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First record of *Gracixalus quyeti* (Amphibia: Anura: Rhacophoridae) from Laos: molecular consistency versus morphological divergence between populations on western and eastern side of the Annamite Range

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Abstract: We report the first country record of the poorly known *Gracixalus quyeti* from Laos based on a recently collected specimen from Khammouane Province, central Laos. While the genetic analysis revealed nearly identical sequences, we found some differences in body ratios and color patterns among the specimen from Laos and the type series from the eastern side of the Annamite Range in Vietnam.

Keywords: *Gracixalus quyeti* - biogeography - distribution - new record - Annamite Range - Laos.

INTRODUCTION

Although the knowledge of amphibian diversity in Laos has strikingly increased within the last two decades, distribution patterns and natural history of many species are still poorly known (Luu *et al*., 2014). Information about the geographic distributions of species is essential for understanding their evolution and ecology and is furthermore crucially required for effective conservation (Nguyen *et al*., 2014; Rowley *et al.*, 2015). The rhacophorid genus *Gracixalus* currently contains 11 species whose ranges are restricted to lowland and montane forests in China, Thailand, Vietnam and Laos (Nguyen *et al.*, 2008; Rowley *et al.*, 2011; Matsui *et al*., 2015; Frost, 2016). Luu *et al*. (2014) recorded *G. supercornutus* (Orlov, Ho & Nguyen, 2004) from Laos, which also represented the first record of the genus *Gracixalus* from this country. However, relatively little is known about this genus, and distribution ranges of species within the genus are largely unknown (Luu *et al*., 2014).

In this study, we report *Gracixalus quyeti*, a recently described and rare species from Phong Nha – Ke Bang National Park (NP) in central Vietnam, for the first time from Hin Nam No National Protected Area (NPA) in central Laos on the western side of the Annamite Range.

MATERIAL AND METHODS

Material examined: A single female specimen of *Gracixalus quyeti* (VNUF A.2014.73) was collected by Vinh Quang Luu, Thomas Calame, Dung Van Phan and Kieusomphone Thanabuaosy during a field survey in May 2014 in Noong Ma Village (17°17.394' N, 106°09.980'

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E, 592 m a.s.l., recorded by a Garmin GPSMAP 60CSx GPS receiver and recorded in datum WGS 84), within Hin Nam No NPA, Boualapha District, Khammouane Province, central Laos (Fig. 1).

Sampling methods: The specimen was caught by hand and anaesthetized in a closed vessel with a piece of cotton wool containing ethyl acetate. After taking photographs, the specimen was fixed in 80% ethanol and subsequently transferred into 70% ethanol for permanent storage. A tissue sample was preserved separately in 95% ethanol. The specimen has been deposited in the collection of the Vietnam National University of Forestry (VNUF), Hanoi, Vietnam.

Molecular analysis: Total genomic DNA was extracted from a tissue sample using a commercially available DNeasy Tissue Kit following manufacturer's instructions (QIAGEN Inc., Valencia, CA, USA). A fragment of 16S gene was amplified using the primer pair 16Sar + 16Sbr (Palumbi *et al.,* 1991). The standard PCR conditions used 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 sequencing. Successful amplifications were purified to eliminate PCR components using GeneJETTM PCR Purification kit (Fermentas, Canada). Purified PCR products were sent to Macrogen Inc. (Seoul, South Korea) for sequencing. The obtained sequence was compared to those available from other species using the BLAST search in GenBank.

Fig. 1. Map showing the distribution of *Gracixalus quyeti*, including the localities of the type series after Nguyen *et al.* (2008) in Quang Binh Province, Vietnam (marked with blue dots) and our first record from Khammouane Province, Laos (marked with a red dot).

Morphological analysis: Determination of morphological characters followed Nguyen *et al*. (2008). Measurements were taken by the first author with a digital caliper to the nearest 0.1 mm. Abbreviations were used as follows: SVL: snout-vent length; HL: head length (from the back of mandible to the tip of snout); HW: head width (across angle of jaws); MN: distance from the back of mandible to the nostril; MFE: distance from the back of mandible to the front of eye; MBE: distance from the back of mandible to the back of eye; IFE: distance between the front of eyes; IBE: distance between the back of eyes; IN: internasal distance; EN: distance from the front of eye to the nostril; EL: horizontal eye diameter; NS: distance from nostril to the tip of snout; SL: distance from the front of eye to the tip of snout; TYD: greatest tympanum diameter; TYE: distance from tympanum to the back of eye; IUE: minimum distance between upper eyelids; UEW: maximum width of upper eyelid. Forelimb: HAL: hand length (from the base of outer palmar tubercle to the tip of fourth toe); FLL: forelimb length (from the elbow to the base of outer tubercle); TFL: third finger length (from the base of the first subarticular tubercle to the tip of third toe); fd1-4: width of discs of fingers I-IV; fw1-4: width of fingers I-IV (measured at the narrowest point of the distant phalanx). Hindlimb: FL: femur length (from vent to knee); TL: tibia length; TW: tibia width; FOL: foot length (from the base of inner metatarsal tubercle to the tip of fourth toe); FTL: fourth toe length (from the base of the first subarticular tubercle to the tip of fourth toe); TFOL: distance from the base of tarsus to the tip of fourth toe; IMT: length of the inner metatarsal tubercle; ITL: inner toe length; td1-4: width of discs of toes I-IV; fw1-4: width of toes I-IV (measured at the narrowest point of the distant phalanx). Webbing: MTTF: distance from the distal edge of metatarsal tubercle to the maximum incurvation of web between third and fourth toes; TFTF: distance from the maximum incurvation of web between third and fourth toes to the tip of fourth toe; MTFF: distance from the distal edge of metatarsal tubercle to the maximum incurvation of web between fourth and fifth toes; FFTF: distance from the maximum incurvation of the web between fourth and fifth to the tip of fourth toe. Webbing formula followed Glaw & Vences (2007). Comparative data were taken from Nguyen *et al.* (2008). Institutional abbreviations are as follows:

