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A new species of the genus *Leptobrachium* (Anura: Megophryidae) from the Gaoligongshan Mountain Range, China

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Abstract

We describe a new species of the genus *Leptobrachium* from the Gaoligongshan Mountain Range, Yunnan Province of China based on molecular and morphological evidences. The new species, *Leptobrachium tengchongense* **sp. nov.**, can be distinguished from its congeners by a combination of the following characters: (1) relatively small size (adult males SVL 41.7–51.5 mm); (2) head width slightly larger than head length; (3) tympanum indistinct; (4) two palmar tubercles oval and distinct, inner one larger than outer one; (5) sexually active males without spines on the upper lip; (6) dorsal skin smooth with distinct network of ridges; (7) dorsum pinkish grey and scattered with irregular black markings; (8) venter dark purplish-gray with numerous small white spots on tubercles, solid white chest; (9) iris bicolored, upper one-third light blue, lower two-third dark brown. With the description of the new species, the number of *Leptobrachium* species currently known from China adds up to ten.

Key words: Spadefoot toad, taxonomy, *Leptobrachium tengchongense* **sp. nov.**, Tengchong County, Yunnan Province

Introduction

Asian spadefoot toads of the genus *Leptobrachium* Tschudi, 1838, are widely distributed from southern China west to northeastern India and Myanmar, through Indochina mainland to peninsular Malaysia, Borneo, Sumatra, Java and Philippines (Frost 2016). At least thirty-four nominal species within the genus are recognized to date, with about half (17) described only in the past decade (Frost 2016). Based on previously phylogenetic studies, species of *Leptobrachium* are divided into two major clades: the Indochina/China clade composed of species from Indochina mainland and China including Hainan Island; the Sundaland/Thailand Clade composed of species from Sundaland, southern Thailand and southern Myanmar (Brown *et al.* 2009; Matsui *et al.* 2010; Wogan 2012). Nine species of the genus are now known from China, i.e., *L. ailaonicum*, *L. boringii*, *L. chapaense*, *L. guangxiense*, *L. hainanense*, *L. huashen*, *L. leishanense*, *L. liui* and *L. promustache*; five of them occur in Yunnan Province (Fei *et al.* 2009, 2012; Frost 2016).

Gaoligongshan (GLGS, hereafter), in western Yunnan Province of China, is a long, north–south running mountain range along the Sino-Burmese border, drained by the Salween River on the east and the Irrawaddy River on the west. For detail description of the geography and major regions of the mountain range, see Chaplin (2005) and Dumbacher *et al.* (2011). During recent fieldwork at the Tengchong Section of Gaoligongshan National Nature Reserve, in the southern region of GLGS, we collected *Leptobrachium huashen*, and another form of *Leptobrachium* species, which is clearly different from *L. huashen* in morphology. Subsequent studies on morphological characters and 16S rRNA mtDNA gene sequences revealed that it represents an undescribed species and distinctly differs from all recognized congeners a combination of morphological characters and molecular divergence; we therefore describe it herein as a new species.

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Materials and methods

Sample Collection. We conducted fieldwork covering different locations and altitude bands at the Tengchong Section of Gaoligongshan National Nature Reserve, Tengchong County, Yunnan Province of China between 2014 and 2015. Specimens were collected between 1900 and 0200 h, fixed and preserved in 80% ethanol, and deposited at The Museum of Biology, Sun Yat-sen University (SYS). The geographic position of the surveyed locality and the distribution of the five currently known *Leptobrachium* species in Yunnan Province are shown in Fig. 1.

DNA extraction and sequencing. Genomic DNA was extracted from the muscle tissue using TIANamp Genomic DNA Kit. We amplified a fragment of the mitochondrial 16S rRNA gene from *Leptobrachium* specimens using the primer pairs L3975 and H4551 (Simon *et al.* 1994). PCR amplifications were performed in a reaction volume of 25 µl containing 100 ng of template DNA, 0.3 mM of each PCR primer and 10 µl Premix EX TaqTM (Takara). The PCR conditions were an initial denaturing step at 95 °C for 4 min, followed by 35 cycles of denaturing at 94 °C for 30 s, annealing at 52 °C for 30 s an extension step at 72 °C for 1 min, and a final extension of 72 °C for 7 min. PCR products were purified with spin columns. The purified products were sequenced with both forward and reverse primers using BigDye Terminator Cycle Sequencing Kit (Thermo Fisher Scientific Inc., Waltham, Mass., USA) according to the guidelines of the manufacturer. The products were sequenced on an ABI Prism 3730 automated DNA sequencer at the ShangHai Majorbio Bio-pharm Technology Co.,Ltd.

