The first clutch was found in stage 14 (neural folds visible); stages 15–18 were observed within the next two days (see Table 1 and Fig. 6 for early developmental stages). Developing bodies of the larvae had a slightly darker coloration than the large, white to cream-colored yolk mass. Stage 19 (gill buds, distinct tail elongation) was photographed on day four; stage 20 (gill circulation, further tail elongation) on day five. The second clutch was found on that day in stage 20. At stage 22 (tail fins transparent), the pigmentation of the larvae darkened slightly and the yolk sac started to reduce. At stage 23, the body coloration had darkened further, and the eyes had significantly darkened. The larvae inside the eggs had grown continuously as the yolk sac further reduced. At stage 25, the external gills had atrophied and the yolk sac was largely absorbed. The intestinal loop was visible on the lateral side of the tadpoles inside the eggs. The grey coloration had extended laterally as dark spots scattered on the sides and the ab-

Fig. 4. Karst forest habitat in Hoa Binh Province. *Photo credit C.T. Pham.*

Table 1. Early developmental stages and morphological characters of *Gracixalus quangi* larvae from Hoa Binh Province; stage diagnostic characters according to Gosner (1960) in italics. n: number of individuals observed at the respective stage; Age (days): day the first clutch was found herein defined as day 1.

Stage	Diagnostic characters	n	Age (days)
14	*Neural folds*	14	1
15–16	*Elongation*, *rotation*, *neural tube*, *gill plates*	13	2
17–18	*Tail bud*, *olfactory pits*, large yolk mass, pigmentation	13	3
19	*Gill buds*, tail elongation, tail separated from yolk mass, tadpoles turned around inside eggs		4
20	*Gill circulation*, *tail elongation*	21	5
21	*Cornea transparent*, *mouth opens*	11	6
22	Tail fins transparent, pigmentation darkens, yolk mass starting to reduce	11	7
23–24	*Labia and teeth differentiate*, *operculum covers gill bases / closes on right*, coloration on body and eyes darkened	11	8–9
25	*External gills atrophied*, *mouth parts obvious*, yolk mass largely resorbed, pigmentation extended	8	10

domen in most larvae. On 4 May 2014 (24 days after the first clutch was found), all larvae had hatched and ingested food regularly; the tadpoles varied from stage 26 to stage 32 (see Table 2 and Figs. 7, 8 for advanced developmental stages). The bodies of the tadpoles were almost entirely translucent, with small dark grey and white pigmented markings, that were concentrated dorsally and became more scattered and isolated towards the sides. Although the dark grey pigments were arranged more densely in some areas, they remained isolated dots, while the white markings merged into conjunctions resembling ice crystals in several individuals. In general, the amount of grey and white pigments varied considerably between the tadpoles, forming a distinct marble pattern in some larvae, while in others only white or almost no grey pigments were found throughout development. The inner organs were clearly discernible and colored white to rosy, the intestinal coils appeared white to light grey. The dark grey and white spots were also found on the tail, where especially the dark pigments were aggregated and formed irregular large blotches in some larvae. Those blotches were mostly found on the tail musculature and sometimes on the upper tail fin, the lower fin of all tadpoles was completely translucent or only covered by few white pigments. The v-shaped myotomes of the tail musculature and blood vessels of the tail were well discernible. The hind limb buds were white. The pupils were black and round, and surrounded by a thin white to light-yellow ring. The iris was light blue and covered by skin with varying amounts of black and white pigments. The keratinized structures of the oral disc were well discernible through the skin. The openings of the nares were surrounded by dark pigmentation. In several individuals, a triangular shaped white spot was found on the snout tip. Around stage 28, the ground coloration of the skin began to turn a pale green in some larvae. At stage 29 (length of hind limb buds > 1½ d), in some individuals the dark grey and white pigmentation had developed further and formed a more distinct pattern, while in others it remained as scattered markings. In most individuals, the white pigments had increased and extended laterally and ventrally. The development of the hind limbs progressed from the foot paddle at stage 31 and stage 37, all toes were separated and the legs began to bend at the knee and foot joints. Dark grey pigmentation marked the toes and limb joints. A larger part of the iris was exposed and no longer covered by skin; the exposed part around the pupil was pale yellow, while the outermost ring was still blue with black pigmentation. At stage 39, the foot was distinctly elongated; the keratinized mouth

