

RESEARCH ARTICLE

Two New Species of *Liuixalus* (Rhacophoridae, Anura): Evidence from Morphological and Molecular Analyses

Shaobo Qin^{1,4}, Yunming Mo², Ke Jiang¹, Bo Cai¹, Feng Xie¹, Jianping Jiang¹, Robert W. Murphy³, Jia-Tang Li^{1*}, Yuezhao Wang^{1*}

1 Chengdu Institute of Biology, the Chinese Academy of Sciences, Chengdu, Sichuan, China, **2** Natural History Museum of Guangxi, Nanning, Guangxi, China, **3** Centre for Biodiversity and Conservation Biology, Royal Ontario Museum, Toronto, Ontario, Canada, **4** College of Life Sciences, Sichuan University, Chengdu, Sichuan, China

☞ These authors contributed equally to this work.

* lijit@cib.ac.cn (JTL); arcib@cib.ac.cn (yw)



OPEN ACCESS

Citation: Qin S, Mo Y, Jiang K, Cai B, Xie F, Jiang J, et al. (2015) Two New Species of *Liuixalus* (Rhacophoridae, Anura): Evidence from Morphological and Molecular Analyses. PLoS ONE 10(8): e0136134. doi:10.1371/journal.pone.0136134

Editor: Zhong-Jian Liu, The National Orchid Conservation Center of China; The Orchid Conservation & Research Center of Shenzhen, CHINA

Received: January 18, 2015

Accepted: July 29, 2015

Published: August 25, 2015

Copyright: © 2015 Qin et al. This is an open access article distributed under the terms of the [Creative Commons Attribution License](https://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Data Availability Statement: All relevant data are within the paper.

Funding: This study was supported by the National Natural Science Foundation of China (31372173, 31411130190, 31101618, 31471964, 31372174), Youth Innovation Promotion Association CAS, and grants from the Ministry of Science and Technology of China (MOST Grant 2011FY120200, Grant 2005DKA21402).

Abstract

Due to small body sizes, superficial similarities in morphologies, and obscure activity behaviors, the phylogeny and taxonomy of species in the genus *Liuixalus* were very troublesome. Some species might comprise a complex of cryptic species. To investigate the species of group, we constructed the matrilineal genealogy of the genus using 16s rRNA mitochondrial DNA sequences. Analyses recovered six well supported matrilineal lines that involved *L. romeri*, *L. ocellatus*, *L. hainanus*, *L. calcarius*, *Liuixalus shiwandashan* **sp. nov.** and *Liuixalus jinxiuensis* **sp. nov.**, though the historical relationships among them remained unresolved. Currently, *Liuixalus* included 4 species, distributed eastwards from northern Vietnam to Hong Kong, China. Based on genealogical and morphological distinctiveness, we described *Liuixalus jinxiuensis* **sp. nov.** from the type locality Mt. Dayao, Jinxiu, China and *Liuixalus shiwandashan* **sp. nov.** from the type locality Mt. Shiwanda, China. A combination of morphological measurements, genetic, bioacoustic and osteological analysis was served to diagnose the new taxa.

Introduction

The genus *Liuixalus* was proposed in 2008 with type species *L. romeri* (Smith, 1953) based on its distinct molecular phylogenetic position [1], which was confirmed by Li *et al.* [2] and Yu *et al.* [3]. All previous studies suggested that *Liuixalus* was a sister clade to all other members in the subfamily Rhacophorinae [1–5]. A small body size, the absence of vomerine teeth, strongly reduced webbing and X-shape dark colored mark on the shoulder served to diagnose *Liuixalus* [6–9]. *Liuixalus romeri* was described from Hong Kong but also found to occur in southern Guangxi. *Liuixalus hainanus* and *L. ocellatus* occurred at Mt. Diaoluo (Diaoloushan), Hainan, China and Mt. Wuzhi (Wuzhishan), Hainan, respectively. Recently, Milto *et al.* [9] described *L. calcarius* and suggested it was endemic to Cat Ba Island, Gulf of Tonkin, Vietnam. Shortly

Competing Interests: The authors have declared that no competing interests exist.

thereafter, Nguyen *et al.* [10] described the new species *L. catbaensis* from the same locality. Based on their similar morphologies, same distributions, and close publication dates, Frost (2015) suggested that *L. catbaensis* was a junior synonym of *L. calcarius*.

