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A new species of the genus *Liuixalus* (Anura: Rhacophoridae) from southern China

JIAN-HUAN YANG[1, 2], DING-QI RAO[3] & YING-YONG WANG[1, 4]

[1]*State Key Laboratory of Biocontrol/The Museum of Biology, School of Life Sciences, Sun Yat-sen University, Guangzhou 510275, Guangdong, China. E-mail: jhyang@kfbg.org*

[2]*Present address: Kadoorie Conservation China, Kadoorie Farm and Botanic Garden, Lam Kam Road, Tai Po, Hong Kong, China. E-mail: jhyang@kfbg.org*

[3]*State Key Laboratory of Genetic Resources and Evolution, Kunming Institute of Zoology, Chinese Academy of Sciences, Kunming 650223, Yunnan, China*

[4]*Corresponding author. E-mail: wangyy@mail.sysu.edu.cn*

Abstract

A new tree frog species of the genus *Liuixalus* was described from Heishiding Nature Reserve, Guangdong Province, China based on a combination of morphological characters and molecular phylogenetic analyses. *Liuixalus feii* **sp. nov.** is distinguished from its congeners by a combination of following characters: small size (SVL 16.2–17.6 mm in adult males and 18.0–18.7 mm in adult females); snout obtusely pointed; tympanum distinct, about half size of eye diameter; nostril closer to eye than to the tip of snout; fingers free of webbing; toe III longer than toe V; toes weakly webbed; tibiotarsal articulation reaching the naris or loreal; dorsal skin smooth and scattered with fine granulars; a subtle longitudinal median ridge present on dorsum; weak skin folds present on dorsal surface of body and thighs; supratympanic fold distinct and curved; ventral surface dull white with more or less irregular dark spots; iris bicolored. The new species appears to be forest-dependent and to date has only been found on the forest floor in primary forests at elevations between 350–800 m. Based on our molecular analyses, we consider *Liuixalus catbaensis* as a junior synonym of *L. calcarius*. Thus, with the description of the new species, the genus *Liuixalus* hitherto contains five recognized species, four of which are endemic to China.

Key words: Rhacophoridae, *Liuixalus*, new species, taxonomy, China

Introduction

The genus *Liuixalus* of the family Rhacophoridae was established by Li *et al.* (2008) on the basis of the monophyly of *Chiromantis romeri* (Smith 1953) and its placement as sister clade to remaining rhacophorines. This newly established genus is currently recognized as a well-supported monophyletic group by subsequent molecular phylogenetic analyses which have proposed the addition of two members from Hainan Island, *L. hainanus* (Liu & Wu 2004) and *L. ocellatus* (Liu & Hu 1973), to the genus (Li *et al.* 2009, 2013; Yu *et al.* 2008, 2009; Pyron & Wiens 2011). Milto *et al.* (2013) described a new species, *Liuixalus calcarius* from Cat Ba Island in northeastern Vietnam, which represents the first record of the genus *Liuixalus* from Vietnam; and they also provided the first morphological diagnosis for the genus *Liuixalus* and discussed its distribution. Shortly thereafter, another new species of the genus, *L. catbaensis*, was described by Nguyen *et al.* (2014) based on a single juvenile specimen also collected from Cat Ba Island, Vietnam; and they also claimed that it was the first report of the genus *Liuixalus* species from Vietnam. Apparently, *L. calcarius* and *L. catbaensis* may be conspecific and therefore *L. catbaensis* was preliminarily listed as a junior synonym of *L. calcarius* by Frost (2015). Thus, at present, four species within the genus are hitherto recognized, i.e., *L. calcarius*, *L. hainanus*, *L. ocellatus* and *L. romeri*. The taxonomic status of *L. catbaensis* remains unclear.

The first record of *Liuixalus* from Guangdong Province of China was reported by Kadoorie Farm and Botanic Garden (2004), from Heishiding Nature Reserve, which identified as "*Philautus ocellatus*" (=*Liuixalus ocellatus*).

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During field surveys in southern China in past few years, we collected a series of specimens of this population of small tree frog species from Heishiding Nature Reserve, Guangdong Province. Our subsequent morphological and molecular phylogenetic studies have revealed that these specimens differ from *L. ocellatus* and other known congeners by a combination of morphological characters and molecular divergence. Herein, we describe these specimens as a new species of *Liuixalus*.

Material and methods

Sampling. All specimens were collected during fieldwork in southern China. Specimens were fixed in 10% formalin before being transferred to 80% ethanol for permanent preservation, and deposited at The Museum of Biology, Sun Yat-sen University (SYS). Muscle tissues were taken and preserved in 99% ethanol prior to fixation for genetic analysis. Samples used for the molecular analyses were obtained from the following specimens: SYS a002157, 2388, 2389 and 2390 from Heishiding Nature Reserve for unnamed species; SYS a002427, 2428 and 2429 from Hong Kong for *Liuixalus romeri*.

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 seven *Liuixalus* 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; 35 cycles of denaturing at 94 °C for 30 s, annealing at 52 °C for 30 s and extending at 72 °C for 1 min, and a final extending step 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.