Fig. 5. Breeding sites of *Gracixalus quangi* in Hoa Binh Province. *Photo credit C.T. Pham.*

Table 2. Developmental stages and morphological characters of *Gracixalus quangi* larvae from Hoa Binh Province from stage 26 to 39; total length (TTL) in mm (mean ± sd; range in parentheses), stage diagnostic characters according to Gosner (1960) in italics. n: number of individuals observed at the respective stage. d: number of days in which stage could be observed within one clutch (dates in parentheses); A: clutch found on 11 April 2014, B: clutch found on 17 April 2014; *= TTL of preserved larva in stage 36 excluded, as part of the tail was missing.

Stage	Diagnostic characters	TTL (mm)	d (A)	d (B)
26–27	Larvae hatched, free-swimming and feeding, blue iris coloration, light yellow ring around pupil, body with dark grey and white pigments in variable concentration, skin mostly translucent, *hind limb bud development* (buds light white)	17.5 ± 2.4 (14.6–21.4), n=6		3 (May 4–6)
28	*Hind limb bud development (l ≥d)*, pale green dorsal ground coloration in some individuals	21.1 ± 2.8 (17.9–23.8), n=6		5 (May 4–8)
29	*Hind limb bud development (l≥1½ d)*; dark grey and white pigments extended in some individuals	21.8 ± 0.6 (21.4–22.2), n=2		5 (May 4–8)
30	*Hind limb bud development (l=2 d)*	20.3 ± 2.0 (18.5–23.5), n=5		7 (May 5–11)
31	*Foot paddle developed*	22.4 ± 2.0 (20.6–25.6), n=5		5 (May 6–10)
32	*Toe indentation 4-5*	24.9 ± 2.9 (22.4–29.9), n=4	2 (May 4–5)	
33	*Toe indentation 3-4*	23.8 ± 2.4 (20.9–26.9), n=6	2 (May 4–5)	4 (May 7–10)
34	*Toe indentation 2-3*	25.6 ± 2.4 (20.8–29.5), n=9	4 (May 4–7)	3 (May 9–11)
35	*Toe indentation 1-2*	26.6 ± 1.2 (25.7–28.6), n=7	9 (May 4–12)	2 (May 9–10)
36*	*Toes 3-5 separated*; forelimbs with elongated fingers discernible under skin of preserved larva	27.3 ± 1.1 (26.1–29.4), n=6	9 (May 6–14)	1 (May 11)
37	*All toes separated*, legs begin to bend at knee and foot joints, dark grey pigmentation on toes and joints, larger part of iris exposed (pale yellow); forelimbs with finger projections discernible under skin of preserved larva	28.2 ± 2.1 (23.3–29,9), n=19	7 (May 7–13)	2 (May 15–16)
38	*Metatarsal tubercle*, toe elongation, toe joints bending; forelimbs with elongated fingers and well developed discs discernible under skin of preserved larvae	27.2 ± 0.2 (27.0–27.3), n=3	2 (May 17–18)	
39	*Subarticular patches* (slightly visible); foot elongation	30.0 ± 0.1 (30–30.1), n=3	2 (May 13–14)	1 (May 21)