Based on fieldwork in Mt. Dayao in 2008 and Mt. Shiwanda in 2013, we collected several specimens of two unknown forms of *Liuixalus*. Located in central Guangxi, southern China, Mt. Dayao is the watershed of the Gui and Liu rivers. Lying in south of Guangxi, Mt. Shiwanda stretches for over 100 km and covers an area of 2600 km². This rolling mountain chain consists of 72 peaks over 500 m and 21 peaks over 1000 m a.s.l. The ecosystem in Guangxi is diverse owing to its particular geographic location and complicated environment. Previous surveys identified about 166 species of mammals, 483 birds, and 1766 Marine animals in Guangxi [11].

Accurate taxonomies form the foundation of conservation. The phylogenetic relationship among the *Liuixalus* requires extensive sampling and robust diagnoses. In this study, we firstly construct matrilineal genealogy of the group by analyses of mitochondrial DNA (mtDNA). The genealogy makes a framework for morphological assessments. The results indicate the taxonomy of the genus *Liuixalus* more clearly and the necessity to describe two new species *Liuixalus shiwandashan* **sp. nov.** and *Liuixalus jinxiuensis* **sp. nov.**. Bioacoustic and osteological analyses enrich the species descriptions in critical ways.

Material and Methods

Ethics Statement

This work was conducted with the permission of the Management Offices of the Mt. Dayao and Mt. Shiwanda Nature Reserve. All animal procedures were approved by the Animal Care and Use Committee of Chengdu Institute of Biology (permission number: 20140401).

Species Sampling and Data Collection

Field work was conducted in April to May 2008 at Mt. Dayao, Guangxi, China, and April to May 2013 at Mt. Shiwanda, Guangxi, China (Table 1). Specimens were ethanol-fixed and deposited in Herpetological Museum of the Chengdu Institute of Biology, Chinese Academy of Sciences (CIB), and Guangxi Zhuang Autonomous Region Museum of Natural History (GXNM). Character data for comparison were taken from specimens and references [6–10].

Molecular Analysis

Taxonomic sampling included 34 sequences. For our sequencing, genomic DNA was extracted from either muscle, or live tissues using the standard phenol-chloroform extract protocol [12]. Primer sequences of Wilkinson *et al.* [13–14] were used to amplify and sequence a fragment that included 1467 aligned nucleotide positions encompassing from 12S to 16S ribosome RNA (rRNA) of the mtDNA genome. Double-stranded polymerase chain reaction (PCR) amplification was carried out using the following parameter: 94°C initial hot start (3 min), then 30 cycles of 94°C denaturation (1 min), 52°C annealing (1 min), and 72°C extension (2 min). Final extension at 72°C was conducted 10 min. PCR products were directly sequenced with an ABI 3730 automated DNA sequencer and in both directions. The resulting sequences were submitted to Blast searching [15] in GenBank to ensure the required sequence had been sequenced. The outgroup taxa (and GenBank Accession Nos.) included *Buergeria japonica* (DQ283055), *Kurixalus hainanus* (DQ283054), *Rhacophorus moltrechti* (DQ283080), *Polypedates megacephalus* (DQ283073), *Chiromantics hansenae* (AB813161), *Buergeria oxycephala* (AB813156), *Rhacophorus rhodopus* (AB813151), *Philautus jinxiuensis* (EU215525), and *Buergeria buergeri* (AB127977).

Table 1. Species used in study. “–” represents the unknown information from Genbank. CIB, Chengdu Institute of Biology, the Chinese Academy of Sciences; KIZ, Kunming Institute of Zoology, the Chinese Academy of Sciences; SCUM, Sichuan University Museum.