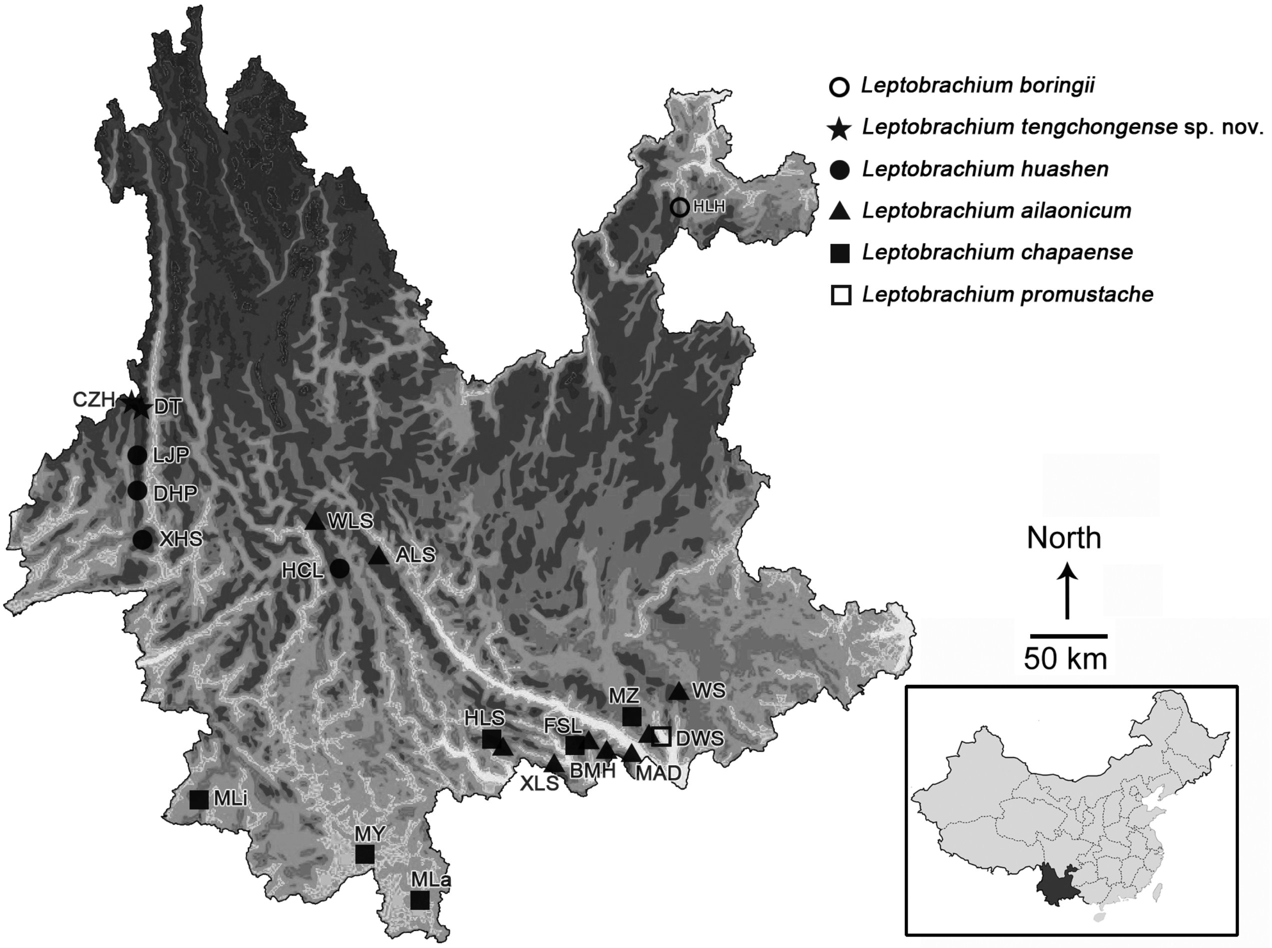

FIGURE 1. Map showing the recorded localities of *Leptobrachium* species in Yunnan Province, China (data from Rao & Wilkinson 2008; Zheng *et al.* 2008; Zhang *et al.* 2010; Fei *et al.* 2009; our data). CZH, Cizhuhe substation of GLGS NNR, Tengchong Co.; DT, Datang substation of GLGS NNR, Tengchong Co.; LJP, Linjiapu substation of GLGS, Tengchong, Co.; DHP, Dahaoping substation of GLGS NNR, Tengchong Co.; XHS, Xiaoheishan, Longling Co.; WLS, Wuliangshan, Nanjing Co.; HCL, Huangcaoling, Jingdong Co.; ALS, Ailaoshan, Jingdong Co.; MLi, Menglian Co.; MY, Mengyang Town, Xishuangbanna; MLa, Mengla County, Xishuangbanna; HLS, Huanglianshan, Lvchun Co.; XLS, Xilongshan, Jinping Co.; FSL, Fenshuiling, Jinping Co.; BMH, Baimahe, Jinping Co.; MAD, Maandi, Jinping Co.; DWS, Daweishan, Pingbian Co.; MZ, Mengzi Co.; WS, Wenshan City.

TABLE 1. Samples and sequences used in the phylogenetic analysis in this study.

Species	Locality	Voucher No.	GenBank No.
(1) *Leptobrachium tengchongense* **sp. nov.**	China, Yunnan, Tengchong, Datang	SYS a004603	KX066876
(2) *Leptobrachium tengchongense* **sp. nov.**	China, Yunnan, Tengchong, Datang	SYS a004604a	KX066877
(3) *Leptobrachium tengchongense* **sp. nov.**	China, Yunnan, Tengchong, Datang	SYS a004604b	KX066878
(4) *Leptobrachium tengchongense* **sp. nov.**	China, Yunnan, Tengchong, Datang	SYS a004604c	KX066879
(5) *Leptobrachium tengchongense* **sp. nov.**	China, Yunnan, Tengchong, Datang	SYS a004604d	KX066880
(6) *L. ailaonicum*	China, Yunnan, Mt. Daweishan	IZCASH30030	EF544222
(7) *L. ailaonicum*	China, Yunnan, Mt. Daweishan	IZCASH30031	EF544223
(8) *L. ailaonicum*	China, Yunnan, Mt. Ailaoshan	IZCASH30046*	EF544224
(9) *L. ailaonicum*	China, Yunnan, Mt. Ailaoshan	IZCASH30027*	EF544225
(10) *L. banae*	Vietnam, Gia Lai, Krong Pa	ROM 32200	EF544229
(11) *L. boringii*	China, Sichuan, Mt. Emei	IZCASH30021*	EF544207
(12) *L. buchardi*	Laos, Champasak, Pakxong District	FMNH 258086	HQ709353
(13) *L. chapaense*	Vietnam, Sa Pa	ROM38038*	EF544227
(14) *L. guangxiense*	China, Guangxi, Mt. Shiwan	200807002*	KC430099
(15) *L. hainanense*	China, Hainan, Diaoluoshan	RDQ120*	EU180885
(16) *L. hasseltii*	Indonesia, C. Java, Purworejo, Kaligesing	KUHE 42818*	AB530423
(17) *L. huashen*	China, Yunnan, Jingdong, Huangcaoling	RDQ183*	EU180852
(18) *L. huashen*	China, Yunnan, Jingdong, Kungang	RDQ157	EU180851
(19) *L. huashen*	China, Yunnan, Tengchong, Dahaoping	RDQ155	EU180853
(20) *L. huashen*	China, Yunnan, Longling, Xiaoheishan	RDQ258	EU180854
(21) *L. huashen*	China, Yunnan, Tengchong, Linjiapu	KFBG 14301	KX066881
(22) *L. ingeri*	Indonesia, Belitung, Tanjung Pandang	MZB Amp1791*	AB530426
(23) *L. leishanense*	China, Guizhou, Leigongshan	IZCASH30003*	EF544199
(24) *L. leucops*	Vietnam, Lam Dong, Lac Duong District	BLS 11838**	HQ709356
(25) *L. liui*	China, Fujian, Mt. Wuyi	IZCASH30006*	EF544182
(26) *L. masatakasatoi*	Laos, Xamneua, Phupan	KUHE 34396	AB530445
(27) *L. mouhoti*	Cambodia, Mondolkiri, O'Rang District, Seima	FMNH 262756**	HQ709357
(28) *L. ngoclinhense*	Vietnam, Kon Tum, Ngoc Linh Mountain	IEBR 2827*	AB530450
(29) *L. promustache*	China, Yunnan, Mt. Daweishan	IZCASH30044*	EF544240
(30) *L. pullum*	Vietnam, Kon Tum, Kon Plong	IEBR 2780	AB530449
(31) *L. rakhinensis*	Myanmar, Rakhine State, Gwa Township	CAS:222296*	JX127250
(32) *L. xanthops*	Laos, Dakchung Plateau	NCSM 78468**	JN711502
(33) *L. xanthospilum*	Vietnam, Gia Lai, Tram Lap	ROM 32177	AB530451
(34) *Oreolalax rhodostigmatus*	China, Guizhou, Da Fang	CIB ZYCA746*	EF397248
(35) *Scutiger chintingensis*	China, Sichuan, Hongya Co.	ROM39065	EF397269