structures were still visible. Toe webbings and toe discs were developed in stage 40 (see Table 3 and Fig. 8 for stages of metamorphs). In stage 41, the coloration on the back and hind limbs had become a pale green, while the skin was still mostly translucent. The spiracle was still present, the vent tube was gone. The eye coloration had become light yellow. In several individuals, the dark interorbital bar, which is also present in adults, appeared. The fore limbs were well discernible under the skin, the oral disc was reduced. Stage 45 (mouth posterior to eye, tail stub) was only documented in one individual which had entered the land section of the tank on 1 June 2014. Fore and hind limbs were light green and speckled with white dots; coloration on the body darker green to olive. Skin was still slightly translucent with inner organs visible. A light ochre to yellow, triangular shaped marking was present on the forehead from the eyes to the snout tip. The pupils were horizontal (round in larval stages); the iris was colored golden-brown, with a bright yellow ring around the pupil.

Assuming that the clutches were collected a few days after egg deposition, the whole embryonal and larval development required about 7–8 weeks.

Larval description. The following larval description is based on a single tadpole (IEBR 4333b) in stage 35 (Figs. 9–11; for measurements see Table 4).

Table 3. Developmental stages and morphological characters of *Gracixalus quangi* metamorphs from Hoa Binh Province; stage diagnostic characters according to Gosner (1960) in italics. n: number of individuals observed at the respective stage. d: number of days in which stage could be observed; dates in parentheses.

Stage	Diagnostic characters	n	d
40	*Foot tubercles*, *vent tube present*, toe webbings and toe discs developed	2	2 (May 19–20)
41	*Forelimbs visible*, *mouthparts atrophy*, *vent tube gone*, spiracle still present, back and hind limbs pale green, iris pale yellow, dark interorbital bar in several larvae	ca. 20	14 (May 19–June 1)
45	*Mouth posterior to eye*, *tail stub*, froglet on land, fore and hind limbs light green, speckled with white dots; body coloration darker green to olive, skin slightly translucent; light ochre to yellow, triangular shaped marking on fore head between eyes and snout tip; pupils horizontal; iris golden-brown, bright yellow ring around pupil	1	1 (June 1)

Coloration in life: Skin translucent, dorsal body surface pale green, laterally becoming whitish-transparent; ventrally completely translucent, inner organs (gills, heart, gut coil) well discernible through the skin. Slight pigmentation consisting of small dark grey dots, density decreasing from dorsal to ventral surface. Body dorsally and laterally marbled with white, ice crystal shaped pigments; on ventral side white marbling is only present in intestinal coil area, while gill area is unpigmented. Spiracular tube is transparent. Soft mouthparts, hind limb, and tail musculature are pale grey to white; tail musculature fades from anterior to posterior, becoming almost transparent in the distal third of tail. V-shaped myosepts of tail and large, red-colored blood vessels in the proximal third of tail are well discernible. Small aggregations of dark dots forming diffuse blotches are present on the proximal third of tail musculature. Tail fins are translucent, with aggregations of the white pigments forming irregular blotches on upper tail fin and in distal quarter of lower tail fin. Ground coloration of iris is pale yellow, with scattered light blue and black pigments; pupil is surrounded by a unicolored, pale yellow ring. Skin which partially covers eyes forms a darker ring around the eye, which is irregularly patterned with bluish and dark grey pigments.

Coloration in preservative: Ground coloration of body and tail musculature yellowish-beige to light apricot, skin distally translucent in dorsal view. Inner organs visible through the skin appear reddish-brown. White pigments on skin are not visible; dorsal and ventral pigmentation consists of small grey spots; pigment aggregations on tail musculature appear darker and almost black in preserved specimen. Tail fins are transparent. Snout region, vent tube, and hind limbs are whitish-translucent; few small dark pigments scattered dorsally on hind limbs. Eyes and keratinized mouth structures are black; pupils are white.

