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A novel third species of the Western Ghats endemic genus *Ghatixalus* (Anura: Rhacophoridae), with description of its tadpole

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Abstract

The Western Ghats biodiversity hotspot is a recognized center of rhacophorid diversity as demonstrated by several recent studies. The endemic genus *Ghatixalus* is represented by two species from two separate high-elevation regions within the Ghats. Here, we describe a third species that can be distinguished by morphological and larval characters, as well as by its phylogenetic placement.

Key words: Rhacophoridae; *Ghatixalus*; New species; Tadpole; Western Ghats; India

Introduction

The rhacophorid genus *Ghatixalus* Biju et al., 2008 was erected to accommodate a phylogenetically distinct clade of two foam-nesting frog species endemic to the montane plateaux of the southern part of the Western Ghats in India. The type species (*Polypedates* [*Ghatixalus*] *variabilis* Jerdon, 1853) had a long history of being taxonomically problematic due to its lack of distinct morphological synapomorphies and the presence of high degree of homoplasy (Biju, Roelants & Bossuyt, 2008). However, recent phylogenetic studies have alluded to its unique generic placement, although maintaining close relationships with other foam nesting genera like *Polypedates* and the Sri Lankan *Taruga* (Biju, Roelants & Bossuyt, 2008; Abraham et al., 2013; Li et al., 2013).

The two currently recognized species of *Ghatixalus* occur respectively on two sides of a prominent 35 km wide geological divide in the Western Ghats, the Palakkad Gap. *Ghatixalus variabilis* occurs in the Nilgiri Hills north of the gap, while *G. asterops* Biju, Roelants & Bossuyt, 2008 is found south of the gap in the Eravikulam and Devikulam Plateaux as well as in the Palni Hills. Both species occupy the highest reaches (1700–2695m ASL) of their respective ranges (Biju, Roelants & Bossuyt, 2008).

The tadpole of *Ghatixalus variabilis* was first described and figured by Annandale (1918), mistakenly identified as that of *Nyctibatrachus pygmaeus* Boulenger, 1882. However, he was quick to rectify his error and rightly called attention to its actual identity (Annandale, 1919), and pointed out that it is one of the most abundant tadpoles in the hill-streams of the higher elevations of the Nilgiri Hills.

During the period of our fieldwork in the southern Western Ghats, we observed a large, distinct hitherto undescribed rhacophorid frog, which was encountered in mid-elevation hill forests. Based on a unique combination of molecular, morphological and larval characteristics, we ascribe this novel species here to the genus *Ghatixalus*. We also furnish here comparative descriptions of its tadpole with those of its congeners.

Materials and Methods

Morphological Analyses of Adult Frogs

We conducted fieldwork in the Western Ghats during 2012–2015 and collected field data and specimens. Morphological data were recorded from specimens, which were euthanized, fixed in 10% neutral buffered formalin (NBF), and later transferred to 70% ethanol for storage. Voucher specimens are deposited in the collection at the Natural History Museum, Thiruvananthapuram (TNHM). Measurements (rounded to 0.1 mm) were made with a Mitutoyo Digimatic caliper and terminologies follow those of Biju et al. (2008) for adult frogs. Live colouration for individual animals was recorded within an hour after collection.

Morphological data consisted of the following morphometric characters: SVL = snout–vent length, from tip of snout to vent; HL = head length, from rear of mandible to tip of snout; HW = greatest head width, at angle of jaw; SL = snout length, from tip of snout to anterior orbital border of eye; IUE = interorbital distance, the shortest distance between the upper eyelids; EL = eye length, horizontal distance between bony orbital borders of eye; UEW = maximum upper eyelid width; EN = eye to nostril distance, i.e. from nostril to anterior orbital border of eye; IBE = internal back of eyes, shortest distance between posterior orbital borders of eyes; IFE = internal front of eyes, shortest distance between anterior orbital borders of eyes; NS = distance from nostril to tip of snout; IN = inter-narial distance, i.e. between internal borders of nostrils; MBE = distance from rear of mandible to posterior orbital border of eye; MFE = distance from rear of mandible to anterior orbital border of eye; MN = distance from rear of mandible to nostril; TYD = largest tympanum diameter; TYE = tympanum to eye distance, i.e. from posterior orbital border of eye to tympanum; FLL = forelimb length, from elbow to base of outer palmar tubercle; HAL = hand length, from base of outer palmar tubercle to tip of fourth finger; ShL = shank length; TL = thigh length; FOL = foot length, from base of inner metatarsal tubercle to tip of fourth toe. All measurements are in millimeters. Because it has been shown that anurans have lost the first (medial) finger of the hand, the remaining four digits are numbered II through V, in contrast to the traditional I through IV (Schubin and Alberch, 1986).