Taxon	Specimen voucher No.	Locality	GenBank accession nos. 16s
<i>Buergeria japonica</i>	UMFS 5821	Taiwan, China	DQ283055
<i>Buergeria buergeri</i>	–	Ota River, Hiroshima prefecture, Japan	AB127977
<i>Buergeria oxycephala</i>	SCUM 050267YJ	Hainan China	EU215524
<i>Rhacophorus rhodopus</i>	SCUM 060692L	Mengyang Jinghong China	EU215531
<i>Polypedates megacephalus</i>	SCUM LJT 73	Yaan Sichuan China	KF053220
<i>Philautus jinxiuensis</i>	KIZ 061210YP	Mt. Dayao Guangxi China	EU215525
<i>Rhacophorus moltrechti</i>	SCUM 061106L	Lianhuachi Taiwan China	EU215543
<i>Chiromantis hansenae</i>	KUHE:34136	Nong Khor, southeastern Siam	AB813161
<i>Kurixalus hainanus</i>	HNNU A1180	Mt. Diaoluo Hainan China	EU215548
<i>Liuxalus ocellatus</i>	–	China	GU120328
<i>Liuxalus ocellatus</i>	–	Mt. Diaoluo, Hainan, China	AB871414
<i>Liuxalus ocellatus</i>	HN 0806046	Mt. Wuzhi, Hainan, China	KC465829
<i>Liuxalus ocellatus</i>	–	Mt. Wuzhi, Hainan, China	AB871417
<i>Liuxalus ocellatus</i>	HN 0806045	Mt. Wuzhi, Hainan, China	GQ285672
<i>Liuxalus ocellatus</i>	–	Mt. Diaoluo, Hainan, China	AB871413
<i>Liuxalus ocellatus</i>	–	Mt. Wuzhi, Hainan, China	AB871419
<i>Liuxalus ocellatus</i>	–	Mt. Wuzhi, Hainan, China	AB871418
<i>Liuxalus ocellatus</i>	–	Mt. Wuzhi, Hainan, China	AB871416
<i>Liuxalus ocellatus</i>	–	Mt. Wuzhi, Hainan, China	AB871415
<i>Liuxalus jinxiuensis</i> sp. nov.	KIZ 060821245	Guangxi, China	EF564535
<i>Liuxalus jinxiuensis</i> sp. nov.	CIB 101060	Mt. Dayao Guangxi, China	KT192635*
<i>Liuxalus jinxiuensis</i> sp. nov.	KIZ 060821246	Guangxi, China	EF564536
<i>Liuxalus calcarius</i>	–	Cat Ba island, Gulf of Tonkin, Vietnam	AB871420
<i>Liuxalus hainanus</i>	HN 0806039	Yinggeling, Hainan, China	KC465827
<i>Liuxalus hainanus</i>	HN 0806040	Yinggeling, Hainan, China	KC465828
<i>Liuxalus hainanus</i>	SCUM 060401L	Diaoluoshan, Hainan, China	GQ285671
<i>Liuxalus hainanus</i>	LJT V15	Diaoluoshan, Hainan, China	KC465826
<i>Liuxalus romeri</i>	–	Hong Kong, China	AB871412
<i>Liuxalus romeri</i>	CIB 10LJT	Hong Kong, China	KT192638*
<i>Liuxalus romeri</i>	CIB 7LJT	Hong Kong, China	KT192637*
<i>Liuxalus romeri</i>	CIB 6LJT	Hong Kong, China	KT192636*
<i>Liuxalus shiwandashan</i> sp. nov.	KIZ 061205YP	Mt. Shiwanda, Guangxi, China	EU215528
<i>Liuxalus shiwandashan</i> sp. nov.	CIB 101061	Mt. Shiwanda, Guangxi, China	KT192633*
<i>Liuxalus shiwandashan</i> sp. nov.	CIB 101054	Mt. Shiwanda, Guangxi, China	KT192634*

* Sequences new to this study.

doi:10.1371/journal.pone.0136134.t001

Alignments first used CLUSTALX 1.81 [16–18] with default parameter followed by visual confirmation and manual adjustments. Nucleotide sites with ambiguous alignments were removed from analyses. Gaps were analyzed as missing data. Our *de novo* were trimmed to 16s rRNA only to match data downloaded from NCBI. The aligned sequences were analyzed using Bayesian inference (BI) in MrBayes 3.12 [19]. Markov Chain Monte Carlo (MCMC) generations used three million iterations and we sampled every 1000th step. The first 25% of the samples were discarded as conservative burn-in. The remaining samples were used to generate a majority-rule consensus tree (Fig 1).