Description in dorsal view: Body shape oval, elongated (maximum body width 0.58 of body length), snout rounded. Widest portion of body at the gill region (posterior to eyes) in dorsal view; with a slight constriction of body contour near location of spiracle opening. Oral disc is positioned anteroventrally and not visible in dorsal view. Eyes are of moderate size (eye diameter 0.23 of body width), directed laterally and positioned dorsolaterally at the first body third (interorbital distance 0.58 of maximum body width), not visible in ventral view. Nares are small, anterodorsally positioned and directed; located nearer to snout tip than to pupil (SN 0.29 of NE); internarial distance is 0.32 of interorbital distance. Tail musculature is moderately developed (width of tail musculature at base 0.42 of maximum body width).

Description in lateral view: Body slightly depressed (maximum body height 0.81 of maximum body width),

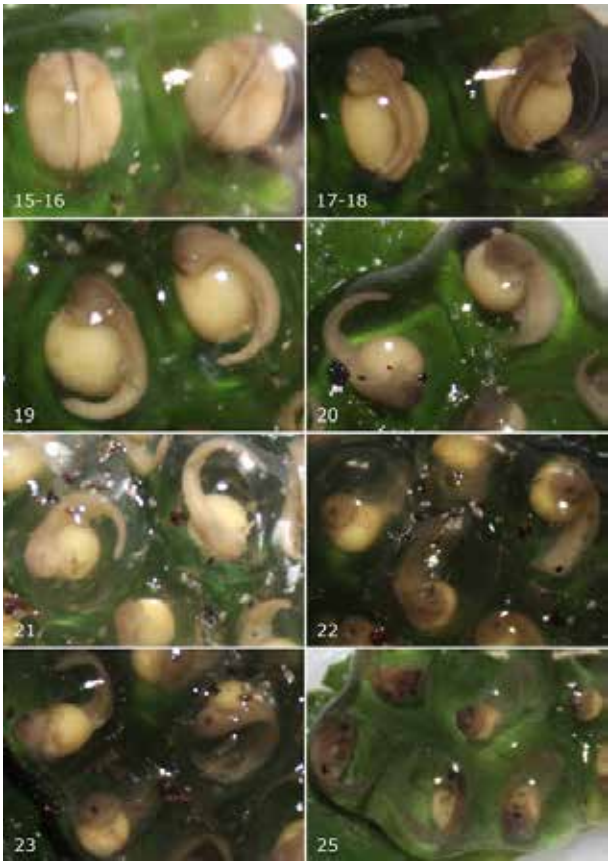

Fig. 6. Early developmental stages of *Gracixalus quangi* from Hoa Binh Province (development stages indicated). *Photo credit C.T. Pham.*

Table 4. Measurements of preserved tadpoles of *Gracixalus quangi* from Hoa Binh Province in mm; (for abbreviations see Material and Methods) *= part of tail missing; ** = specimen used for genetic analysis with tail and part of body missing.

Specimen	IEBR 4333a	IEBR 4333b	IEBR 4333c	ZFMK 101032	IEBR 4333d	ZFMK 101033
Stage	?	35	36	37	38	38
BH	2.6	4.2	4.4	3.8	4.0	5.0
BL	-	9.2	8.5	8.6	9.9	9.7
BS	-	3.5	2.4	3.2	3.4	3.2
BW	4.3	5.3	5.9	4.7	5.7	5.9
ED	0.8	1.2	1.8	1.3	1.6	1.6
ES	1.5	1.7	1.9	1.8	1.9	1.9
IND	1.2	1.0	1.3	1.2	1.4	1.1
IOD	1.9	3.1	3.8	3.2	3.7	3.4
LFH	-	1.1	-	0.6	0.5	0.8
MTH	-	4.1	-	3.6	3.7	3.6
NE	1.2	1.4	1.8	1.4	1.5	2.0
ODW	1.6	2.2	2.4	2.1	2.1	2.1
SN	0.3	0.4	0.5	0.5	0.7	0.5
SS	4.3	5.7	6.1	5.4	6.5	6.5
TAL	-	16.5	-	17.6	17.4	17.3
TMH	-	2.8	3.0	2.9	3.0	2.5
TMW	-	2.2	2.9	2.5	2.0	2.2
TTL	6.9	25.7	19.9	26.2	27.3	27.0
UFH	-	1.4	-	1.2	1.1	1.1
LTRF	4(2-4)/3	5(2-5)/3	5(2-5)/3	5(2-5)/3(1)	5(2-5)/3(1)	5(2-5)/3(1)