Morphological Analyses of Tadpoles

Tadpoles were monitored and recorded till metamorphosis in May. Two tadpoles (identification confirmed with DNA sequence data) in developmental stages 37–39 (Gosner, 1960) were taken for measurements after being directly fixed in 10% NBF and preserved in a 1:1 mixture of 70% ethanol and 10% NBF. Terminology used to describe external features of tadpoles follows standard references (Altig & McDiarmid, 1999a). Measurements were as follows; total length (TL), body length (BL), tail length (TAL), body height (BH), eye length (EL), tail muscle height (TMH), maximum tail height (MTH), upper tail fin height (UTF), lower tail fin height (LTF), nasal snout distance (NSD), eye-nasal distance (END), inter-nasal distance (IND), inter orbital distance (IOD), snout-spiracle distance (SSD), head width (HW), body width (BW), tail muscle width (TMW).

Molecular Phylogenetic Analyses

We extracted total genomic DNA from muscle samples of four individuals representing the three species of the *Ghatixalus* genus with a Qiagen DNeasy kit. Each extraction was amplified for the genes of interest through standard PCR (Palumbi, 1996) and sequencing protocols to generate sequence data for a target region of 970bp of 12S and 570bp 16S ribosomal RNA fragments, and 320bp of RHOD and 1300bp of RAG1 nuclear loci (San Mauro et al., 2004; Frost et al., 2006). The sequences were deposited in GenBank under the accession numbers KT359618–KT359621, KT359624–KT359627, KT359630–KT359637. The combined sequence data from GenBank and our sequencing outcomes were aligned using the default parameters of the MUSCLE algorithm (Edgar, 2004). We did not partition by stems and loops, but the protein-coding loci were partitioned by codon. Data for two species of *Ghatixalus*, 14 species representing four foam-nesting and one bubble-nesting rhacophorid genera, and one outgroup rhacophorid species, were downloaded from GenBank (accession numbers provided in Appendix) and a molecular phylogeny was constructed, for which the final concatenated alignment consisted of 2024 bp. We estimated phylogeny using Maximum Likelihood (ML) under the GTR+ Γ model by selecting the best of 200 independent best-tree searches in RAxMLv7.2.8 (Stamatakis, 2006), using the rapid-bootstrapping (BS) algorithm with 1000 replicates, and we consider BS > 70 to represent strong support. Genetic distances were calculated using PAUP* 4.0 (Swofford 2001).

Results

The results of our phylogenetic analyses (Fig. 1) reveal that *Ghatixalus* still forms a monophyletic clade with the addition of a third novel species. The novel species, which forms a sister lineage with strong support (BS = 93) to the clade of *G. variabilis* + *G. asterops*, is described below. Uncorrected p-distances calculated from the mitochondrial (12S + 16S) alignment show that the new species is between 4.7–6.2% divergent from *G. asterops* and between 6–8.1% divergent from *G. variabilis*.