Phylogenetic analyses. Sequences of all *Liuixalus* species available from GenBank were included in the genetic analysis (Table 1). We used *Gracixalus gracilipes* (type species of *Gracixalus*) and *Philautus aurifasciatus* (type species of *Philautus*) as outgroups. The resulting 28 sequences were first aligned using the ClustalW algorithm (Thompson *et al.* 1994) in MEGA6.06 (Tamura *et al*. 2013), with default parameters, the alignment was further checked and manually revised if necessary. Indels were removed before phylogenetic analyses. The General Time Reversible model assuming a gamma-shaped distribution across sites (Felsenstein 2004) was selected as the best-fit nucleotide substitution model using Akaike's Information Criterion (Akaike 1974) in jModelTest 1.0 (Posada & Buckley 2004). The alignment was analyzed using maximum likelihood (ML) implemented in MEGA 6.06 (Tamura *et al.* 2013), and Bayesian inference (BI) using MrBayes 3.12 (Ronquist & Huelsenbeck *2003*). For ML analysis, the bootstrap consensus tree inferred from 1000 replicates was used to represent the evolutionary history of the taxa analyzed. Branches corresponding to partitions reproduced in less than 50% of the bootstrap replicates were collapsed. For BI analysis, two independent runs, each comprising four Markov Chain Monte Carlo simulations were performed for one million iterations and sampled every 1000th step. The first 25% of the samples were discarded as burn-in. Convergence of the Markov Chain Monte Carlo simulations was assessed by checking the average standard deviation of split frequencies between two runs using Tracer v.1.4 (http://tree.bio.ed.ac.uk/software/tracer/). Apart from phylogenetic tree-based methods, we also calculated pairwise distances between the 28 sequences by calculating uncorrected *p*-distance in MEGA 6.06.

Morphological characters Measurements followed Fei *et al.* (2009) and were taken with digital calipers to the nearest 0.1 mm: SVL = snout-vent length; HL = head length from tip of snout to the articulation of the jaw; HW = head width at the greatest cranial width; SL = snout length, from tip of snout to the anterior corner of the eye; IND = internasal distance; IOD = interorbital distance; UEW = maximum upper eyelid width; ED = eye diameter, from the anterior corner of the eye to posterior corner of the eye; TD = tympanum diameter; TED = tympanum-eye distance, from anterior edge of tympanum to posterior corner of the eye; LAHL = length of lower arm and hand; HAL = hand length, from the carpal-metacarpal articulations to the tip of the longest finger; HLL = hindlimb length; TIB = tibial length; LFT = length of foot and tarsus; FL = foot length; FDW III = finger III disk width, at the widest part of the pad of III; TDW IV = toe IV disk width. Webbing formulae are given following Savage (1975). Sex was determined by the presence of nuptial pads, as well as dissection. Comparative morphological data of four known species of *Liuixalus* were obtained from the literature: Liu *et al*. 1973 (for *L. ocellatus*); Liu *et al*. 2004 (for *L. hainanensis*); Fei *et al*. 2009 (for *L. ocellatus* and *L. romeri*); Milto *et al*. 2013 (for *L. calcarius*).

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

Species	Locality	Voucher No.	Genbank No.
(1) *Liuixalus feii* **sp. nov.**	China: Heishiding NR, Guangdong	SYS a002157	KT198731
(2) *Liuixalus feii* **sp. nov.**	China: Heishiding NR, Guangdong	SYS a002388	KT198732
(3) *Liuixalus feii* **sp. nov.**	China: Heishiding NR, Guangdong	SYS a002389	KT198733
(4) *Liuixalus feii* **sp. nov.**	China: Heishiding NR, Guangdong	SYS a002390	KT198734
(5) *Liuixalus catbaensis*	Vietnam: Cat Ba Island	VNMN 3684	AB871420.1
(6) *Liuixalus calcarius*	Vietnam: Cat Ba Island	NAP-03581	KT198738
(7) *Liuixalus calcarius*	Vietnam: Cat Ba Island	NAP-03582	KT198739
(8) *Liuixalus calcarius*	Vietnam: Cat Ba Island	NAP-03584	KT198740
(9) *Liuixalus calcarius*	Vietnam: Cat Ba Island	NAP-03587	KT198741
(10) *Liuixalus romeri*	Hong Kong	SYS a002427	KT198735
(11) *Liuixalus romeri*	Hong Kong	SYS a002428	KT198736
(12) *Liuixalus romeri*	Hong Kong	SYS a002429	KT198737
(13) *Liuixalus romeri*	Hong Kong	CIB 20080048	AB871412.1
(14) *Liuixalus* cf. *romeri*	China: Mt. Shiwan, Guangxi	KIZ 061205YP	EU215528.1
(15) *Liuixalus hainanus*	China: Diaoluoshan NR, Hainan	LJT V15	KC465826.1
(16) *Liuixalus hainanus*	China: Diaoluoshan NR, Hainan	SCUM 060401L	GQ285671.1
(17) *Liuixalus ocellatus*	China: Diaoluoshan NR, Hainan	-	AB871413.1
(18) *Liuixalus ocellatus*	China: Diaoluoshan NR, Hainan	-	AB871414.1
(19) *Liuixalus ocellatus*	China: Wuzhishan NR, Hainan	-	AB871415.1
(20) *Liuixalus ocellatus*	China: Wuzhishan NR, Hainan	-	AB871416.1
(21) *Liuixalus ocellatus*	China: Wuzhishan NR, Hainan	-	AB871417.1
(22) *Liuixalus ocellatus*	China: Wuzhishan NR, Hainan	-	AB871418.1
(23) *Liuixalus ocellatus*	China: Wuzhishan NR, Hainan	-	AB871419.1
(24) *Liuixalus ocellatus*	China: Wuzhishan NR, Hainan	HN0806045	GQ285672.1
(25) *Liuixalus ocellatus*	China: Hainan	-	GU120328.1
(26) *Liuixalus ocellatus*	China: Wuzhishan NR, Hainan	HN 0806046	KC465829.1
(27) *Gracixalus gracilipes*	Vietnam: Lao Cai Province	MNHN 1999.592	AY880504
(28) *Philautus aurifasciatus*	Indonesia: Java	ZRC.1.5266	AY141850

Results

Molecular relationships. The Bayesian inference (BI) tree based on 510 bp fragment of the 16S rRNA gene strongly supports the placement of the rhacophorid specimens from Heishiding Nature Reserve, Guangdong Province, China in the genus *Liuixalus* (1.00 Bayesian posterior probability, Fig. 1) and substantially separates these samples from two outgroup genera by a large uncorrected sequence divergence (p = 13.6% from *Gracixalus gracilipes* and p = 15.9% from *Philautus aurifasciatus*, Table 2).