with maximum height approximately on spiracle axis, snout rounded. Tubular spiracle sinistral, laterally positioned at second third of body (distance from snout tip to opening of spiracle 0.61 of body length), opening posterolaterally and visible in dorsal view; spiracular opening is oval. Outer wall of the medial vent tube is attached to lower tail fin; cloacal aperture is dextral. Tail relatively long (tail length 0.64 of total length) and of moderate height (maximum height of tail, including fins, is 0.25 of tail length), v-shaped mysepta of tail musculature are well discernible (tail muscle height 0.67 of maximum body height, and 0.68 of maximum tail height). Tail musculature almost parallel at proximal half, then gradually tapering to end of tail, not reaching tail tip. Upper fin originates at body-tail junction, lower fin inserts axially parallel, connecting with abdomen. Point of maximum tail height is located at the second half of the tail; dorsal fin is higher than ventral fin (maximum height of upper fin 0.34 of maximum tail height; maximum height of lower fin 0.79 of maximum height of upper tail fin), tail tip is narrowly rounded.

Oral disc anteroventrally positioned, of nearly trapezoidal shape and laterally emarginated (oral disc width 0.42 of maximum body width). Oral disc framed by finger-shaped marginal papillae, upper labium with large medial gap. Lower labium and lower half of upper labium with one row of submarginal papillae (about 50 submarginal papillae under posterior keratodont row P3). Labial tooth row formula 5(2-5)/3; A5 greatly reduced. About 43 keratodonts per 0.5 mm. Jaw sheaths black, slightly serrated. Upper jaw sheath forming smooth arc, lower jaw sheath V-shaped.

Measurements (in mm): BH 4.2; BL 9.2; BS 3.5; BW 5.3; ED 1.2; ES 1.7; IND 1.0; IOD 3.1; LFH 1.1; MTH 4.1; NE 1.4; ODW 2.2; SN 0.4; SS 5.7; TAL 16.5; TMH 2.8; TMW 2.2; TTL 25.7; UFH 1.4.

Variation within the larvae series. Oral disc: Generally uniform in all preserved specimens; except for a medial gap in P1 in stage 37 and 38 tadpoles (LTRF 5(2-5)/3[1]). A5 is very short in all specimens examined and not yet present in the specimen used for genetic comparisons (IEBR 4333a), which was preserved in an earlier larval stage. The shape of the tail fins, especially the tail tip, varies from broadly to narrowly rounded / bluntly pointed. The coloration is highly variable; especially the amount and composition of blotches formed by the dark grey and white pigments. The nares are surrounded by dark grey ridges in some larvae, and sometimes a distinct white, nearly triangular fleck is present reaching from the nares to the snout tip. The greenish dorsal coloration is more intense in the advanced stages.

Proportions vary as follows (ratios for the tadpole for genetic comparison are excluded here, as this specimen was preserved in an early larval stage, and the tail and part of the body were dissected for molecular analy-