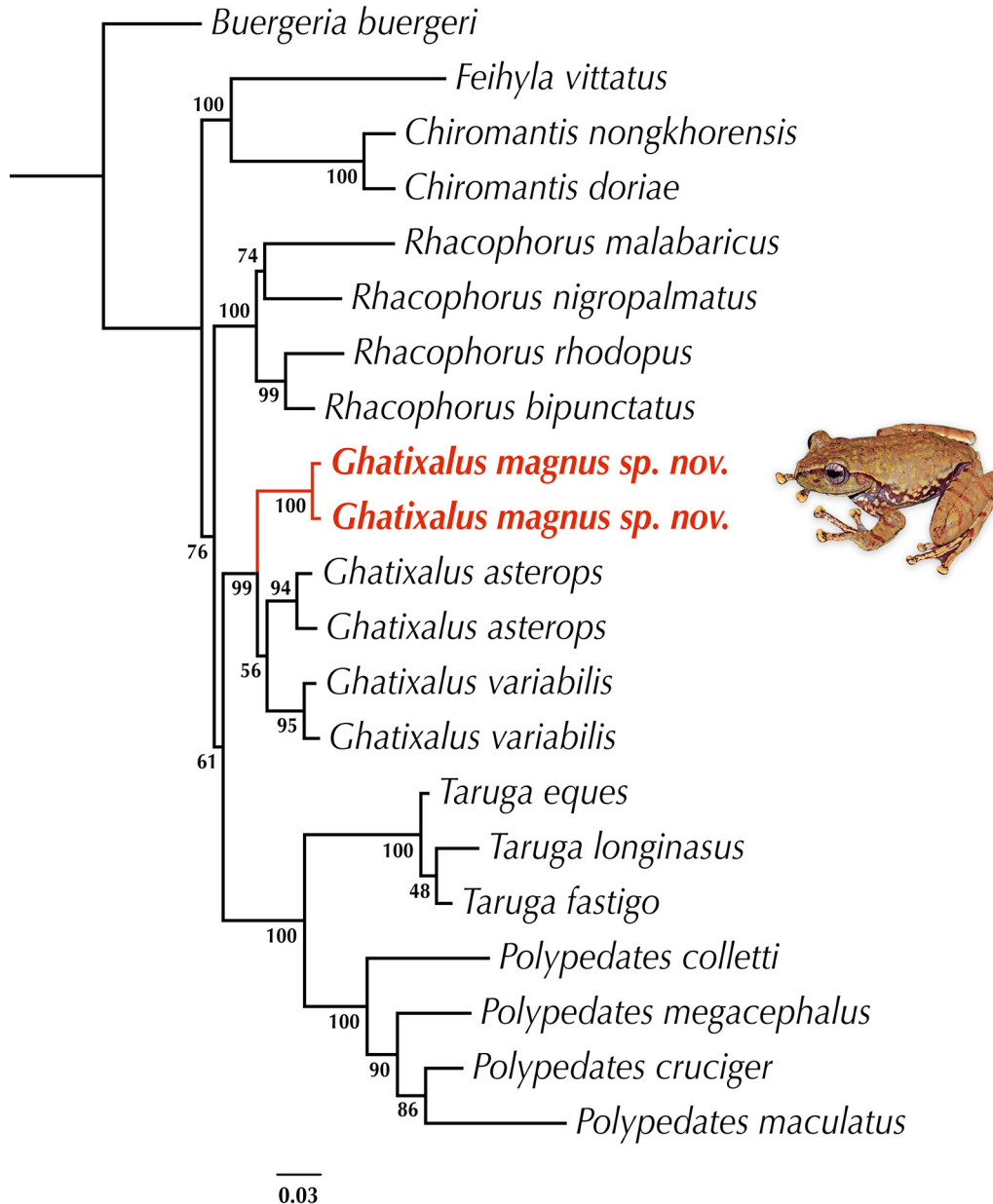


FIGURE 1. Maximum-likelihood (ML) tree based on two ribosomal RNA mitochondrial (12S & 16S) and two nuclear (RAG1 & RHOD) gene sequences showing the relationship of *Ghatixalus magnus* sp. nov. to congeneric species, and other foam-nesting Rhacophoridae.

Ghatixalus magnus sp. nov.

(Figs. 2C, 3, Tables 1 & 2)

Holotype: TNHM (H) 15.5.20/80, adult male (81.90 SVL), collected from near a forest stream, 10° 7'53.75"N, 76°59'57.20"E, at elevation 1440m ASL in Kadalar, Idukki district, Kerala, India by Anil Zachariah on 05 May 2015.

TABLE 1. Morphometric measurements (in mm) of adult male specimens of *Ghatixalus* spp. For abbreviations, see Materials and methods.

Species	Sex	Museum #	SVL	HW	HL	SL	EL	EN	IFE	IBE	IUE	UEW	TYD	TYE	FLL	HAL	SHL	TL	FOL	MN	MBE	MFE	NS	IND
<i>Ghatixalus magnus</i>	M	TNHM(H)	81.90	31.02	27.84	13.19	8.16	8.12	16.78	25.76	11.38	6.75	4.75	2.57	19.67	25.88	39.84	41.87	36.01	25.05	8.14	15.24	6.21	6.78
		15.5.20/80																						
<i>Ghatixalus magnus</i>	M	TNHM(H)	71.54	28.53	25.34	12.25	7.61	7.34	15.32	24.83	11.29	5.94	4.44	1.98	18.28	25.41	39.46	41.51	36.94	21.4	8.31	15.11	5.01	6.41
		14.8.01/81																						
<i>Ghatixalus asterops</i>	M	TNHM(H)	48.81	17.82	15.76	8.61	6.9	4.46	9.62	11.12	7.85	4.82	3.23	2.21	11.12	17.92	25.26	23.82	21.32	12.71	5.34	9.47	2.65	4.71
		12.6.18/68																						
<i>Ghatixalus variabilis</i>	M	TNHM(H)	45.13	15.32	13.9	7.91	4.84	3.83	8.32	13.53	5.56	3.51	2.91	1.23	9.98	13.23	22.56	21.25	21.13	10.32	4.31	6.9	2.78	3.52
		12.6.18/67																						

TABLE 2. Morphometric measurements (in mm) of tadpole specimens (Stage 39) of *Ghatixalus* spp.. For abbreviations, see Materials and methods.

Species	TL	BL	TAL	BH	EL	TMH	MTH	UTF	LTF	NSD	END	IND	IOD	SSD	HW	BW	TMW
<i>Ghatixalus magnus</i> sp. nov.	69.70	21.48	48.01	10.53	1.88	8.13	13.21	3.34	4.17	3.42	6.90	2.66	5.79	14.93	12.32	12.45	8.13
<i>Ghatixalus variabilis</i>	47.38	15.63	31.75	6.53	1.72	4.75	5.73	2.82	1.32	2.19	1.58	1.05	3.09	8.62	7.74	7.90	4.88
<i>Ghatixalus asterops</i>	51.00	16.87	34.96	7.49	1.84	6.16	9.29	3.13	1.74	3.23	2.63	1.57	5.20	10.96	9.29	8.06	5.48

Paratype: TNHM (H) 14.8.01/81, adult male (76.91 SVL), collected from near a forest stream, 10° 7'54.95"N, 76°59'57.89"E, at elevation 1409 m ASL in Kadalar, Idukki district, Kerala, India by Robin Abraham on 09 July 2014.