There is no genetic divergence between the paratypes of *L. calcarius* and the holotype of *L. catbaensis* in the molecular analyses; therefore we conclude that the *Liuixalus catbaensis* is a junior synonym of *L. calcarius*.

The observed intraspecific genetic distances within the genus *Liuixalus* in our analysis were not high, varying from p = 1.3% to 3.2%, with the least distance found between *L. ocellatus* from Hainan and *L. romeri* from Hong Kong (p = 1.3%), and the greatest distance found between *L. hainanus* from Hainan and *L.* cf. *romeri* from Mt. Shiwan, Guangxi (p = 3.2%). The *Liuixalus* sp. from Heishiding Nature Reserve differed from all other known species within the genus *Liuixalus* by a genetic distance 1.5% to 2.8%, with the lowest value p = 1.5% observed in the comparison with the sequences of *L. romeri* from Hong Kong, as well as of *L. ocellatus* from Hainan. Although not high, this value is notably higher than the smallest intraspecific distance between two recognized species *L. ocellatus* from Hainan and *L. romeri* from Hong Kong (*p* = 1.3%). While 3% value of *p*-distance at the gene fragment examined is proposed as a good indication for candidate new species, intraspecific distances can be as low as 1–2% in some cases (eg. Vassilieva *et al.* 2014).

TABLE 2. Uncorrected *p*-distances among the *Liuixalus* species based on a 16S rRNA fragment.

	(1–4)	(5)	(6–9)	(10–13)	(14)	(15–16)	(17, 19–20, 22–24)	(18, 21, 25–26)	(27)
Liuixalus feii **sp. nov.**, (1–4)	-								
L. catbaensis, (5)	0.017	-							
L. calcarius, (6–9)	0.017	0.000	-						
L. romeri, (10–13)	0.015	0.019	0.019	-					
*L.*cf. *romeri*, (14)	0.021	0.025	0.025	0.011	-				
L. hainanus, (15–16)	0.028	0.030	0.030	0.025	0.032	-			
L. ocellatus, (17, 19–20, 22–24)	0.017	0.021	0.021	0.015	0.021	0.021	-		
L. ocellatus, (18, 21, 25–26)	0.015	0.019	0.019	0.013	0.019	0.023	0.002	-	
*Gracixualus gracilipe*s, (27)	0.136	0.133	0.133	0.138	0.144	0.133	0.129	0.127	-
Philautus aurifasciatus, (28)	0.159	0.159	0.159	0.163	0.165	0.153	0.155	0.155	0.155