ZFMK: Zoologisches Forschungsmuseum Alexander Koenig, Bonn, Germany; VNUH: Vietnam National University, Hanoi, Vietnam.

RESULTS

Ecology of examined specimen: The Laotian specimen was found at night (20:30) sitting on a branch of a shrub, ca. 0.5 m above an outcrop at an elevation of 592 m above sea level. The air temperature was 27.3°C and the relative humidity was 80%. The locality was surrounded by limestone cliffs and karst vegetation, mainly consisting of species of Ebenaceae, Dracaenaceae, Arecaeae, Meliaceae, and Moraceae.

Molecular analysis: The obtained sequence had 656 bps. Comparative analysis of the obtained sequence with those from GenBank (ZFMK 82999: EU871429.1, VNUH 160706: EU871428.1) showed 99% similarity between the newly collected specimen from Laos (VNUF A. 2014.73) and the holotype (ZFMK 82999) as well as the paratype (VNUH 160706) of *Gracixalus quyeti* from Vietnam. Specifically, the new sequence was different from EU871429.1 in one position and EU871428.1 in two positions.

Morphological analysis: The morphological diagnosis coincided with the original description of *G. quyeti* from central Vietnam (Nguyen *et al.,* 2008) in the following characters: Small rhacophorid (ZFMK 82999 SVL 34 mm, VNUF A. 2014.73 SVL 31.4 mm), vomerine teeth absent. Snout rounded, longer than the diameter of eye. Nostrils closer to tip of snout than to eye. Pupil oval and horizontal. Tympanum distinct, rounded and wider than disc of finger III. Dorsal surface of head and body and upper part of flanks with small sharp tubercles. A dark pattern forming an inverse Y, triangular spot between eyes bifurcating into two bands continuing posteriorly onto the back. Brown marbling on margin of throat and throat. Webbing moderately developed: Ii(1)-(2)iIIe(0.5)-(1 2/3)iIIIe(0.5)-(2)iIVe(2)-(0.5)iV.

Despite the high genetic correspondence, we also noted some differences in morphology between the examined specimen from Laos and the type specimens of *G. quyeti* (Nguyen *et al.*, 2008): *Body ratios:* Head wider than long, in contrast to longer than wide in the type series. Forelimb and hand length slightly shorter, the latter approximately 27% of SVL (30% in the holotype and 28% in the paratype). Length of finger III approximately 16% of SVL and remarkably shorter than in the specimens from Vietnam (24% in the holotype and 19% in the paratype). Hind limb approximately 1.45 times longer than SVL, shorter than in specimens from Vietnam (holotype: 1.6, paratype: 1.7). Tibia 1.5 times thinner than in the holotype. Length of toe IV 23% of SVL, remarkably shorter than in the holotype (37%) and slightly shorter compared to the paratype (25%) (Tab. 1). *Coloration in life:* Dorsal surface of head and body is greenish beige to grayish light-brown with a grayish dark-brown blotching pattern as described above, in contrast to brownish to moss-green with a dark brown pattern in the adult holotype and moss-green with an indistinct pattern in the subadult paratype. Forelimb and dorsal part of hindlimbs are beige to grayish light-brown with grayish dark-brown bands, versus moss-green with dark brown bands in the holotype and the subadult paratype. The ventral surface can only be described based on the preserved specimen: Belly, chest and throat slightly white to yellowish white with brown marbling on margin of throat and throat, versus background color more yellowish in the preserved holotype and more bluish in the living paratype (Fig. 2A-B).