Remark: ** represents sequences from type specimens; * represents sequences from its type locality.

Phylogenetic analyses. In addition to the newly collected specimens, sequences of all *Leptobrachium* species belonging to the Indochina/China clade, as well as three representatives of the Sundaland/Thailand Clade, i.e., *L.*

hasseltii, *L. ingeri* and *L. rakhinensis,* were included in the genetic analysis. *Scutiger chintingensis* and *Oreolalax rhodostigmatus* were used as outgroups (Table 1). The resulting sequences were aligned by ClustalW 1.83 (Thompson *et al.* 1994) under default settings and then checked by eye and adjusted if necessary. Phylogenetic trees were constructed using maximum likelihood (ML) and Bayesian inference (BI). Maximum likelihood analysis was conducted in RAxML v8.2.4 (Stamatakis 2014). Confidence intervals were determined with 1000 bootstrap replicates utilizing the rapid bootstrap option under the GTRGAMMA substitution model. Bayesian analyses were performed in MrBayes 3.2.6 (Ronquist *et al.* 2012) under the GTR+I+G model as suggested by the Akaike Information Criterion implemented in jModelTest 2.1.2 (Darriba *et al.* 2012). We employed two separate MCMC runs, each with four Metropolis-coupled chains. The analyses were run for 2.5 million generations, with parameters and topology sampling every 1000 generations. The stationary phase was detected using Tracer 1.6 (Rambaut *et al.* 2014). The first 1000 trees were discarded as burn-in and posterior probabilities were determined from the remaining trees. We also calculated the uncorrected pairwise genetic distances using MEGA 6.06 (Tamura *et al.* 2013).

Morphological data. Measurements were taken by JHY to the nearest 0.1 mm with dial calipers: snout-vent length (SVL); head length from tip of snout to rear of jaws (HDL); maximum head width (HDW); snout length from tip of snout to anterior corner of eye (SNT); eye diameter (EYE); upper eyelid width (UEW); interorbital distance (IOD); internasal distance (IND); forearm length, from elbow to base of outer palmar tubercle (LAL); manus length from tip of third digit to base of outer palmar tubercle (HND); tibia length (TIB); femur length (TGH); tarsus length (TSL); pes length from tip of fourth toe to base of inner metatarsal tubercle (FTL); inner metatarsal tubercle length (IML); and inner metatarsal tubercle width (IMW). Webbing formulae followed Savage (1975). Labial tooth row formulae of tadpoles follows Dubois (1995) and developmental stages of tadpoles were determined according to Gosner (1960). Sex was determined by direct observation of calling in life. Comparative morphological data of *Leptobrachium* species were obtained from the literature (Bourret 1937; Lathrop *et al.* 1998; Matsui *et al.* 1999; Ohler *et al.* 2004; Fei *et al.* 2005, 2009, 2012; Orlov 2005; Rao *et al.* 2006; Stuart *et al.* 2006, 2011, 2012; Bain *et al.* 2009; Sondhi & Ohler 2011; Matsui 2013).

Results

Molecular relationships. Predictably, the unknown *Leptobrachium* species from the northern section of Tengchong was recovered within the Indochina/China clade of *Leptobrachium* species based on 511 bp fragment of the 16S rRNA gene in both BI tree and ML analysis (Fig. 2), with a sister relationship to the clade composed of *L. ailaonicum*, *L. promustache*, *L. leishanense*, *L. luii* and *L. boringii*. For the uncorrected pairwise divergence in the 16S rRNA gene fragment, the observed interspecific genetic distances within *Leptobrachium* species in our analysis ranged from $p = 1.2\%$ (between *L. guangxiense* and *L. hainanense*) to 15.9% (between *L. rakhinensis* and *L. promustache*). The unknown *Leptobrachium* species was found to be closely related to *L. ailaonicum* ($p = 2.3$–3.1%) and *L. leishanense* ($p = 2.5\%$); these levels of divergences are equivalent to or higher than those observed between several pairs of well-distinguished species of *Leptobrachium* (see Table 2). Intraspecific genetic variations in this gene fragment for two closely distributed species, *L. huashen* and *L. ailaonicum*, were 0.0–0.6% and 0.0–0.8% respectively. As the observed molecular differences are corroborated by differences in morphology (see below), we consider the unknown *Leptobrachium* sp. from the Tengchong of GLGS to be a new species and describe it herein.

Leptobrachium tengchongense sp. nov.

Holotype. SYS a004603, adult male, calling on the floor in shrub about 3 m from a rocky stream. The stream was 3–5 m wide, flowing pass mature montane evergreen broadleaf forest at Datang substation of the Tengchong Section of Gaoligongshan National Nature Reserve, Tengchong County, Yunnan Province, China (25°44'26.23" N, 98°41'45.74" E, 2060 m above sea level), collected at 2100 h on 13 March 2015 by J.H. Yang.

Paratypes. SYS a004623–4627, five adult males, from the same locality as holotype, collected at 2000–0130 h on 17–18 March 2016 by J.H. Yang. SYS a004628–4632, five adult males, from Cizhuhe substation of the Tengchong Section of Gaoligongshan National Nature Reserve (25°46'16.85" N, 98°37'54.27" E, 2240 m above sea level; 25°47'22.33" N, 98°37'33.60" E, 2510 m above sea level), collected at 2000–0130 h on 20–21 March 2016 by J.H. Yang.