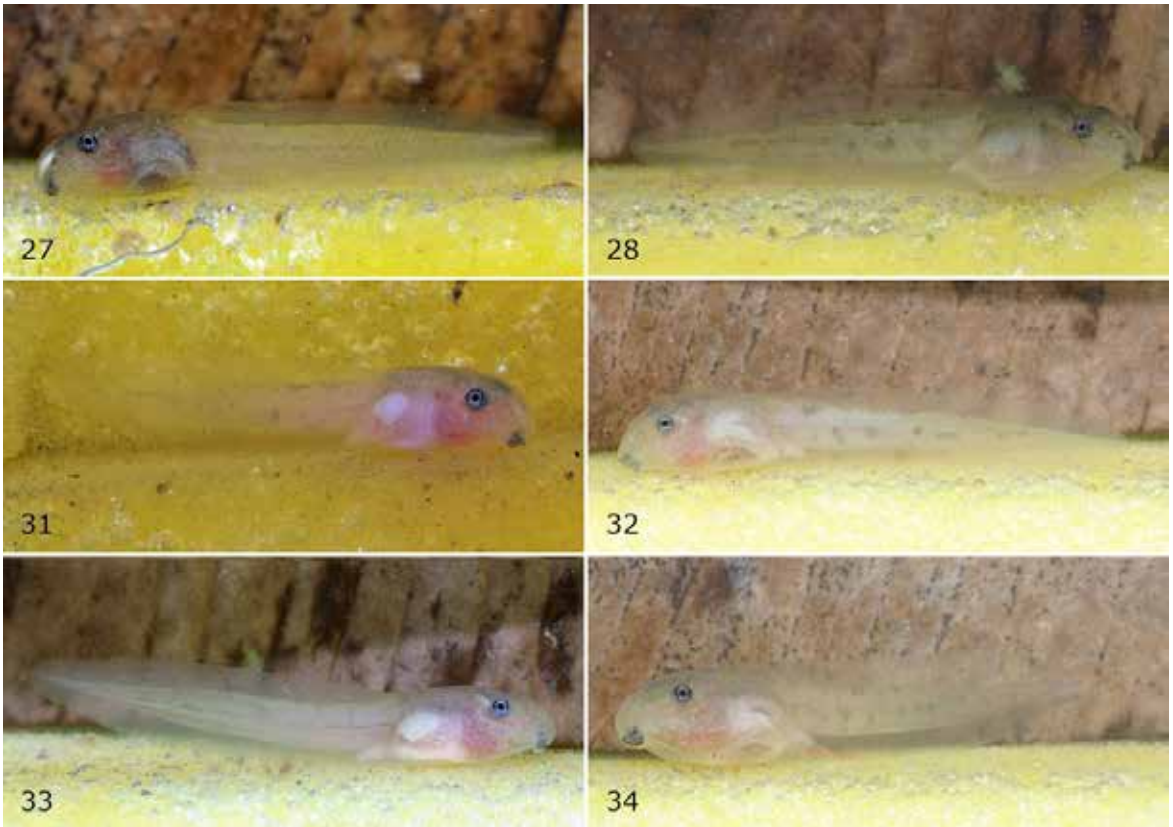

Fig. 7. Advanced developmental stages of *Gracixalus quangi* from Hoa Binh Province (development stages indicated). *Photo credit T. Ziegler.*

Fig. 8. Advanced developmental stages of *Gracixalus quangi* from Hoa Binh Province (development stages indicated). *Photo credit T. Ziegler and T.D. Tran.*