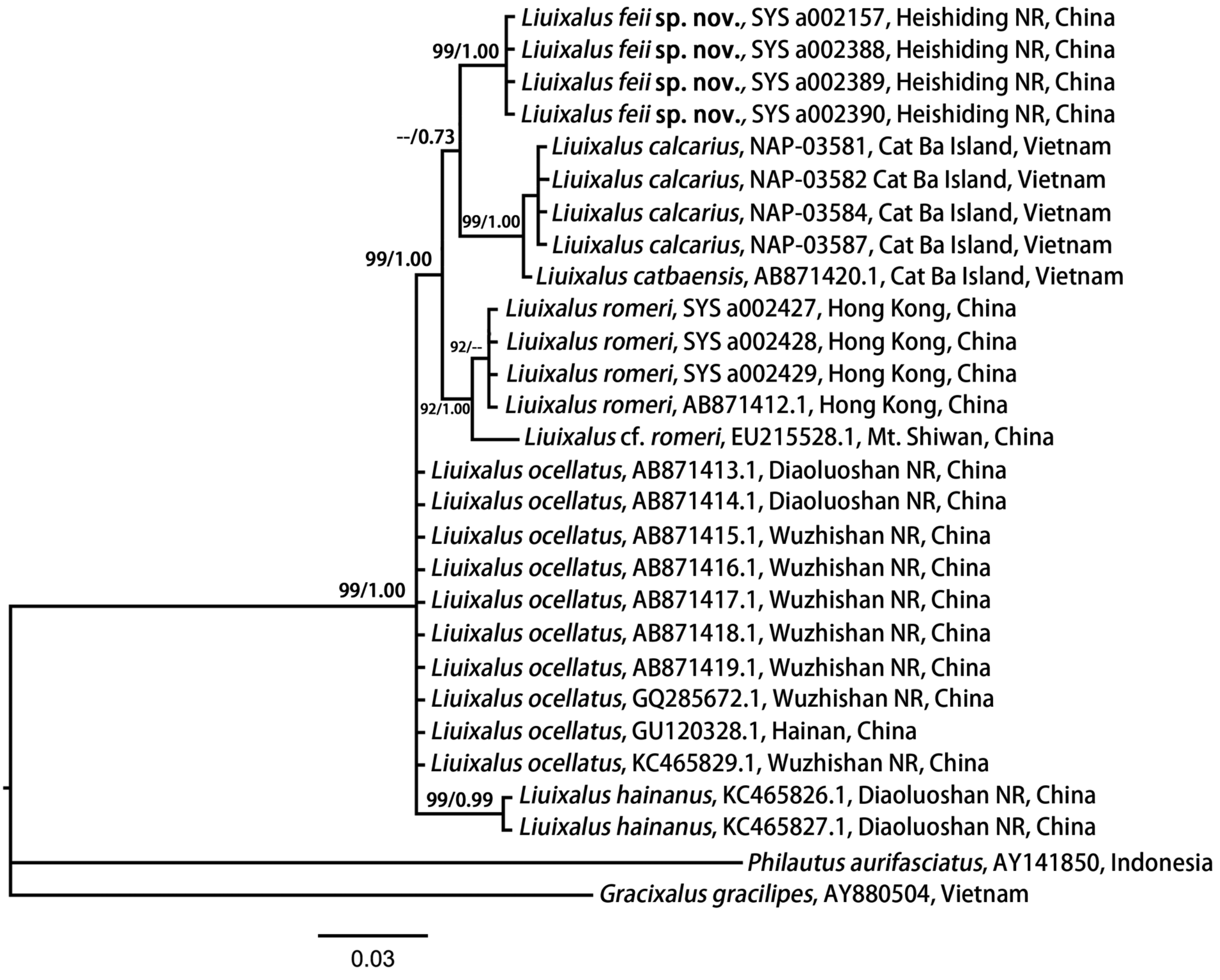

FIGURE 1. Bayesian inference (BI) tree derived from partial DNA sequences of the mitochondrial 16S rRNA gene sequences. Numbers above branches are bootstrap support for maximum likelihood analyses (>70 retained) and numbers below branches indicate Bayesian posterior probabilities (> 70% retained).

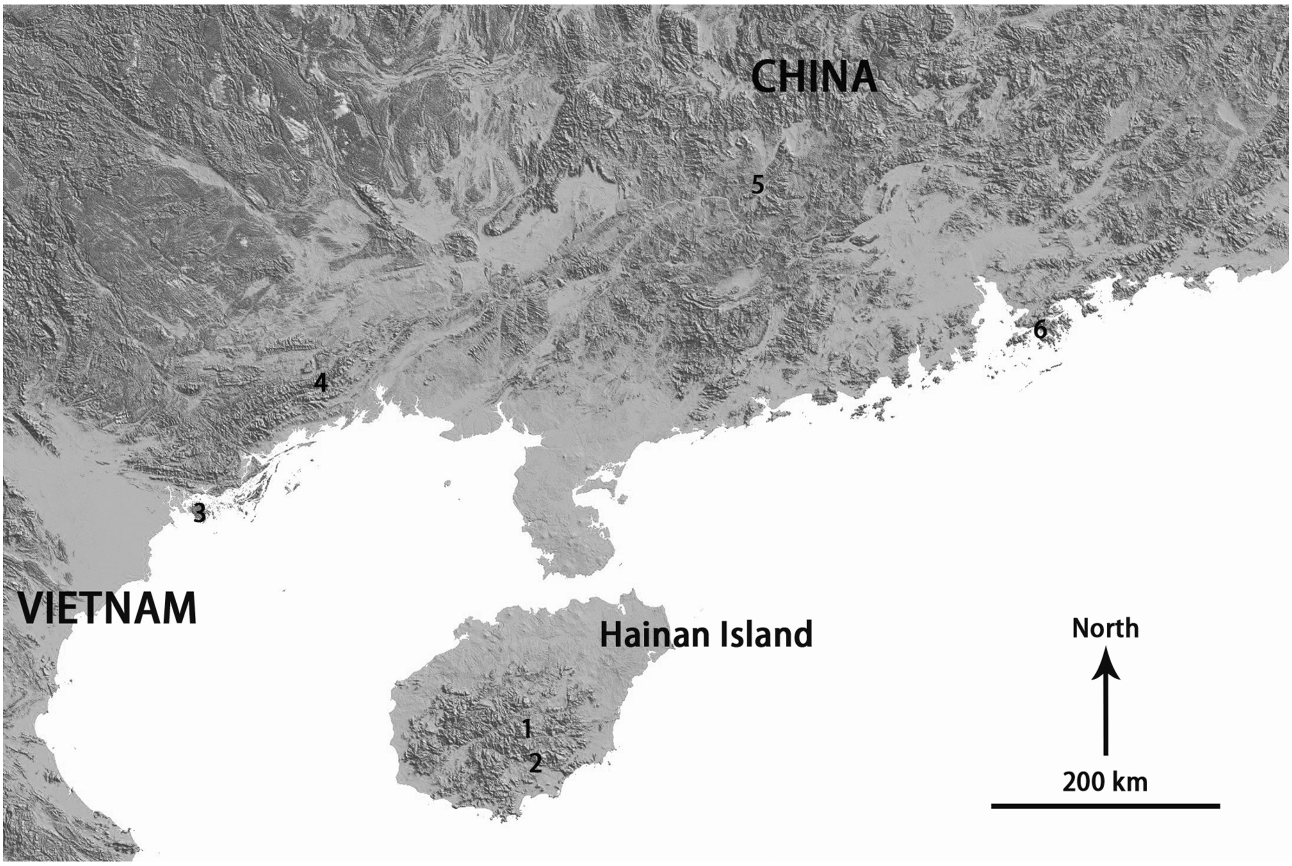

FIGURE 2. Map showing the distribution of of *Liuixalus* species and populations included for phylogenetic analyses in this study. 1: Wuzhishan NR, Hainan; 2: Diaoluoshan NR, Hainan; 3: Cat Ba Island, Vietnam; 4: Mt. Shiwan, Guangxi; 5: Heishiding NR, Guangdong; 6: Hong Kong.

Liuixalus feii sp. nov.

Holotype. SYS a002389, adult male, from Heishiding Nature Reserve, Fengkai County, Guangdong Province, China (23°27'10.4" N, 111°53'15.4" E, 550 m a.s.l.; Fig. 2), collected by Yu-Long Li and Jian Zhao at night on 6 October 2013.

Paratypes. Four adult males and two adult females from the same locality as holotype: SYS a000454, adult female, collected by Jian-Huan Yang on 29 May 2009; SYS a002158, adult male, collected by YLL and JZ on 1 June 2013; SYS a002388, adult female, and a002390, adult male, same data as holotype; SYS a003049–3050, adult males, collected by Ying-Yong Wang and Zu-Yao Liu on 15 July 2014.