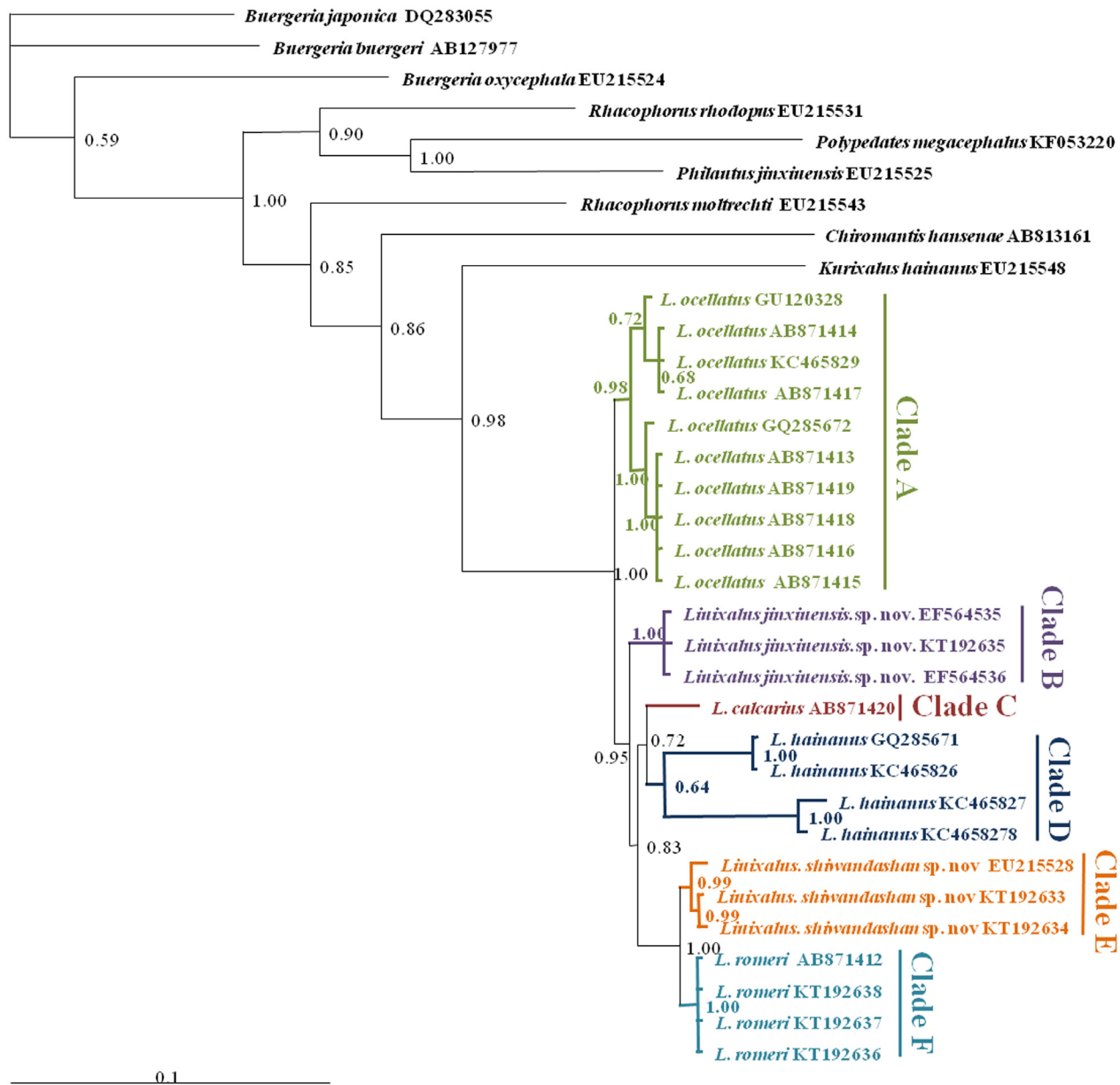


Fig 1. Bayesian inference tree. This tree was inferred from the mtDNA dataset. All of the Bayesian posterior probabilities (BPP) and NCBI numbers were shown.