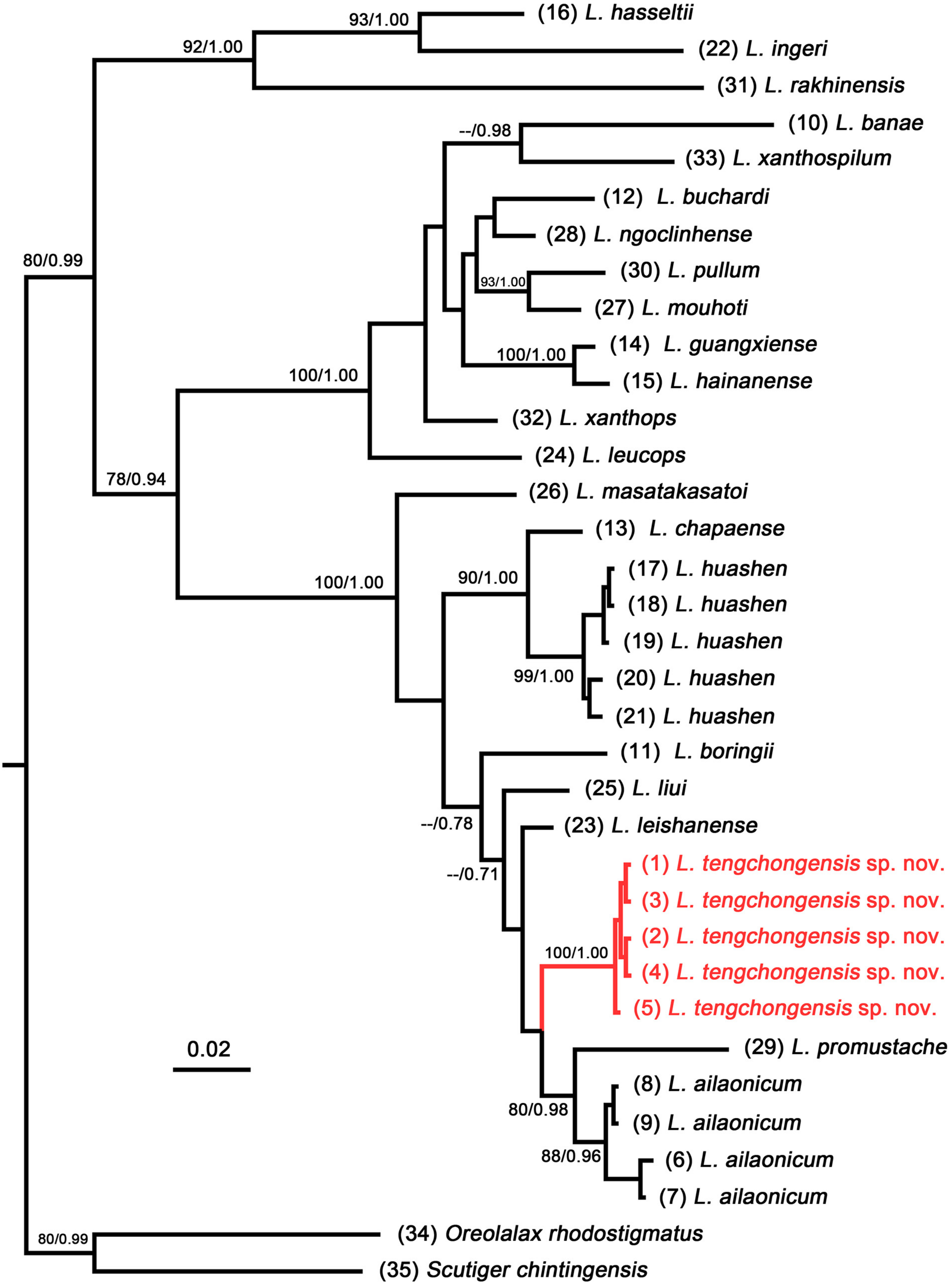

FIGURE 2. Bayesian inference (BI) tree derived from partial sequences of the mitochondrial 16S rRNA gene. Numbers above branches are bootstrap support values for maximum likelihood analyses (>70 retained) and numbers below branches indicate Bayesian posterior probabilities (> 70% retained).

TABLE 2. Uncorrected *p*-distances (%) among the *Leptobrachium* species and outgroups based on a 16S rRNA fragment. (To be continued)

	(1–5)	(6–9)	10	11	12	13	14	15	16	(17–21)
(1–6) *Leptobrachium* sp.	0.0									
(6–9) *L. ailaonicum*	2.3–3.1	0.0–0.8								
(10) *L. banae*	12.8	13.0–13.5	–							
(11) *L. boringii*	4.8	4.6–4.8	13.0	–						
(12) *L. buchardi*	10.6	11.0–11.6	7.9	11.6	–					
(13) *L. chapaense*	5.0	4.3–4.6	13.3	5.8	11.8	–				
(14) *L. guangxiense*	10.4	11.2–11.8	7.7	11.4	4.8	11.6	–			
(15) *L. hainanense*	10.8	11.0–11.6	7.7	11.4	5.2	12.0	1.2	–		
(16) *L. hasseltii*	11.4	11.4–11.8	13.3	12.6	11.2	11.6	12.0	12.6	–	
(17–21) *L. huashen*	4.8–5.0	4.6–5.0	13.0–13.3	5.4–5.8	12.0	2.5–2.7	11.4	11.8	12.0–12.2	0.0–0.6
(22) *L. ingeri*	13.7	13.5–13.7	13.5	15.3	12.8	13.9	13.9	14.5	6.6	13.9
(23) *L. leishanense*	2.5	2.1–2.3	13.0	3.7	12.0	3.7	11.8	12.2	12.0	3.3–3.7
(24) *L. leucops*	10.1	11.2–11.8	8.9	11.2	4.6	10.8	6.0	6.2	11.6	11.2
(25) *L. liui*	3.5	3.3–3.7	14.1	4.1	12.6	5.2	12.4	12.8	12.4	4.6–4.8
(26) *L. masatakasatoi*	4.3	5.0–5.8	12.8	4.8	11.6	5.4	11.0	11.4	12.0	5.0–5.2
(27) *L. mouhoti*	9.0	10.8–11.0	7.7	10.6	4.1	10.8	4.3	4.6	12.0	11.2
(28) *L. ngoclinhense*	10.1	10.6–11.2	6.0	10.8	2.9	11.0	3.7	4.1	11.0	11.2–11.4
(29) *L. promustache*	4.8	3.7–4.1	13.5	5.8	11.6	6.8	12.4	12.2	13.3	7.2–7.5
(30) *L. pullum*	10.4	11.0–11.4	7.9	11.2	4.3	11.0	5.4	5.2	12.6	11.8
(31) *L. rakhinensis*	14.3	14.5–14.9	14.5	15.1	14.7	13.5	15.3	15.5	11.2	12.8–13.3
(32) *L. xanthops*	9.9	10.1–10.8	7.0	10.6	4.1	10.8	4.8	4.6	11.2	11.2
(33) *L. xanthospilum*	11.8	12.2–12.6	7.9	12.8	6.6	12.4	8.1	8.5	12.2	13.3
(34) *Oreolalax rhodostigmatus*	11.4	11.4–11.6	13.3	12.4	11.6	10.4	11.8	11.8	12.2	11.4
(35) *Scutiger chintingensis*	12.8	12.0–12.2	14.5	12.6	12.4	12.0	12.6	12.2	12.6	12.2