Etymology. The specific epithet "*feii*" is a patronymic noun in the genitive singular; derived from the name of Professor Liang Fei of the Chengdu Institute of Biology, Chinese Academy of Sciences, China, in recognition of his long-term great contribution to the advancement of amphibian research in China. For the common name, we suggest "Fei's Small Tree Frog" in English, "Fei Shi Xiao Shu Wa" in Chinese.

Diagnosis. The new species is assigned to the genus *Liuixalus* by presenting the following characters: body size small; tympanum well distinct and about half the eye diameter; fingers without webbing; toes weakly webbed; hindlimb relatively long, tibio-tarsal articulation reaching the naris or loreal; discs on the digits well developed, discs of toes slightly smaller than disks of fingers; dorsum of body with a X-shaped marking (Milto *et al*. 2013). *Liuixalus feii* **sp. nov.** is distinguished from its congeners by a combination of following characters: small size (SVL 16.2–17.6 mm in adult males and 18.0–18.7 mm in adult females); snout obtusely pointed; tympanum distinct, about half size of eye diameter; nostril closer to eye than to the tip of snout; fingers free of webbing; toe III longer than toe V; toes weakly webbed; tibiotarsal articulation reaching the naris or loreal; dorsal skin smooth and scattered with fine granulars; a subtle longitudinal median ridge present on dorsum; weak skin folds present on dorsal surface of body and thighs; supratympanic fold distinct and curved; ventral surface dull white with more or less irregular dark spots; iris bicolored.

FIGURE 3. Holotype SYS a002389 of *Liuixalus feii* **sp. nov.** in preservative. A: dorsal view; B: ventral view; C: lateral view of head and body; D: ventral view of right hand; E: ventral view of left foot.

Description of holotype. SYS a002389, adult male, body size small (SVL 16.8 mm), head length slightly greater than head width (HL 6.6 mm; HW 5.7 mm); snout obtusely pointed in dorsal view and profile, projecting beyond margin of the lower jaw; interorbital region flat; canthus rostralis distinct and rounded; loreal region slightly oblique and concave; nostrils oval, slightly protuberant, slightly closer to eye than to tip of snout; interorbital distance slightly greater than internasal distance; pupil horizontal; tympanum distinct, 48.0% of eye diameter, distant from eye by half of its own diameter (ratio of TED/TD 0.5); tympanic rim weakly elevated relative to skin of temporal region; interorbital distance wider than the upper eyelid; pineal ocellus absent; vomerine teeth absent; choanae rounded, at margins of mouth roof; tongue oblong, attached anteriorly and notched posteriorly; supratympanic fold distinct and somewhat curved, extending from behind eye to shoulder basis.

Forelimbs moderately robust, relative length of fingers I < II < IV < III (Fig. 3D); tips of fingers II, III, IV with well-developed disks with distinct circummarginal grooves, disks relatively wide compared to finger width (disk of finger III 216% wider than width of finger III), third finger disk width 68.4% of tympanum diameter; tip of finger I round and without distinct disk; fingers free of webbing; subarticular tubercles prominent, rounded, formula 1, 1, 2, 2; outer palmar tubercle weakly divided into two; accessory palmar tubercles indistinct; no nuptial pads observed on dorsal surface of fingers.

Hindlimbs long and slender, 171% of SVL; tibiotarsal articulation reaching the naris when adpressed along the body; tibia length 54% of SVL; relative length of toes I < II< V < III < IV (Fig. 3E); tips of toes with poorly developed disks with distinct circummarginal grooves, disks smaller than those of fingers; toe weakly webbed, webbing formula I 2–2½ II 2–3¼ III 2⅓–4 IV 3⅓–1¾ V; subarticular tubercles distinct, rounded, formula 1, 1, 2, 3, 2; inner metatarsal tubercle low, oval, distinct, approximately 0.8 mm in length; outer metatarsal tubercle very small and indistinct.

Dorsal surface of head and body smooth and scattered with fine granules, those on flanks and eyelids somewhat prominent; a subtle longitudinal median ridge present on dorsum; weak skin folds present on dorsal surface of body and thigh; ventral surface of thighs and abdomen with flat granules, throat smooth.

FIGURE 4. Paratypes specimens of *Liuixalus feii* **sp. nov.** in preservative, note the variation in dorsal and ventral patterns: SYS a002158, SYS a002388, SYS a002390, SYS a003049, SYS a003050 (from left to right).

Coloration of holotype in preservative. Dorsal surface pale brown with a dark X-shaped marking from behind the eyes, consisting of two crooked dark lines along body not in contact medially; a dark interorbital narrow bar between the eyes extending to the upper eyelids; dark transverse bars present on dorsal surface of fore and hind limbs: two on the lower arm, four on the thigh, three on the tibia; ventral surface dirty-white, scattered with few irregular dark spots on chest and venter; lateral of head dark brown, supratympanic fold distinctly darker; lower lip pale white with conspicuous dark bars; a remarkable dark blotch present on the anterior portion of shoulder joint; iris bicolored, upper third pale white, lower two third dark brown.

Variation. All six paratypes match the overall characters of the holotype (measurements of type series see Table 3). All type specimens have a dark X-shaped marking on dorsum composed of two crooked dark lines, these lines are usually separated but are in contact medially in SYS a002158; the dark spots on the ventral surface are somewhat distinct in SYS a002388, 2390, 3049, 3050 (see Figs. 3–5 for coloration variation). No significant differences between males and females were revealed, however females are slightly larger than males (SVL 18.0–18.6 mm versus 16.2–17.6 mm). Of the five males, only SYS a003050 possesses distinct nuptial pad on the posterodorsal surface of finger I.