doi:10.1371/journal.pone.0136134.g001

Morphological Analysis

Measurements were taken with dial calipers with a precision of 0.1 mm under binocular dissecting microscope by one person to preclude variation owing to researcher-bias. The following measurements and abbreviations were used: SVL = snout-vent length; A-G = axilla to groin, distance from posterior base of forelimb at its emergence from body to anterior base of hind limb at its emergence from body; HW = head width at the greatest cranial width; HL = head length from the rear of the lower jaw to the tip of the snout; HD = head depth, greatest

transverse depth of head, taken beyond interorbital region; UEW = upper eyelid width: greatest width of upper eyelids; IOD = interorbital distance; ED = horizontal diameter of eye; TD = horizontal diameter of tympanum; ESL = tip of snout-eye distance; TED = tympanum-eye distance from anterior edge of tympanum to posterior corner of the eye; IND = inter-narial distance: distance between nostrils; END = eye to nostril distance: distance from anterior corner of eye to nostril; FLL = length of forelimb from tip of disk of finger III to axilla; FFL = first finger length; TFL = third finger length; FTD = maximal diameter of disc of finger III; and NPL = nuptial pad length.

Bioacoustics Analysis

Calls were collected at Mt. Shiwanda, Guangxi, China from April to May 2013 using a Nikon P310 recorder, and at our sampling locality Mt. Dayao from April to May 2008 using a Canon. The calls were frequently heard at 19:00–23:00. Spectrograms of male calls were generated using Avisoft-SAS lab lite software with a 22.05 kHz sampling frequency and 16-bit precision.

Osteological Analysis

The skeletons of one adult male from each locality were analyzed as cleared and stained specimens prepared according to Wassersug's [20–21] protocol. The terminology for cranial and postcranial osteology followed Trueb [22–23], Heyer [24] Lynch [25], and Trueb *et al.* [26].

Nomenclatural Acts

The electronic edition of this article conforms to the requirements of the amended International Code of Zoological Nomenclature, and hence the new names contained herein are available under that Code from the electronic edition of this article. This published work and the nomenclatural acts it contains have been registered in ZooBank, the online registration system for the ICZN. The ZooBank LSIDs (Life Science Identifiers) can be resolved and the associated information viewed through any standard web browser by appending the LSID to the prefix "<http://zoobank.org/>". The LSID for this publication is: urn:lsid:zoobank.org:pub:38D7B0EB-90FF-4817-9579-E0C101119749. The electronic edition of this work was published in a journal with an ISSN, and has been archived and is available from the following digital repositories: PubMed Central, LOCKSS.

Result

Sequence Variation

The aligned rDNA gene fragments from *Liuixalus* consisted of 1467 nucleotide positions before trimming. The posterior 869 nucleotide positions were retained for genealogical reconstructions. The fragments contained 484 constant and 385 potentially phylogenetically informative characters. Plots of transitions and transversions showed a linear relationship, thus, giving no indication of saturation effects. Consequently, all nucleotide positions were used for genealogical inference.

Phylogenetic Analysis

Likelihood value of the 50% majority consensus tree was $\ln L = -5885.820$. The standard deviation of split frequencies among the four BI runs was 0.002966. The following relationships were indicated as being well supported and reliable:

1. Monophyly of *Liuixalus* was strongly supported (BPP = 1.00).

2. Matriline A included *L. ocellatus* from the different localities: Mt. Wuzhi, Hainan, Mt. Diaolu, Hainan and a vague locality (BPP = 0.98). This lineage was the sister-group to all the other matriline (B, C, D, E and F), as association that received moderate support (BPP = 0.95).
3. Matriline B, which contained *Liuixalus jinxiuensis* **sp. nov.** from Jinxiu, Guangxi, China, received strong nodal support (BPP = 1.00). It formed the sister-group to matriline C, D, E, and F. Matriline C included *L. calcarius* from the type locality Cat Ba island, Gulf of Tonkin, Vietnam. Matriline D contained *L. hainanus* from the Mt. Diaolu, Hainan and Yinggeling, Hainan. Matriline C and D were moderately supported sister species (BPP = 0.72). Finally, strongly supported sister matriline (BPP = 1.00) E and F contained *Liuixalus shiwandashan* **sp. nov.** from Mt. Shiwanda, Guangxi, China and *L. romeri* from the type locality Hong Kong, respectively.