……*continued on the next page*

TABLE 2. (Continued)

	22	23	24	25	26	27	28	29	30	31	32	33	34	35
(1–6) *Leptobrachium* sp.														
(6–9) *L. ailaonicum*														
(10) *L. banae*														
(11) *L. boringii*														
(12) *L. buchardi*														
(13) *L. chapaense*														
(14) *L. guangxiense*														
(15) *L. hainanense*														
(16) *L. hasseltii*														
(17–21) *L. huashen*														
(22) *L. ingeri*	–													
(23) *L. leishanense*	14.7	–												
(24) *L. leucops*	12.2	11.6	–											
(25) *L. liui*	15.3	2.3	12.2	–										
(26) *L. masatakasatoi*	14.5	4.3	11.0	5.2	–									
(27) *L. mouhoti*	13.3	11.0	4.8	11.6	11.0	–								
(28) *L. ngoclinhense*	13.0	10.8	4.6	11.8	10.4	2.3	–							
(29) *L. promustache*	15.5	4.6	12.2	5.6	7.0	11.2	11.6	–						
(30) *L. pullum*	14.7	11.6	5.2	12.2	11.4	2.1	3.3	11.0	–					
(31) *L. rakhinensis*	13.3	14.1	14.1	14.7	15.1	14.3	14.1	15.9	14.5	–				
(32) *L. xanthops*	13.3	11.0	5.0	11.2	10.6	3.3	2.9	11.2	3.9	14.3	–			
(33) *L. xanthospilum*	13.9	12.8	7.9	13.7	12.4	6.6	5.6	12.6	6.8	15.1	6.4	–		
(34) *Oreolalax rhodostigmatus*	13.3	11.6	11.6	12.6	11.4	11.8	11.0	11.0	11.4	14.9	11.8	13.9	–	
(35) *Scutiger chintingensis*	14.5	12.6	12.2	13.7	12.8	12.0	11.6	13.3	12.0	13.9	11.8	14.3	9.9	–

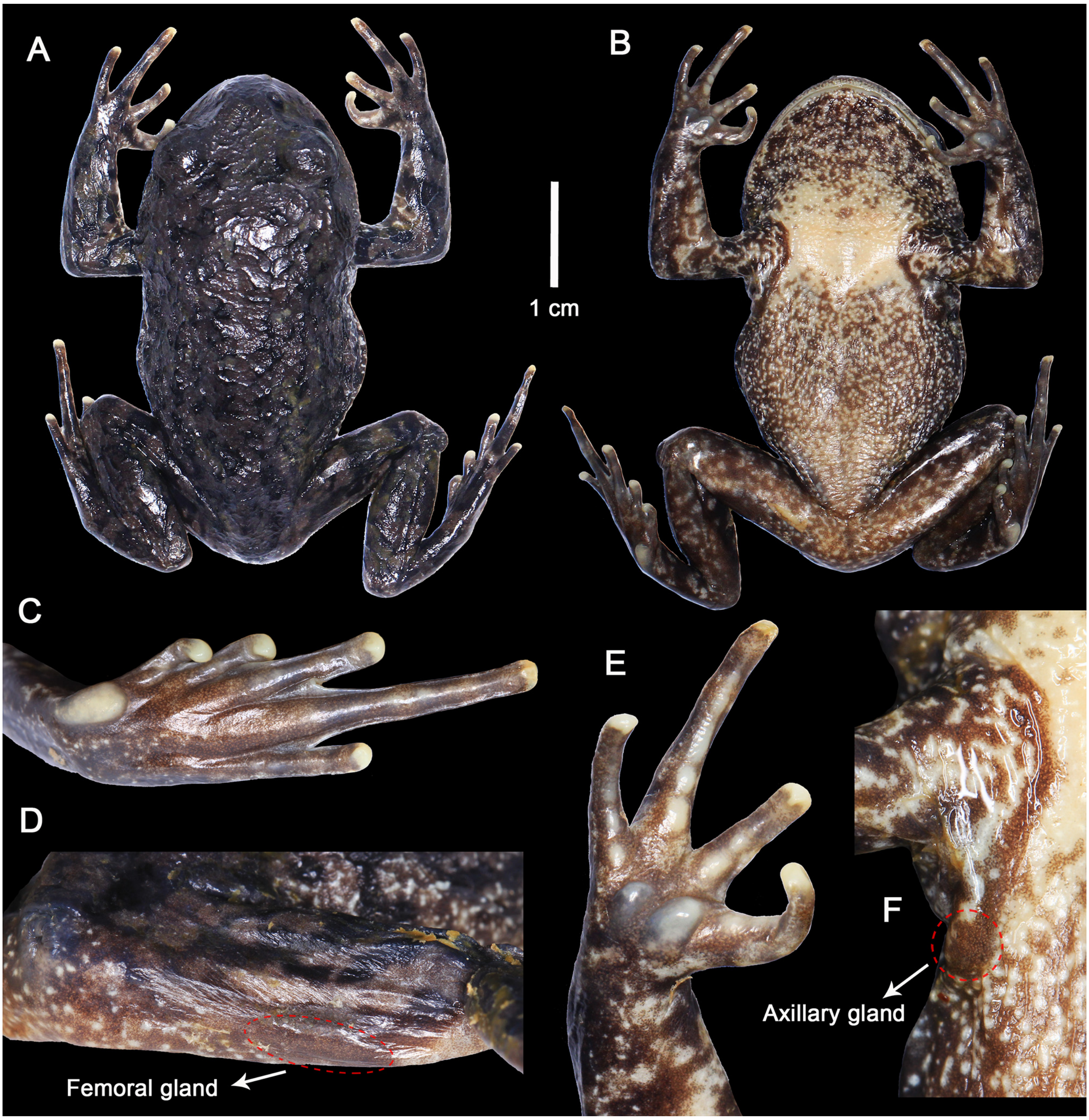

FIGURE 3. Holotype of *Leptobrachium tengchongense* **sp. nov.** (SYS a004603) in preservative: (A) dorsal view; (B) ventral view; (C) plantar view of the left foot; (E) posteroventral surface of thigh, note the imperceptible femoral gland; (D) volar view of the right hand; (F) ventral surface of right chest, note the indistinct axillary gland. Photos by J.H. Yang.