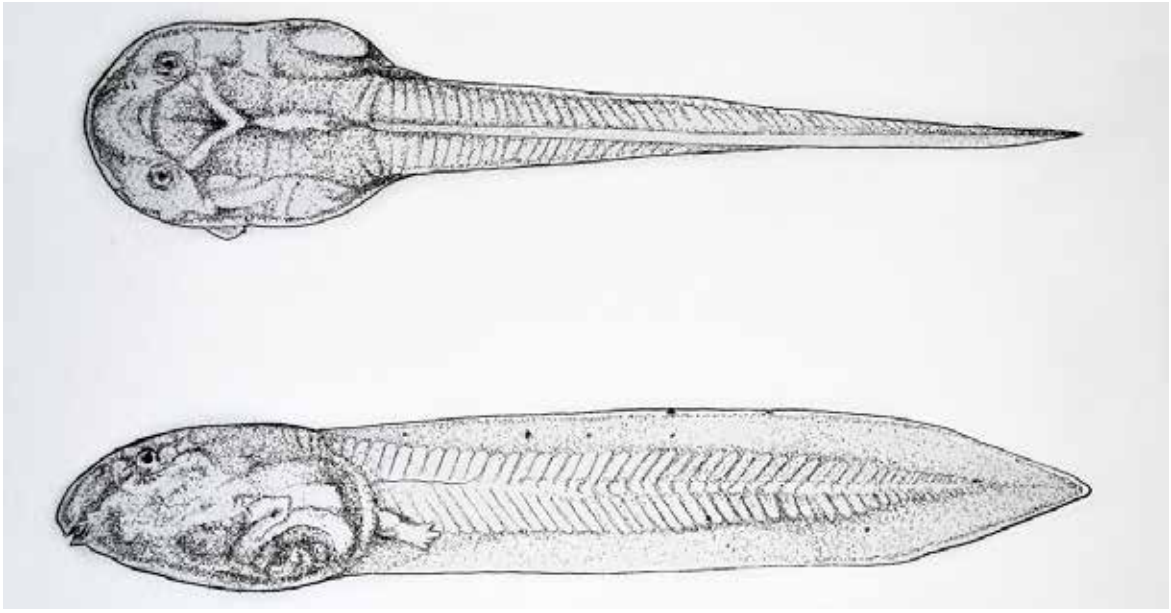

Fig. 9. Drawing of a larva of *Gracixalus quangi* from Hoa Binh Province (IEBR 4333b) in development stage 35 in dorsal and lateral view. *Drawing by C. Niggemann.*

ses): BW 0.54–0.69 of BL; ED 0.27–0.3 of BW; IOD 0.58–0.68 of BW; SN 0.25–0.47 of NE; IND 0.32–0.38 of IOD (0.63); TMW 0.35–0.53 of BW; BH 0.70–0.85 of BW; TAL 0.64–0.66 of TTL, TMH 0.63–0.76 of BH; TMH 0.69–0.81 of MTH; UFH 0.30–0.33 of MTH; LFH 0.46–0.73 of UFH; ODW 0.36–0.45 of BW.

In the four preserved stage 36–38 tadpoles, the forelimbs in different stages of development were well discernible under the skin in ventral view (see Table 3).

Tadpoles of *Gracixalus quangi* are exotroph: lentic: benthic larvae of Orton's Type IV.

Discussion

In this study, we confirmed the findings of Rowley et al. (2015) that *Gracixalus quangi* deposits its eggs in clear jelly clumps on the surface of live leaves, near the leaf tip, in moderate heights above non flowing water. Clutch sizes recorded herein accorded well with observations by Rowley et al. (2015). The maximum height of clutches above the water surface was <50 cm according to Rowley et al. (2015), while we found them at heights of up to 70 cm. Rowley et al. (2011, 2015) examined tadpoles up to stages 24 and 26, respectively, which generally corresponded well with our findings (e.g., large yolk mass in embryonic stages). Rowley et al. (2011, 2015) assumed the actual tooth row formula could only be observed in advanced stages, which we herein confirm.

Grosjean (2005) recommended assessing tadpoles of stages 32–40 for larval descriptions possessing the complete set of taxonomically relevant characters in this range of development. The keratodont row formula 5(2-5)/3[1] was constant in our specimens in stages 35–38; while the individual used for genetic comparison, which was preserved in an earlier stage, was lacking the fifth tooth row in the upper labium. However, as this tooth row was greatly reduced in all analyzed specimens and this individual was obtained from the same egg clutch and corresponded well morphologically with the other specimens

Fig. 10. Drawing of the oral field of a larva of *Gracixalus quangi* from Hoa Binh Province (IEBR 4333b) in development stage 35: natural view on top, schematic drawing below). *Drawing by C. Niggemann.*

Fig. 11. Larva of *Gracixalus quangi* from Hoa Binh Province (IEBR 4333b) in development stage 35 in life (photo taken 4 May 2014). *Photo credit T. Ziegler.*

photographed in comparable stages, it does not belong to a different species. The gap in P1, which was present in the specimens in stages 37 and 38, is likely to represent intraspecific variation rather than being caused by ontogenetic atrophy of the oral disc, which usually occurs in later stages; however, the mechanism of mouth part atrophy is poorly studied and it is not known whether defined patterns of tooth loss within the tooth rows occurs during ontogeny (McDiarmid and Altig 1999).