Diagnosis: *Ghatixalus magnus* sp. nov. can be easily differentiated from its two congeners by the following combination of characters: adult male large, SVL 79.40 ± 1.0 , N = 2 (vs. SVL 46.67 ± 2.0 , N = 7 in *G. variabilis*; SVL 41.93 ± 2.0 , N = 4 in *G. asterops*); eye to nostril distance subequal to eye diameter (vs. eye to nostril distance shorter than eye diameter in *G. variabilis* and *G. asterops*); antero-ventral parts of thighs smooth and postero-ventral regions of thighs areolate (vs. ventral surface of thighs areolate in both *G. variabilis* and *G. asterops*); and larger distance from tympanum to eye distance, TYE 2.57 ± 1.0 (vs. 1.23 in *G. variabilis*; 1.30 in *G. asterops*). *G. magnus* sp. nov. also differs from *G. asterops* in that the supratympanic fold extends from the posterior corner of the eye to below the level of the forearm (vs. supratympanic fold extends from the posterior corner of the eye and ending just above the level of the forearm).

Description of Holotype: A large-sized adult male, SVL 81.90 (Fig. 2C, 3); head length (HL 27.84) slightly less than width (HW 31.02); snout acutely rounded in ventral aspect, rounded in lateral profile, greatly protruding; snout length (SL 13.19) greater than eye diameter (EL 8.16); canthus rostralis rounded, loreal region obtusely flat; nostrils closer to the tip of the snout than to front of eye; eye to nostril distance (EN 8.12) subequal to eye diameter; interorbital distance (IUE 11.38) greater than upper eyelid width (UEW 6.75); pupil oval, horizontal; tympanum distinct, rounded, TYD 4.75, 1.8 times larger than distance from tympanum to eye (TYE 2.57); supratympanic fold distinct, extending from behind the outer canthus to just below the level of the forearm; symphyseal knob W-shaped; lanceolate tongue without lingual papilla; choanal opening oval; pair of vomerine ridges present, directed inwards with a gap between, bearing seven minute round teeth each, odontophores oblique and widely separated between choanae; vocal sac single, median and subgular.



FIGURE 2. *Ghatixalus magnus* sp. nov.; **A.** general habitat comprising mid-montane wet forest; **B.** stream habitat in July near where holotype and paratype were collected; **C.** holotype in life; **D.** paratype in life.