DISCUSSION

The new record of *Gracixalus quyeti* from Hin Nam No NPA, Laos is approximately 80 km straight line distance apart from the type locality in Quang Binh Province,

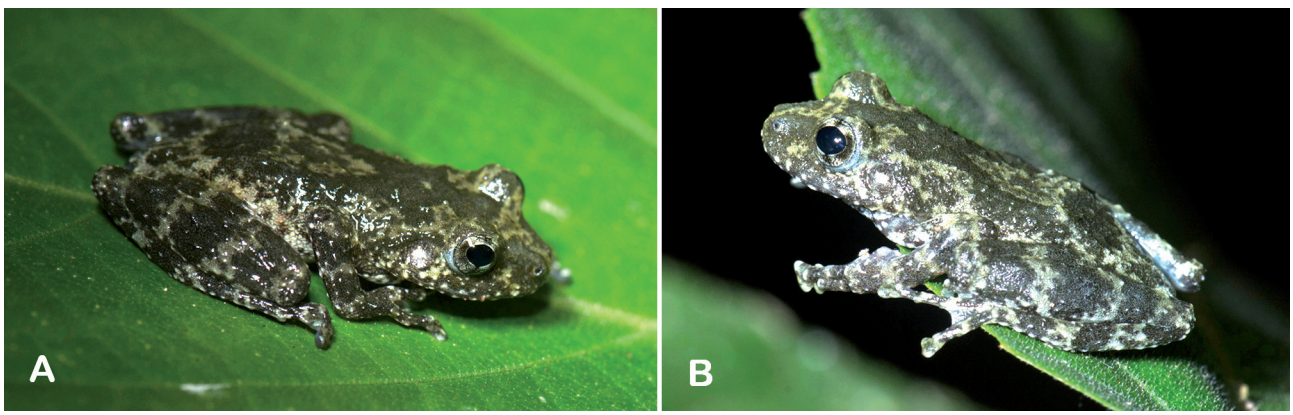

Fig. 2. Female of *Gracixalus quyeti* from Hin Nam No NPA, central Laos. (A) Dorsolateral view. (B) Lateral view. Photos: T. Calame.

Table 1. Morphological characters of the newly collected specimen of *Gracixalus quyeti* from Laos in comparison with the type series from Vietnam (after Nguyen *et al*., 2008; measurements in mm, abbreviations defined in the text).

	VNUF A. 2014.73	ZFMK 82999	VNUH 160706
	female	female, holotype	subadult, paratype
	Hin Nam No NPA, central Laos	Phong Nha – Ke Bang NP central Vietnam	Phong Nha – Ke Bang NP, central Vietnam
SVL	31.4	34.0	22.0
HL	10.3	12.5	8.2
HW	11.73	11.4	7.4
MN	10.3	11.2	7.4
MFE	6.96	8.4	5.4
MBE	3.86	4.5	3.3
IFE	5.45	6.9	4.3
IBE	8.5	11.0	6.8
IN	3.08	3.0	2.1
EN	2.9	3.5	2.5
EL	4.0	4.8	3.2
NS	1.34	2.1	1.3
SL	4.22	5.4	3.8
TYD	2.0	2.5	1.6
TYE	0.92	1.0	0.7
IUE	3.89	4.1	2.4
UEW	2.36	3.0	1.8
HAL	8.4	10.3	6.1
FLL	6.4	7.2	4.9
TFL	4.97	8.1	4.2
fw 1-4	0.31/0.36/0.38/0.36	0.6/0.9/1/0.9	-
fd 1-4	0.48/0.83/0.99/1	1/1.6/1.9/1.7	1.2 (fd 3)
FL	15.68	18.5	11.5
TL	17.61	20.0	11.8
TW	2.57	4.2	2.0
FOL	12.21	15.2	13.9
FTL	7.1	12.5	5.6
TFOL	20.47	24.8	8.1
IMT	0.82	1.3	0.8
ITL	3.83	4.2	1.9
MTTF	7.1	9.4	3.9
MTFF	7.3	9.8	4.7
TFTF	4.71	5.6	3.0
FFTF	4.38	5.0	2.8
tw 1-5	0.28/0.29/0.36/0.49/0.4	0.8/0.9/1.1/1.3/1.1	-
td 1-5	0.35/0.66/0.6/0.96/0.8	0.5/0.6/0.7/0.9/0.8	-

central Vietnam. It is likely that *G. quyeti* is more widespread across the Annamite's extensive limestone areas of central Vietnam and central Laos. The species might potentially be endemic to these fragmented lowland and montane forest habitats, while its occurrence seems to be relatively rare within this presumed distribution range.

All records of *G. quyeti* were derived from different elevations in the limestone area within the transition zone between the northern and central Annamite Mountains, a semiconnected array of hills and forested limestone karst outcrops. The Annamite Range generally experiences a tropical wet monsoon climate, while the eastern oceanic Phong Nha – Ke Bang NP receives more precipitation

and has lower average temperatures than Hin Nam No on the western side in the rain shadow of the Annamite Range (Timmins & Trinh 2001; Sterling *et al.*, 2006; Bain & Hurley, 2011). Taking into account the climatic differences between both sides of the Annamite Range and the lack of knowledge about the genus *Gracixalus*, further research must clarify if the shown morphological differences in coloration and body ratios may possibly reflect ecological adaptations to different environments, evolutionary driven morphs or likely just reflect variation within this poorly known species. Still, our finding elevates the number of amphibian species recorded from Laos to 100, and the number of rhacophorid species known to occur in Laos to 34. Although the species number of amphibians known from Laos has nearly doubled during the last 15 years from 58 to 100 recorded species, the amphibian species richness of Laos is still underestimated (Stuart, 1999; Teynié *et al.*, 2014; Luu *et al.*, 2014; Frost, 2016). In particular big gaps remain in our knowledge of the Annamite's amphibian ecology and distribution.

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