While Gosner (1960) defined tadpoles in stages 20–25 as "hatchlings," the larvae of *G. quangi* remained longer inside the eggs. A lack of data acquisition in late April prevented photographing larvae directly after hatching, but since tadpoles of the first clutch remained inside the eggs in stage 25 on day 10 and the first free swimming larvae were documented in stages 26–27 on day 24, we assume that hatching occurred in stage 25 and the maximum period of this stage was 13 days. A delayed hatch may be advantageous for avoiding predation inside the water body in early hatchling stages, as tadpoles in these stages are quite active and able to swim when they drop into the water. This could also explain the relatively large yolk mass during the embryonic stages, which was completely resorbed at the time of hatching. However, the time of hatching seems to be somewhat variable, as Rowley et al. (2011) found hatched larvae in stage 24.

The transfer of the eggs and the different climatic conditions in the Me Linh Station (i.e., relatively constant temperatures of 25–28 °C versus fluctuations between 19–28 °C in the field) could have led to a delayed hatch in our study. Rowley et al. (2015) also discussed the possible influence of the substrate on which eggs were deposited (live vs. dead leaves) on the embryonic development. In general, the number of days the tadpoles were found in a certain stage varied between 0.5–1 day for the embryonic stages and between 2–14 days for the larval and metamorph stages, but due to the small sample sizes in some stages, these values may not be representative of the general development. The hatchling in stage 26 described by Rowley et al. (2015) was relatively small compared to our observations (10.6 mm TTL vs. 15.8 mm as the smallest length measured in stage 26 here).

The hatchling size could be influenced by the time of hatching and various environmental parameters such as water temperature, larval rearing density, and food availability, which were possible influences both on the timing of larval development and growth in other anuran tadpoles (e.g., McDiarmid and Altig 1999; Álvarez and Nicieza 2002). We found larvae from each clutch with a maximum developmental distinction of five stages per day and size differences up to 8.3 mm per stage, indicating that variation in developmental time and growth within a clutch reared under the same conditions occurs at a certain level. The development of the forelimbs is

clearly visible in several of the photographed and preserved tadpoles from stage 36 onwards due to the transparency of the ventral skin. There is no defined sequence for forelimb development in Gosner's staging system compared to hind limb development, but according to McDiarmid and Altig (1999) forelimb development lags behind that of the hind limbs by about one stage. However, the fingers were noticeably further elongated and shaped in the larva preserved in stage 36 than in the one in stage 37 (see Table 2), suggesting there is also some variation in development before forelimbs emerge.

The white spot on the snout tip, which was listed as a diagnostic character for tadpoles of the genus *Gracixalus* by Delorme et al. (2005), included only the two species *G. supercornutus* and *G. gracilipes*. However, it was not detected in the study of Rowley et al. (2011), who suggested it could be either a variable feature among species within the genus, or it is present only in more well-developed tadpoles. As we found tadpoles with and without the white spot on the snout, it seems to be a variable feature and not related to ontogenetic change. In general, we found the coloration during the larval stages to be quite variable (see Figs. 7, 8).

Finally, our first record of *G. quangi* from a karst forest environment indicates a broader adaptation potential of the species to different habitats than previously thought, and thus also implies the possibility of a wider distribution range.

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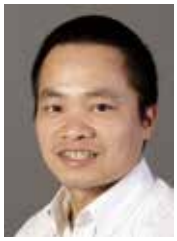

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