Referred specimens. SYS a004604, seven tadpoles (a004604a–g), collection data identical to holotype, not assigned as types.

Etymology. The specific epithet, "*tengchongense*", is in reference to the type locality, Tengchong County of Yunnan Province, China.

Diagnosis. Assigned to the genus *Leptobrachium* on the basis of having head width larger than tibia length; skin above with a network of ridges; oval and large axillary glands present; extremities of digits rounded; breeding males lacking spines on fingers and breast; and iris bicolored (Dubois & Ohler 1998). The new species, *Leptobrachium tengchongense* **sp. nov.**, can be distinguished from other congeners by a combination of following characters: (1) relatively small size (adult males SVL 41.7–51.5 mm); (2) head width slightly larger than head length; (3) tympanum indistinct; (4) two palmar tubercles oval and distinct, inner one larger than outer one; (5)

sexually active males without spines on the upper lip; (6) dorsal skin smooth with distinct network of ridges; (7) dorsum pinkish grey and scattered with irregular black markings; (8) venter dark purplish-gray with numerous small white spots on tubercles, solid white chest; (9) iris bicolored, upper one-third light blue, lower two-third dark brown.

Description of holotype. Adult male, SVL 45.3 mm. Habitus moderately stocky; body tapering to groin. Head broad and depressed; head length (HDL 18.4 mm) slightly shorter than head width (HDW 19.9 mm). Snout rounded in dorsal view, sharply sloping in profile, barely projecting beyond lower jaw in profile; nostril slightly closer to eye than to tip of snout, below canthus; internarial distance (IND 4.8 mm) shorter than interorbital distance (IOD 6.0 mm); canthus rostralis distinct; lores oblique, moderately concave; eye large, slightly projecting from side of head, diameter (EYE 6.7 mm) shorter than snout length (SNT 7.8 mm), interorbital distance slightly greater than upper eyelid width (UEW 5.0 mm); no pineal ocellus; tympanum round, nearly invisible, tympanum diameter (TYM 2.5 mm) about 37.3% eye diameter and slightly greater than distance between tympanum and eye (TED 2.0 mm); tongue heart-shaped, notched posteriorly; large, slit-like vocal sac openings on floor of mouth near lateral margin of tongue; vomerine teeth absent.

Forelimb slender. Fingers moderately slender, without webbing. Tip of fingers blunt, that of fingers I slightly swollen; relative finger lengths II = IV < I < III; two oval palmar tubercles not in contact, inner larger than outer; inner palmar tubercle larger than tip of fingers I; low callous tissue on ventral surface of fingers; nuptial pad absent. Hindlimb slender and relatively short; heels not meeting when limbs are held at right angles to body; tibiotarsal articulation of adpressed limb reaching to the posterior margin of jaws. Toes moderately slender; webbing poorly developed, formula: I 2–2¼ II 1½–3 III 2–3½ IV 4–2 V. Tips of toes similar to those of fingers; relative toe lengths I<II<V<III<IV; toes with lateral fringes; subarticular tubercles obscure, but elongate, replaced by low callous tissue; distinct, oval, inner metatarsal tubercle, length (IML 3.0 mm) about 76.9 % distance between tip of toe I and tubercle; no outer metatarsal tubercle.

Skin above smooth with distinct network of ridges, lower flank scattered with small tubercles; no spines on upper lip; low supratympanic ridge from posterior edge of eye to axilla; temporal region granular; tubercles on sacrum absent; sparsely granulated around vent; chin, chest and belly densely covered with white small tubercles; limbs with distinct ridges dorsally and smooth ventrally; a large, round axillary gland (2.5 mm in diameter) present on ventrolateral surface slightly posterior to insertion of forelimb with body, slightly visible (Fig. 3F); a flat, oblong femoral gland (4.5 mm in length) present on distal half of posteroventral surface of thigh, not in white color and imperceptible (Fig. 3D).

Color of the holotype in life. Dorsum pinkish grey, head slightly brighter, with distinct, black irregular markings; upper flank like dorsum, lower flank like belly, with small white spots on small tubercles; dorsal limbs with distinct wide black bandings; lower two-third of iris dark brown, upper one-third of iris light blue; black streak under canthus and supratympanic fold; a small black spot present on lower anterior corner of eye; ventral surface of body and limbs dark purplish-gray except the chest remarkably white; numerous minute white spots on tubercles on chin, chest, and belly; axillary gland and femoral gland not in white color and imperceptible (Fig. 3D, F).

Color of the holotype in preservative. Color in preservative dark gray-brown, dark markings on dorsum and bandings on limbs still visible. Upper part of iris and scleral arc faded to white.

Variation. All ten paratypes match the overall characters of the holotype (for measurements of the type series see Table 3). The femoral gland imperceptible in the holotype and other nine paratypes, but is dull white and slightly visible in a single paratype SYS a004627; the axillary gland is distinctly visible in five paratypes (SYS a004623, a004624, a004627, a004630 and a004632), but barely visible or imperceptible in the other five paratypes and holotype.

Advertisement call. A single, pulsed note of approximately 0.13 s duration similar to other *Leptobrachium* for which the advertisement call is known. A full description of the advertisement call will be presented elsewhere (Yang *et al.* in prep).

Tadpoles. The following description based on specimen SYS a004604a preserved in 96 % ethanol: stage 40, SVL 31.6 mm, total length 91.2 mm, tail height 18.6 mm; body elliptical in dorsal view, snout rounded; eye moderate, positioned dorsolaterally, not visible in ventral view; spiracle tube short with a free distal end, positioned laterally; tail musculature strong, relatively straight anteriorly and gradually tapering posteriorly, almost reaching the tip; the fin relatively high, tip of tail pointed; oral disk ventral, labial tooth row formula: 1:(5+5)/(4+4). Body and tail light brown, with a wide light V-shaped marking at posterior body, the base of the V slightly extending to the upper fin; dorsal and lateral body scattered with moderate dark brown spots; tail with large dark brown blotches in the proximal half and small dark spots in the distal half.