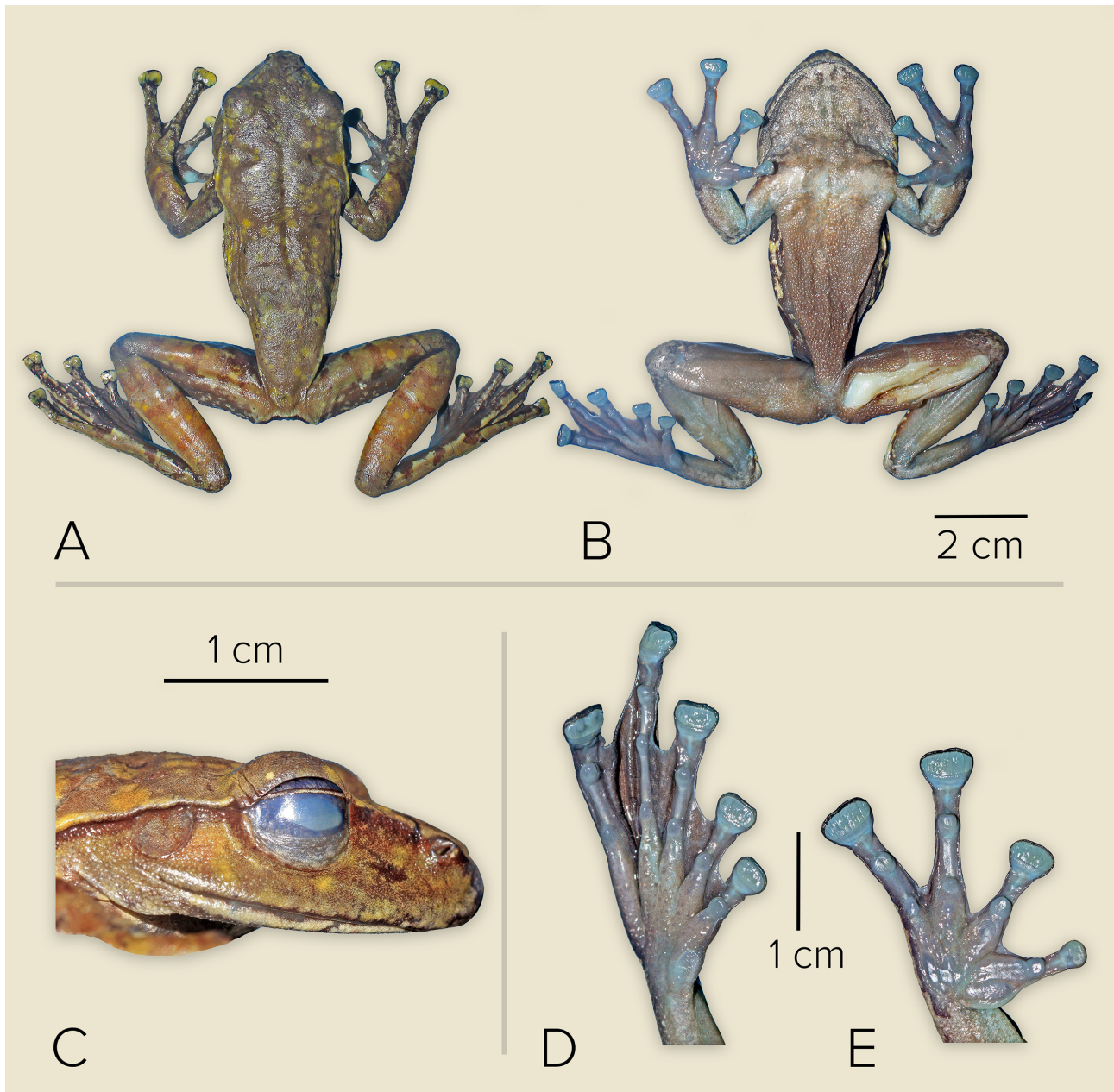


FIGURE 3. Holotype of *Ghatixalus magnus* sp. nov.; **A.** dorsal view; **B.** ventral view; **C.** lateral view of head; **D.** ventral view of foot; **E.** ventral view of hand.

Forelimbs (FLL 19.67) shorter than hand (HAL 25.88); fingers with moderate lateral dermal fringe and bearing large truncate discs with distinct circum-marginal grooves; relative length of fingers: $II < III < V < IV$; tips of fingers enlarged with disks; subarticular tubercles prominent, rounded, and single on second subarticular tubercle on finger II, weakly developed; supranumerary tubercles present; palmar tubercle prominent, elongate; webbing between fingers prominent; male secondary sexual character: nuptial pads present on fingers II and III, more pronounced on finger III.

Hind limbs long, thighs (TL 41.87) longer than shanks (SHL 39.84); toes long and fully webbed, webbing extending from the disk of toe V and reaching slightly above the distal subarticular tubercle of toe IV, and from the distal subarticular tubercle of toe IV to the disc of toe III; relative length of toes: $I < II < III < V < IV$; subarticular tubercles well developed, rounded, single on all toes; supernumerary tubercles present.

Skin of dorsum smooth to shagreen; skin of snout, interorbital region and upper eyelids smooth; sides of head finely shagreen, posterior region of tympanum slightly granular; upper parts of flanks finely shagreen, lower parts

slightly granular; dorsal parts of forelimbs and hindlimbs finely shagreen; throat, chest and belly areolate; postero-ventral regions of thighs areolate, anterio-ventral parts of thighs smooth; ventral surface of shanks and tarsus smooth to granular.