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

	SYS a002158	SYS a002389	SYS a002390	SYS a003049	SYS a003050	Mean ± SD (N=5)	SYS a000454	SYS a002388	Mean ± SD (N=2)
sex	Male	Male	Male	Male	Male		Female	Female	
SVL	16.3	16.8	17.0	16.2	17.6	16.78±0.57	18.7	18.0	18.35±0.49
HL	6.2	6.6	6.6	6.2	6.8	6.48±0.27	7.2	6.7	6.95±0.35
HW	6.1	5.7	6.0	5.6	6.3	5.94±0.29	6.2	6.2	6.20±0.00
SL	2.4	2.7	2.6	2.4	2.9	2.60±0.21	2.9	2.7	2.80±0.14
IND	1.9	2.0	2.0	2.0	2.2	2.02±0.11	2.3	2.1	2.20±0.14
IOD	2.3	2.1	2.0	2.1	2.4	2.18±0.16	2.5	2.3	2.40±0.14
UEW	1.2	1.4	1.3	1.1	1.1	1.22±0.13	1.3	1.1	1.20±0.14
ED	2.0	2.1	2.2	2.2	2.3	2.16±0.11	2.5	2.2	2.35±0.21
TD	0.9	1.0	0.9	0.9	1.0	0.94±0.05	1.0	0.9	0.95±0.07
TED	0.6	0.5	0.6	0.6	0.6	0.58±0.04	0.7	0.6	0.65±0.07
LAHL	7.9	8.0	7.4	7.8	8.1	7.84±0.27	7.4	8.1	7.75±0.49
HAL	3.9	4.2	4.3	4.1	4.2	4.14±0.15	3.9	4.3	4.10±0.28
HLL	26.9	28.7	27.0	27.8	28.3	27.74±0.79	28.7	29.9	29.30±0.85
TIB	8.3	9.1	9.0	8.7	9.0	8.82±0.33	9.4	9.6	9.50±0.14
LFT	11.9	12.6	12.1	11.9	12.0	12.10±0.29	12.1	13	12.55±0.64
FT	7.3	7.8	7.2	7.3	7.7	7.46±0.27	7.8	7.6	7.70±0.14
FDW III	0.5	0.7	0.5	0.5	0.7	0.58±0.11	0.5	0.6	0.55±0.07
TDW IV	0.5	0.5	0.5	0.5	0.6	0.52±0.04	0.5	0.5	0.50±0.00
HL/SVL	0.38	0.39	0.39	0.38	0.39	0.39±0.00	0.39	0.37	0.38±0.01
HL/HW	1.02	1.16	1.10	1.11	1.08	1.09±0.05	1.16	1.08	1.12±0.06
SL/SVL	0.15	0.16	0.15	0.15	0.16	0.15±0.01	0.16	0.15	0.15±0.00
ED/SL	0.83	0.78	0.85	0.92	0.79	0.83±0.05	0.86	0.81	0.84±0.03
TD/ED	0.45	0.48	0.41	0.41	0.43	0.44±0.03	0.40	0.41	0.40±0.01
TED/TD	0.67	0.50	0.67	0.67	0.60	0.62±0.07	0.70	0.67	0.68±0.02
LAHL/SVL	0.48	0.48	0.44	0.48	0.46	0.47±0.02	0.40	0.45	0.42±0.04
HLL/SVL	1.65	1.71	1.59	1.72	1.61	1.65±0.06	1.53	1.66	1.60±0.09
TIB/SVL	0.51	0.54	0.53	0.54	0.51	0.53±0.01	0.50	0.53	0.52±0.02

Distribution and natural history. The new species, *Liuixalus feii* **sp. nov.**, is currently known from Heishiding Nature Reserve, Fengkai County, Guangdong Province, China (Fig. 2). All individuals recorded were found on leaf-litter on the forest floor in well-preserved primary forest at elevations between 350–800 m.

In its type locality, calls of males of *Liuixalus feii* **sp. nov.** have been heard from April to October. A small tree hole in the roots of a large tree (the hole opening approximately 28 mm wide, 64 mm high and 78 mm deep; water depth inside the hole about 20 mm), 10 cm above the ground, was found containing eight free-swimming tadpoles on 2 May 2010, as well as an adult male calling nearby. We assume these to belong to the new species, but have not confirmed it. All individuals of the new species were found on the forest floor and not closed to streams or pools; and no individual was recorded along the streams, ponds or other kinds of wetlands during our surveys in the nature reserve. Therefore, the new species appears to be a forest-dependent species and is likely a phytotelm-breeder, breeding in water accumulated in tree or bamboo holes in the forest.

Comparisons. *Liuixalus feii* **sp. nov.** differs from *L. calcarius* by having nostril closer to eye than to tip of snout (reverse condition in *L. calcarius*); tibiotarsal articulation reaching the naris (versus beyond the tip of snout in *L. calcarius*); toe III longer than toe V (versus toe III length equal to toe V in *L. calcarius*); throat of males dull white (versus dark colored in males in *L. calcarius*). *Liuixalus feii* **sp. nov.** differs from *L. hainanus* by having a relatively smaller tympanum in males (mean ratio of TD/SVL 0.056 in *L. feii* **sp. nov.** versus 0.081 in *L. hainanus*); relatively shorter hindlimbs in males (mean ratio of HLL/SVL 1.65 in *L. feii* **sp. nov.** versus 1.68 in *L. hainanus*); relatively shorter tibias in males (mean ratio of TIB/SVL 0.53 in *L. feii* **sp. nov.** versus 0.64 in *L. hainanus*); tibiotarsal articulation reaching the naris (versus beyond the tip of snout in *L. hainanus*); the absence of beige speckling on the dorsum (versus present in *L. hainanus*, Fig. 6); the distinct difference in eye color pattern (iris bicolor in *L. feii* **sp. nov.** versus iris yellowish overall with distinct small dark spots above and below pupil in *L. hainanus*). *Liuixalus feii* **sp. nov.** differs from *L. ocellatus* by having relatively smaller tympanum in males (mean ratio of TD/SVL 0.056 in *L. feii* **sp. nov.** versus 0.078 in *L. ocellatus*); relatively shorter snout (mean ratio of SL/SVL 0.15 in *L.* feii **sp. nov.** versus 0.18 in *L. ocellatus*); relatively longer hindlimbs in males (mean ratio of HLL/SVL 1.66 in *L. feii* **sp. nov.** versus 1.63 in *L. ocellatus*); tip of 1st finger not enlarged and without disk (versus with tip of 1st finger with distinct disk in *L. ocellatus*). *Liuixalus feii* **sp. nov.** differs from *L. romeri* by having relatively smaller tympanum in males (mean ratio of TD/SVL 0.056 in *L. feii* **sp. nov.** versus 0.088 in *L. romeri*); snout longer than eye diameter in males (mean ratio of ED/SL 0.85 in *L. feii* **sp. nov.** versus 1.07 in *L. romeri*); tibiotarsal articulation reaching the naris (versus reaching the tip of snout in *L. romeri*); fingers free of webbing (versus weakly webbing present between fingers III and V in *L. romeri*); toes weakly webbed (versus toes 1/3 webbed in *L. romeri*).