FIGURE 4. (A) Holotype of *Leptobrachium tengchongense* **sp. nov.** (SYS a004603) in life, inserted picture depicts the iris coloration of holotype in life; (B) *Leptobrachium huashen* from Linjiapu substation of Gaoligongshan NNR, inserted picture depicts the iris coloration in life. Photos by J.H. Yang.

FIGURE 5. (A) Paratype of *Leptobrachium tengchongense* **sp. nov.** (SYS a004625) in life from Datang Substation of GLGS, inserted picture depicts the microhabitat; (B) paratype of *Leptobrachium tengchongense* **sp. nov.** (SYS a004628) in life from Cizhuhe Substation of GLGS, inserted picture depicts the microhabitat. Photos by J.H. Yang.

Distribution and natural history. *Leptobrachium tengchongense* **sp. nov.** is so far known from Datang substation and Cizhuhe substation of the Tengchong Section of Gaoligongshan National Nature Reserve, Tengchong County, Yunnan Province of China. The new species was found mainly on the riparian forest floor, near shallow, clear-water rocky streams in well-preserved montane evergreen broadleaf forest at elevations between

2000–2500 m. Most adult males were found calling with their bodies buried in the moist topsoil and heads hidden under leaf litter, making them difficult to detect (Fig. 5A). A few males were calling amongst rock crevices (Fig. 5B). No females were detected during the surveys.

We conducted field surveys in the type localities of the new species, namely Datang and Cizhuhe substations of GLGS, between April 2014 and March 2016. A few males were heard calling during the survey on 13–14 March 2015 in Datang, and only one calling adult male (holotype) was collected; but during the surveys on 17–18/20–21 March 2016 in Datang and Cizhuhe respectively, calling males of the new species were ubiquitous along streams and riparian forests, and ten calling adult males were collected from both localities. In March 2016, montane forests between 2200–2900 m a.s.l. were surveyed in Cizhuhe, but mating calls and individuals of the new species were only detected from the lower elevation band (i.e. 2200–2510 m a.s.l.). No calls and specimens of the new species were detected during the survey on 26–27 April 2014 in Datang and 18–19 May 2015 in Cizhuhe, except the tadpoles that were found in the stream in both localities in all surveys. Sympatric herpetofauna recorded in the locality during the survey included the Spiny stream toad *Bufo tuberospinius* (Yang, Liu & Rao), Yunnan spiny frog *Nanorana yunnanensis* (Anderson), Glandular horned toad *Megophrys glandulosa* Fei, Ye & Huang, Anderson's frog *Odorrana andersonii* (Boulenger), Chaochiao brown frog *Rana chaochiaoensis* Liu, Large-eyed bamboo snake *Pseudoxenodon macrops* (Blyth), and Leonard's Keelback *Rhabdophis leonardi* (Wall).

Comparison. We compared *Leptobrachium tengchongense* **sp. nov.** to all other recognized species of *Leptobrachium* from China and Indochina, all of which formed a monophyletic clade in molecular studies (Brown *et al.* 2009; Matsui *et al.* 2011; this study): i.e., *L. ailaonicum*, *L. banae*, *L. boringii*, *L. buchardi*, *L. chapaense*, *L. guangxiense*, *L. hainanense*, *L. huashen*, *L. leishanense*, *L. leucops*, *L. liui*, *L. masatakasatoi*, *L. mouhoti*, *L. ngoclinhense*, *L. promustache*, *L. pullum*, *L. xanthops* and *L. xanthospilum*. We also compared *L. tengchongense* **sp. nov.** with the newly described *L. bompu* from India of which comparative 16S mtDNA data was unavailable from the GenBank. Unrelated Sunderland/Thailand clade taxa (occur in southern Myanmar, Thailand, Peninsular Malaysia, Sumatra, Java, Bali, Borneo, and the Philippines) are omitted from comparisons for simplicity.

By the absence of spines on the upper lip in sexually active males, *Leptobrachium tengchongense* **sp. nov.** distinctly differs from six other congeners, i.e., *L. ailaonicum, L. boringii, L. leishanense, L. liui*, *L. ngoclinhense* and *L. promustache*, all of which process spines on the upper lip in sexually active males and were formerly referred to the genus *Vibrissaphora* (Rao *et al.* 2006; Fei *et al.* 2005, 2009). By having a bicolored iris with upper one-third light blue, *Leptobrachium tengchongense* **sp. nov.** can be distinguished from *L. bompu* (iris uniformly grey-blue), *L. chapaense* (iris uniformly dark brown), *L. huashen* (iris uniformly dark brown, [see discussion below]), *L. leishanense* (upper part of iris light green), *L. leucops* (upper part of iris white), *L. liui* (upper part of iris light green), *L. mouhoti* (iris black with an orange-yellow crescent on outer margin), *L. ngoclinhense* (iris uniformly dark brown), *L. pullum* (upper part of iris red), *L. xanthops* (upper part of iris pale yellow), and *L. xanthospilum* (upper half of iris white). By having a relatively smaller body size (SVL 41.7–51.5 mm in adult males), *Leptobrachium tengchongense* **sp. nov.** can be distinguished from the larger *L. ailaonicum* (68.5–87.6 mm in males), *L. banae* (57.2–70.0 mm in males), *L. boringii* (69.8–89.0 mm in males), *L. guangxiense* (53.5–58.1 mm in males), *L. leishanense* (69.0–95.7 mm in males), *L. liui* (67.8–90.5 mm in males), *L. masatakasatoi* (55.2 mm in male), *L. ngoclinhense* (77.1 mm in male) and *L. xanthospilum* (62.8–73.4 mm in males). By having inner palmar tubercle larger than outer one, *Leptobrachium tengchongense* **sp. nov.** can be distinguished from *L. buchardi*, *L. ngoclinhense*, *L. pullum* and *L. hainanense* (versus two palmar tubercles subequal in size in former three species, and outer one slightly larger or equal to the inner one in *L. hainanense*). *Leptobrachium tengchongense* **sp. nov.** further differs from *L. guangxiense* and *L. hainanense* by having an indistinct tympanum (versus distinct in both species).