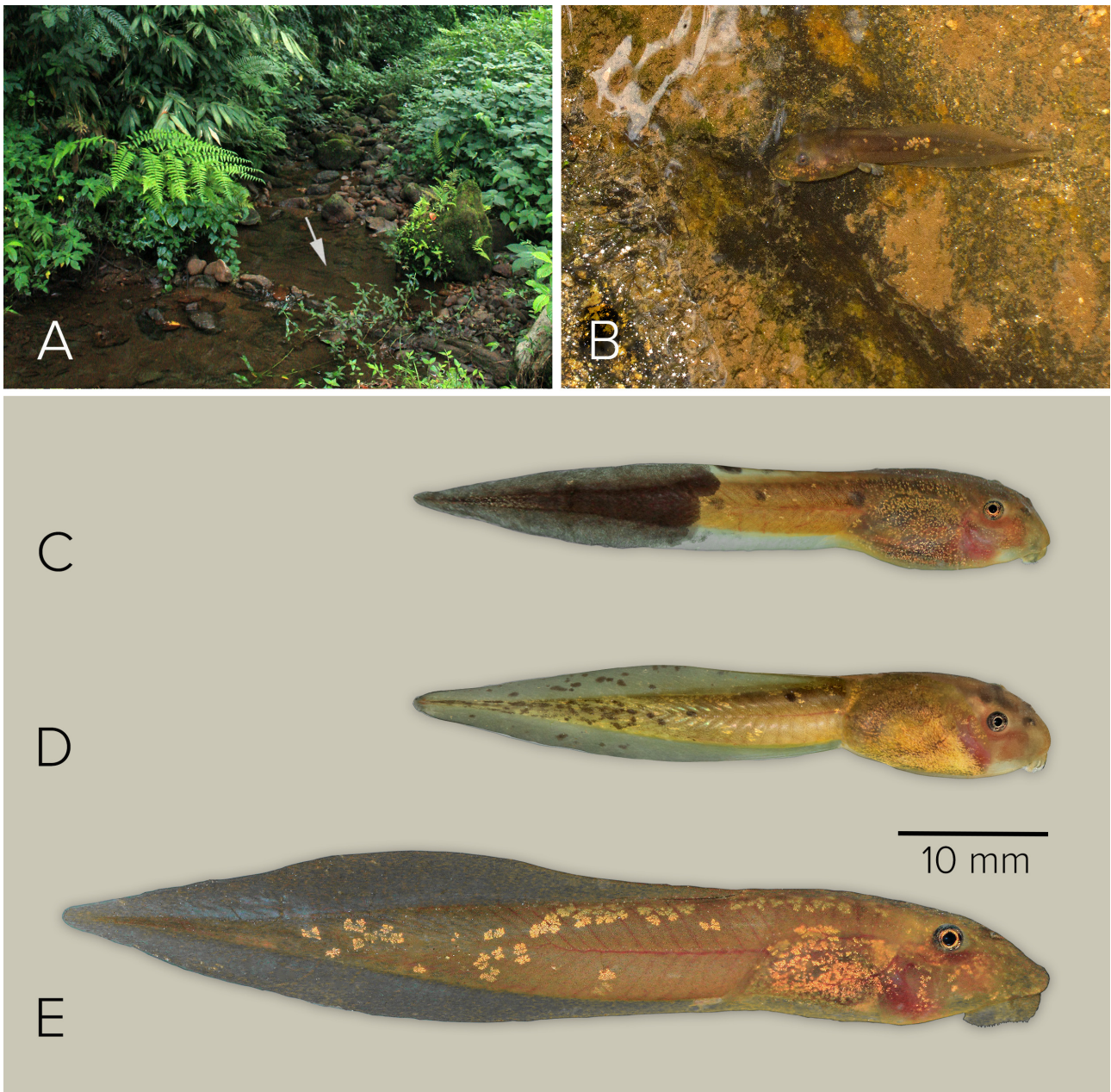


FIGURE 4. **A.** Stream pool habitat in May; **B.** stage 41 *Ghatixalus magnus* sp. nov. tadpole in stream pool; live tadpoles (Stage 38) of **C.** *G. variabilis*; **D.** *G. asterops*; **E.** *G. magnus* sp. nov.

Coloration of Holotype in Life: Dorsum pale rusty-yellowish, reticulated with numerous small bright-yellow irregular blotches; lateral side chocolate brown with creamy reticulations and purplish-turquoise blotches on the groin; loreal region and tympanum pale yellow with a narrow bright yellow band on the canthus rostralis and the upper margin of the tympanic fold converging at the snout tip; iris purplish-grey, with numerous black venations; limbs rusty-yellow with dark brown cross-bands, fingers and toes with cross-bands, discs yellowish, posterior side of thigh brown without reticulation, but a few, scattered yellow blotches; venter dull creamy to grey white; foot and hand light turquoise blue, webbing bluish-brown.

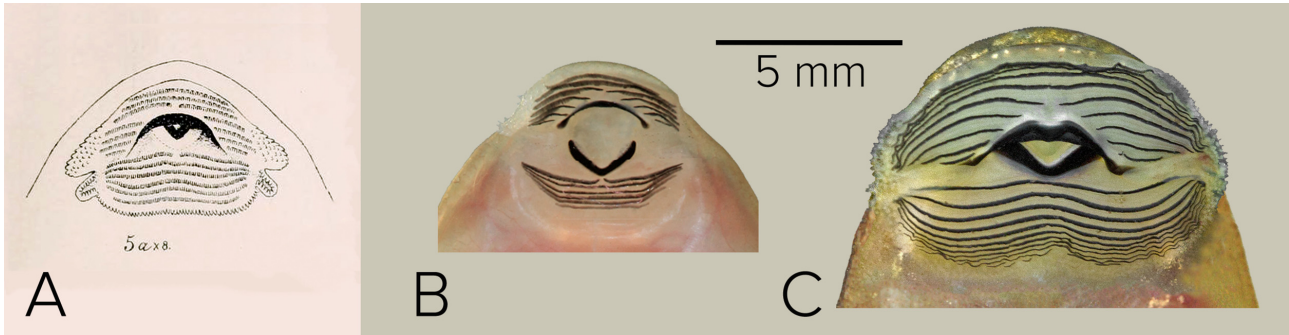


FIGURE 5. Oral morphology of tadpole of **A.** *Ghatixalus variabilis* (illustration reproduced from Annandale, 1918); **B.** *G. asterops*; **C.** *G. magnus* sp. nov.

Coloration of Holotype in Preservative: Dorsum dark brown with dull-yellow irregular blotches; lateral sides dark brown with numerous irregular dull-yellow blotches which extend from behind the arms till the groin; tympanum yellowish-brown, upper eyelid grey; venter brownish-grey, lighter at the throat and chest, darkening towards the posterior; hands and feet pale blue.