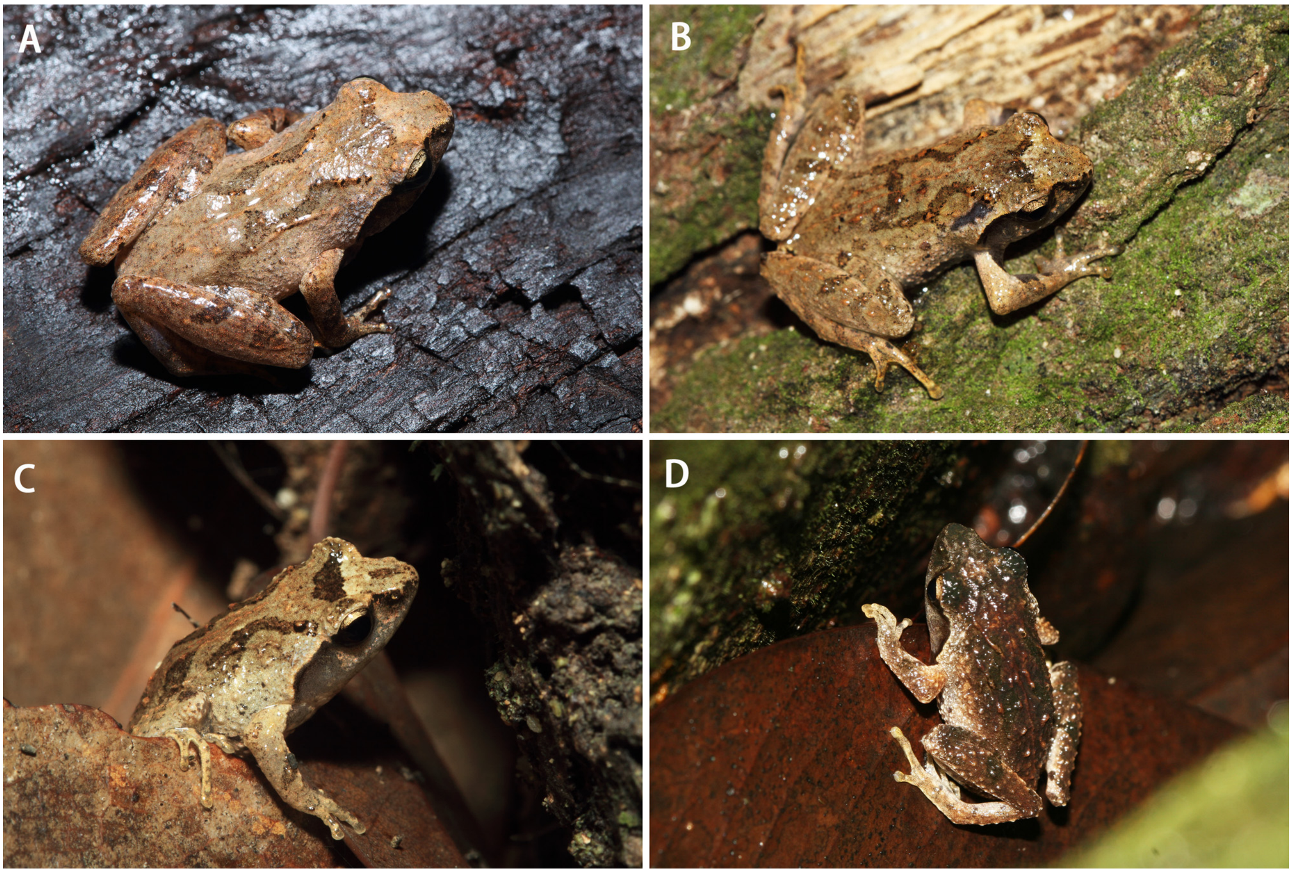

FIGURE 5. *Liuixalus feii* **sp. nov.** from its type locality, showing the color variation in life. A: SYS a003049, adult male; B: SYS a002158, adult male; C: adult female, photographed on 2 May 2010, not collected; D: adult male, photographed on 29 May 2009, not collected.