Besides those differences mentioned above, *Leptobrachium tengchongense* **sp. nov.** further differs from the phylogenetically closely related congeners without spines on the upper lip in adult males, namely *L. chapaense*, *L. huashen* and *L. masatakasatoi* (Fig. 2), by having a pinkish grey dorsal surface of body in life (versus brown in life in *L. chapaense* and *L. huashen*, Fig. 4.), presence of a distinct network of ridges on the dorsum (versus absent in *L. masatakasatoi*); inner metatarsal tubercle larger than first toe (versus smaller in *L. masatakasatoi*); presence of a dark black spot on lower anterior corner of eye (versus absent in *L. chapaense* and *L. huashen*), relatively wide black bands on the dorsal surface of the limbs (versus relatively narrower bands in these species); and large and distinct cream spots on lower flanks absent (versus present in these species).

TABLE 3. Measurements (mm) of type specimens of *Leptobrachium tengchongense* **sp. nov.**. Abbreviations defined in text.

	SYS	SYS	SYS	SYS	SYS	SYS	SYS	SYS	SYS	SYS	SYS	Mean ± SD
	a004603	a004623	a004624	a004625	a004626	a004627	a004628	a004629	a004630	a004631	a004632	(N=11)
	Holotype	Paratype	Paratype	Paratype	Paratype	Paratype	Paratype	Paratype	Paratype	Paratype	Paratype	
Sex	Male	Male	Male	Male	Male	Male	Male	Male	Male	Male	Male	
SVL	45.3	51.5	48.0	45.9	47.9	50.5	48.2	48.9	41.7	48.6	50.3	47.89±2.76
HDL	18.4	20.7	19.4	18.7	19.4	20.2	19.7	20.8	17.5	18.9	20.2	19.45±1.02
HDW	19.9	21.7	20.4	20.0	20.2	21.2	20.9	22.3	18.2	20.5	21.9	20.65±1.14
SNT	7.8	8.4	8.1	7.9	7.7	8.1	8.1	7.9	7.0	8.2	8.5	7.97±0.40
EYE	6.7	7.4	6.8	6.2	6.5	6.9	6.7	6.9	5.4	6.5	6.4	6.58±0.50
UEW	5.0	5.6	5.1	5.0	5.0	5.3	5.2	5.8	4.8	4.8	4.6	5.11±0.35
IOD	6.0	6.0	6.7	6.1	6.3	6.9	6.6	6.3	6.5	6.6	6.6	6.42±0.30
IND	4.8	5.1	5.1	4.7	4.6	4.9	5.1	5.2	4.7	5.2	5.1	4.95±0.22
LAL	14.1	14.4	14.8	13.7	13.6	14.4	13.7	14.1	12.6	14.3	14.0	13.97±0.58
HND	11.9	13.5	12.9	12.6	12.4	13.7	12.0	13.3	11.5	13.2	13.2	12.75±0.72
TIB	16.9	19.0	17.5	17.2	17.6	18.8	17.6	18.4	15.9	17.9	18.0	17.71±0.88
TGH	20.6	22.2	20.9	20.1	20.6	22.0	20.4	21.6	16.9	20.3	21.6	20.65±1.44
TSL	9.8	9.8	9.2	9.7	9.4	9.5	9.9	9.8	9.3	10.0	10.7	9.74±0.41
FTL	18.3	20.4	19.6	18.4	18.3	20.9	18.5	20.0	16.6	20.0	20.3	19.21±1.28
IML	3.0	3.4	3.4	2.7	2.9	3.7	3.0	3.3	2.6	3.6	3.6	3.20±0.38
IMW	1.5	1.8	1.5	1.3	1.5	1.6	1.5	1.8	1.3	1.6	1.7	1.55±0.17

Discussion

Iris coloration in life has been selected as an important diagnostic character for species identification of the genus *Leptobrachium* (Matsui 1999; Hamidy & Matsui 2010; Sondhi & Ohler 2011; Stuart *et al.* 2011, 2012; Wogan 2012). The original description of *L. huashen* (Fei *et al.* 2005) noted the species has a bicolored iris (upper part blue and lower part dark brown); however all individuals of *L. huashen* we discovered from the southernmost GLGS have a uniformly dark brown iris in life (Fig. 4B), while having morphometrics and molecular characters fitting the *L. huashen* sensu stricto from Jingdong County of Yunnan (type locality) (Fig. 2). Noticeably, Mr. Liang Fei of Chengdu Institute of Biology, the authority of *L. huashen*, stated that data on iris coloration of *L. huashen* in the original description (Fei *et al.* 2005) were provided by the collector (types collected in July 1958), not by direct observation by himself (Liang Fei pers. comm. on 7 Oct 2015 with JHY). Thus, we assume the iris coloration of the types of *L. huashen* is actually entirely dark brown, not bicolored as documented before. Furthermore, *L. huashen* is phenotypically and phylogenetically very closely related to sensu stricto *L. chapaense* from Sa pa of Vietnam (Rao & Wilkinson 2008; Fei *et al.* 2009) which has a uniformly dark brown iris (Bourret 1937; Ohler *et al.* 2004), so *L. huashen* may share the same iris coloration.

Several species of *Leptobrachium* characterized as having spines on the upper lip in sexually active males, also known as moustache toads, were formerly assigned to the genus *Vibrissaphora* (Liu 1945; Dubois & Ohler 1998; Fei *et al.* 2005, 2009). However recent phylogenetic studies revealed that *Vibrissaphora* is non-monophyletic as two species of *Leptobrachium* without spines on the upper lip (i.e., *L. chapaense* and *L. huashen*) nested in the clade of *Vibrissaphora* (Rao & Wilkinson 2008; Zheng *et al.* 2008; Matsui *et al.* 2010). *Leptobrachium tengchongense* **sp. nov.** is also nested in the clade with *Vibrissaphora* (see Fig. 2), which further supports the previously finding that the presence of spines on the upper lip is not a homologous character and *Vibrissaphora* is actually a junior synonym of *Leptobrachium* at the genus level.

Our new finding of *Leptobrachium tengchongense* **sp. nov.** and most other new discoveries in GLGS (Guo & Deng 2009; Yang *et al.* 2016; Yang *et al.* unpublished data) highlight the hidden biodiversity of GLGS, and the fact that its herpetofauna is undoubtedly underestimated. Future herpetological exploration will hopefully continue to discover new species/records in the region. Interestingly, according to our preliminary field surveys, the two species of *Leptobrachium* found at Tengchong Section of GLGS, i.e., *L. huashen* and *L. tengchongense*, have non-overlapping distributions: the former species occurs in southernmost parts of GLGS while the new species is only recorded north of *L. huashen* without sympatry. Geographic barriers to species distribution within GLGS require additional investigation.

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