FIGURE 6. Late metamorphic stages of *Ghatixalus magnus* sp. nov.; **A.** Stage 42; **B.** Stage 44; **C.** Froglet.

Etymology: The specific name is latin for 'great', alluding to its larger size as compared to its congeners.

Distribution and Natural History: *Ghatixalus magnus* sp. nov. has been recorded in the highlands of the southern Western Ghats between the Palakkad Gap and the Shencottah Gap. We found this species in forest near hill streams in Valparai and Parambikulam (in the Anamalai Range), Kadarlar (in the High Ranges), Upper Manalar (in the High Wavy Hills) and near Pandianthodu (in the Pandalam Hills) (Fig. 7). The species occurs in mid-montane rainforests at elevations between 1350 to 1800m ASL.

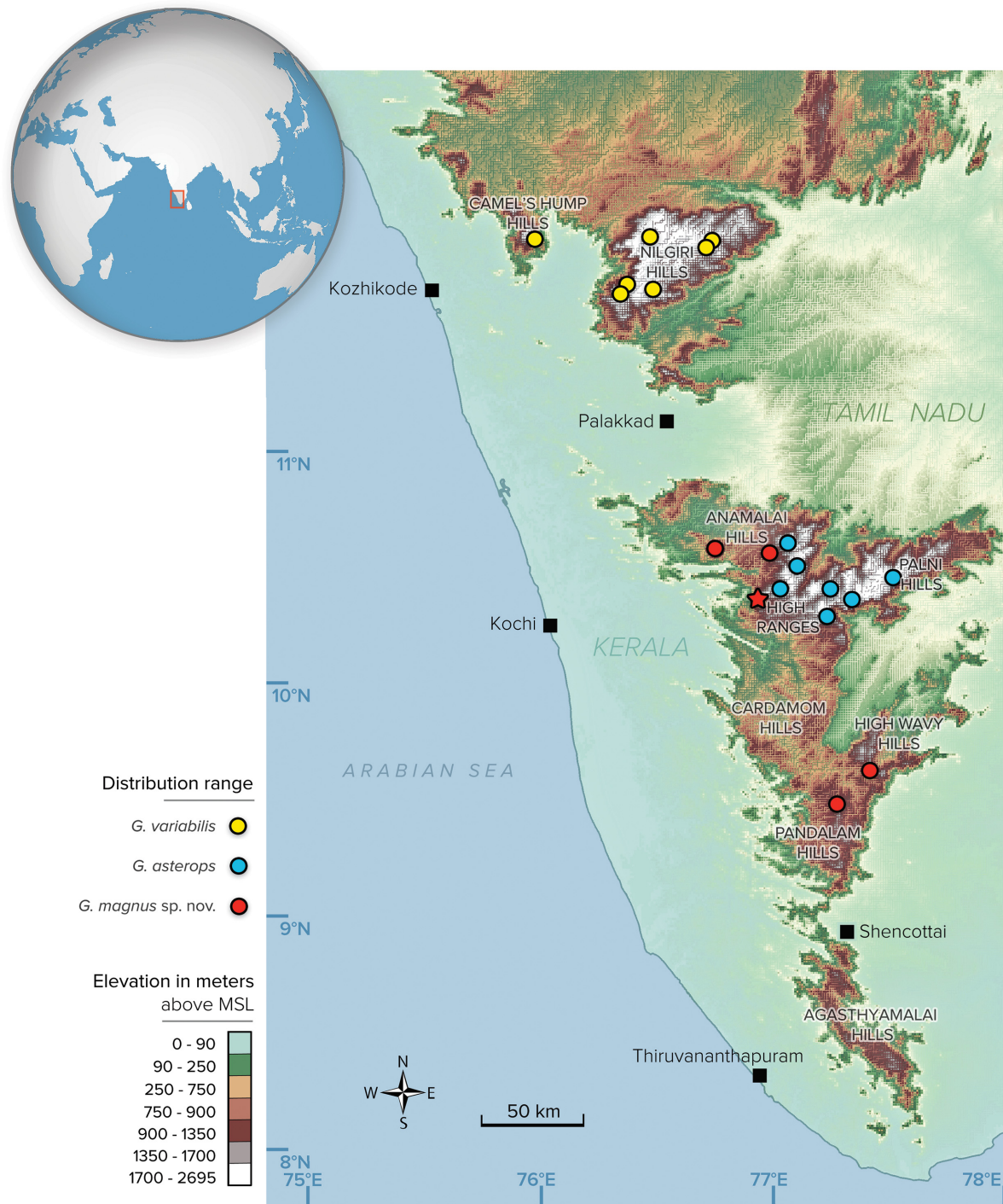


FIGURE 7. Map showing distribution of different *Ghatixalus* spp.; type locality of *G. magnus* sp. nov. marked by red star.