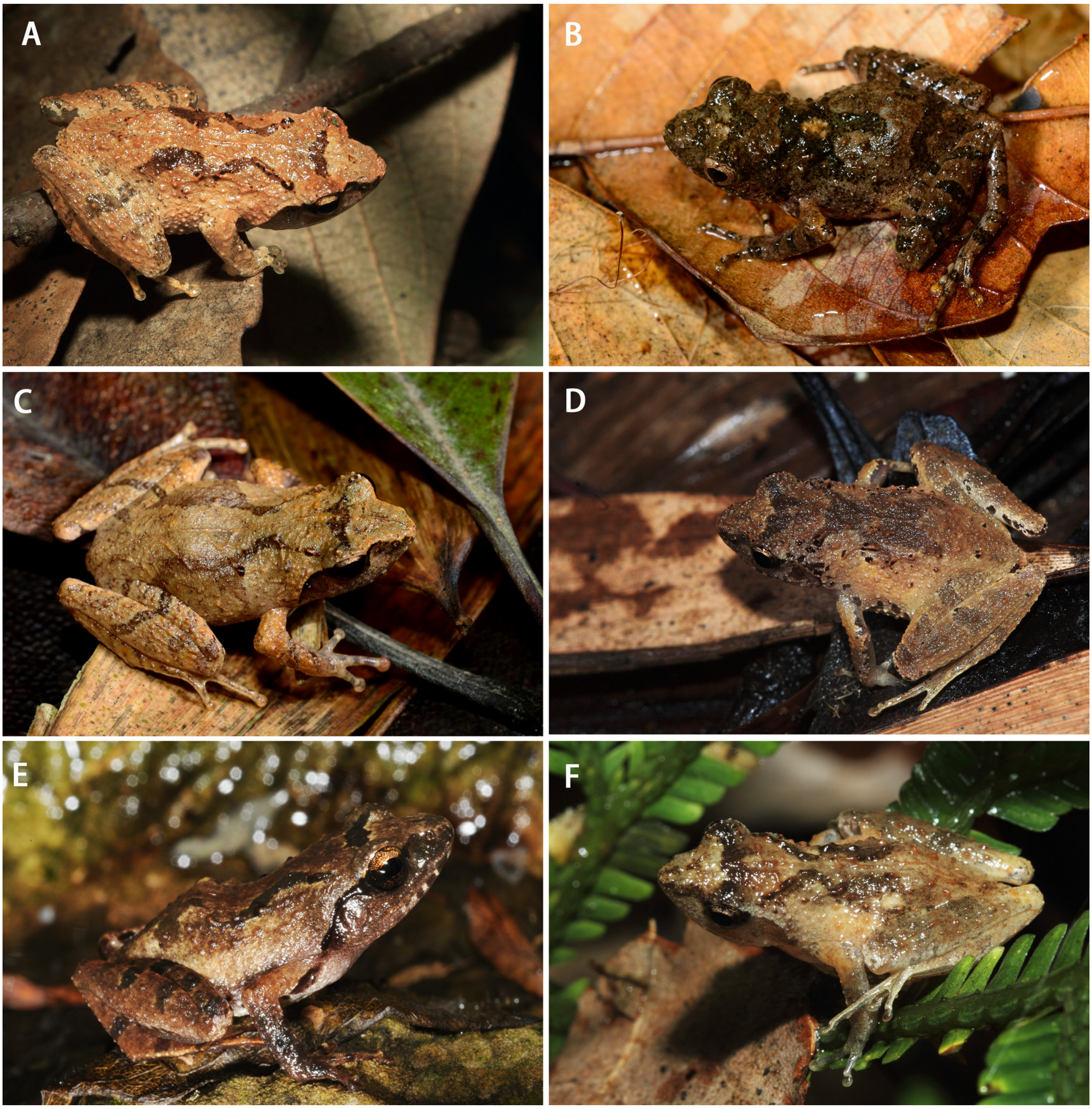

FIGURE 6. A: a male individual of *Liuixalus feii* **sp. nov.**, photographed on 2 May 2010, not collected; B: *L. hainanus* from Diaoluoshan, NR Hainan (type locality); C: *L. ocellatus* from Wuzhishan NR, Hainan (type locality); D: *L. romeri* from Hong Kong (type locality); E: paratype of *L. calcarius* from Cat Ba Island, Vietnam; F: *L.* cf. *romeri* from Mt. Shiwan, Guangxi.

Discussion

Besides genetic divergence, a confirmed candidate species needs to be supported by ecological evidence and/or a diagnostic morphological difference (Vassilieva *et al*. 2014). The genetic distances revealed here between species of *Liuixalus* at the 16S gene fragment examined are often small. For example, *L. ocellatus* from Hainan and *L. romeri* from Hong Kong have only 1.3% divergence at this gene fragment. However there are also examples of valid morphologically different species that have low genetic distances in other complexes of related species in Rhacophoridae, e.g. *Pseudophilautus pleurotaenia* and *P. hoipolloi* (p = 0.5%), *P. asankai* and *P. hoffmanni* (p = 1.0%), *P. decoris* and *P. mittermeieri* (p = 1.4%), *P. dilmah* and *P. hankeni* (p = 1.6%) (Wickramasinghe *et al.* 2015), as well as species in other families such as *Kalophrynus cryptophonus* and *K. honbaensis* (p = 1.69%, Vassilieva *et al.* 2014). Hence we consider the *Liuixalus feii* from Heishiding Nature Reserve can be treated as a recognized species, which is also supported by morphological evidence, as stated above.

Liuixalus cf. *romeri* from Mt. Shiwan, Guangxi and *L. romeri* s. stricto from Hong Kong (the type locality of *L. romeri*) display a 1.1% *p*-distance at the 16S gene fragment analysed. Taking into account the fact that the two localities are greatly geographically separated (ca. 650 km), the *Liuixalus* population from Mt. Shiwan may be an undescribed species; but further morphological and ecological evidence is needed to clarify the taxonomic status of this population.

With the description of *L. feii* and the formal synonym of *L. catbaensis* under *L. calcarius*, the genus *Liuixalus* currently contains five recognized species with disjunct geographic distributions: Cat Ba Island of Vietnam, southwestern and central Guangxi, western Guangdong to Hong Kong, and Hainan Island (Fig. 2). We assume that species of the genus *Liuixalus* were widely distributed in the southern Guangxi and Guangdong Provinces in the past; deforestation over the past decades in southern China has resulted in the range of these forest-dependent species becoming fragmented. Currently unknown populations of *Liuixalus* may be discovered in non-disturbed forests from mid to high elevations in southern Guangxi and Guangdong during future herpetological exploration in the region.

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