Tadpole description: Three tadpoles at Stages 37–40 were collected from a rocky stream pool on 05 May 2015. Sample tissue from a tadpole was DNA sequenced and analyzed to determine its species status. Description is based on a single tadpole at Stage 38 (Fig. 4E). The large exotrophic tadpole is depressed and is yellowish-brown, interspersed with golden iridophores, which occur as small specks on the body and as larger blotches on the tail. The ventral and ventrolateral body sides are pale yellowish-brown and less pigmented. Eyes are golden-brown and of moderate size, positioned dorsolaterally and directed more laterally than anteriorly, not visible in ventral view. Naris equidistant from both snout and eye. Spiracle sinistral, ventrolaterally positioned at midbody. Myotomes of the tail musculature are well developed. Tail fin high, pointed at the end. Upper fin higher than lower fin. Oral disc

anteroventrally positioned, of oval shape in expanded state, and emarginated laterally. Numerous marginal papillae frame the oral disc ventrally and laterally, but absent on the upper labium. Small submarginal papillae cover the entire inner rim of oral disc. Upper jaw sheath narrow and stretched into a wide U-shaped arch, and lower jaw sheath is narrower and V-shaped. Labial tooth row formula (LTRF) is 10 (6-10)/10(10) (Fig. 5C). In contrast, LTRF of both *G. variabilis* and *G. asterops* is 7(3-7)/6(1); this matches the old formula for *G. variabilis* (2:5+5/1+1:5) described by Annandale (1918). Advanced stages of development have been recorded as well (Fig. 6).

Discussion

With the description of *Ghatixalus magnus* sp. nov., there are now three nominal species in this Western Ghats endemic genus of montane frogs. This novel species potentially represents the largest known rhacophorid species in peninsular India, attaining an average male body size of 76 mm (range 71–82 mm, N = 2). The species occurs in mid-elevation hill forests (1350–1800 m ASL) between the two most prominent geological gaps of the Western Ghats, namely the Palakkad and Shencottah Gaps.

Apart from its large size and colour, *Ghatixalus magnus* differs from *G. asterops* and *G. variabilis*, which are sister to each other, in tadpole morphology. Whereas the two latter species have tadpoles largely adapted to montane, rocky streams, with the presence of a moderate oral sucker to help them adhere to the substrate in the rheophilic stream sections, the tadpole of *G. magnus* was found in stream pools in the rainforest. Since we found advanced tadpoles at stages 38–42 in the relatively dry month of May, prior to the southwest monsoon, it is unlikely that the large oral appendage of *G. magnus* tadpoles serves to provide anchorage, since flow rates are very slow in the preceding summer months when much of the tadpole's development takes place. The tadpoles of *Ghatixalus* are also unique among the Rhacophoridae for having the greatest number of labial tooth rows on both jaws, from 7/6 to 10/10 (the average for the family is 5/3) (Altig & McDiarmid, 1999b).

Given the attention amphibian taxonomists have been giving the Western Ghats over the past couple of decades, it comes as a surprise that a large and widespread rhacophorid species such as *Ghatixalus magnus* remained unnoticed for so long. This underscores the significance of the need to continue exploration and research even in areas that are assumed to be well-studied.

Acknowledgments

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APPENDIX. Sequences of rhacophorids downloaded from Genbank for this study.

Species	12S	16S	RAG1	RHOD
<i>Buergeria buergeri</i>	AY880478	AY880444	AB728271	AB728288
<i>Chiomantis doriae</i>	GQ204772	KR827718	GQ285779	GQ204657
<i>Chiomantis nongkhorensis</i>	KC465788	KR827721	GQ204604	GQ204659
<i>Feihyla vittata</i>	GQ204773	GQ204722	GQ285774	GQ285793
<i>Ghatixalus asterops</i>	EU178098	EU178091	-	-
<i>Ghatixalus variabilis</i>	EU178099	EU178092	-	-
<i>Polypedates colletti</i>	KC961243	KC961080	GQ204579	AB728285
<i>Polypedates cruciger</i>	GQ204746	GQ204692	GQ204570	GQ204632
<i>Polypedates maculatus</i>	GU191106	GU191116	GQ204576	GQ204637
<i>Polypedates megacephalus</i>	KC465836	KR828006	EU924517	GQ285791
<i>Rhacophorus bipunctatus</i>	JX219445	LC010569	EU924518	GQ204652
<i>Rhacophorus malabaricus</i>	GU136096	GU136112	DQ347202	AY880656
<i>Rhacophorus nigropalmatus</i>	JN705327	JN705362	GQ204592	GQ204646
<i>Rhacophorus rhodopus</i>	JX219439	LC010604	EU924532	EU215560
<i>Taruga eques</i>	EU178095	GQ204689	GQ204571	GQ204633
<i>Taruga fastigo</i>	AY141802	GQ204690	GQ204572	GQ204634
<i>Taruga longinasus</i>	GQ204745	GQ204691	GQ204573	GQ204635