The genealogy was assumed to represent historical relationship of the species, i.e. resolution of a paternal genealogy would yield the same six lineages. Given prior recognition of most taxa, the assumption was not rejected. Thus, the genealogy served to reject the H_0 of conspecificity of all six matriline due to evolutionary cohesiveness within the current taxonomy. Consequently, we were required to describe two new species so that the taxonomy replicated historical relationships.

Species Accounts

Liuixalus shiwandashan sp. nov.

Li, Mo, Jiang, Xie & Jiang

urn:lsid:zoobank.org:act:DD566B0F-1ABE-443A-A73E-FA9FB9EE957E

Holotype. CIB 101052, an adult male (Figs 2 and 3) from Mt. Shiwanda, Guangxi, China (21.72064°N 107.5427°E, elevation 937m a.s.l.), collected by Jia-Tang LI and Bo CAI on 24 April 2013.

Paratypes (12). CIB 101053, CIB 101055–101059, CIB 101061, adult males, and CIB 101050, CIB 101051, CIB 101054, adult females, collected together with the holotype.

Etymology. The new species was named after the type locality, Mt. Shiwanda.

Diagnosis. *Liuixalus shiwandashan* **sp. nov.** is associated with *Liuixalus* in having a small body-size; head relatively broad; obtusely pointed snout; the right and left heel obviously overlap with each other; and a distinctly granulate “)(” pattern on the dorsum skin and upper eyelid.

Liuixalus shiwandashan **sp. nov.** is distinguished from all other species of *Liuixalus* by the following combination of morphological characters: 1) fingers with prominent dermal fringes; 2) flat tubercles on the ventral surface; 3) tibia-tarsal articulation reach (female) or extend beyond (male) nostril; 4) skin on dorsum smooth with sparse and compressed warts; 5) lateral side varicose in male; 6) nuptial pad covered by small whitish spines, present on the first and second fingers.

Description of the Holotype. Adult male (Figs 2 and 3), body length 17.14 mm. Further measurement provided in Table 2. Body slender; head longer than wide (HL/HW = 1.18); snout rounded, slightly protruding, projecting beyond jaw slightly; canthus rostral rounded, loreal region oblique and slightly concave; eye large, horizontally longer than distance from eye to nostril; tympanum distinct and medium sized, larger than half eye width; vomerine teeth absent, tongue large, oval, without papillae; dorsum skin smooth with a few, widely dispersed, inconspicuous tubercles; ventral skin verrucose.



Fig 2. *Liuxalus shiwandashan* sp. nov., dorsal view. In life, male, from Mt. Shiwanda.

doi:10.1371/journal.pone.0136134.g002

Forelimb long, length from tip of disk of finger III to axilla accounts for 48.77% of SVL. Finger slender, with strongly reduced interdigital web; dermal fringe prominent; relative lengths $I < II < IV < III$; tips rounded, enlarged; subarticular tubercles indistinct; inner metatarsal tubercle elliptical and prominent; outer metatarsal tubercle large but flat.

Hind-limbs long and slender; TBL 9.62 mm accounting for 56.13% of SVL; relative toes length $I < II < III = V < IV$; rounded discs on toes smaller than on fingers; webs between toes rudimentary; dermal fringe obvious; inner metatarsal tubercle flat and distinctively smaller than exterior metatarsal tubercle.

Coloration of holotype in life. Dorsal and lateral body yellowish brown; dorsum with brown “)”-shape pattern from behind the head to the sacrum; puce subtriangular markings lying in the interorbital region. Dorsal part of limb yellowish and decorated with brown



Fig 3. *Liuxalus shiwandashan* sp. nov., dorso-lateral view. In life, male, from Mt. Shiwanda.

doi:10.1371/journal.pone.0136134.g003

transverse bands. Throat, chest and belly immaculate yellowish white to white with sparse dark blotching; ventral surface of limbs transparent gray. Under jaw decorated with black stripe. Iris dark charcoal gray.

Coloration of holotype in preservative. In general, brightness reduces and tends to grayish-brown. Snout and dorsum brownish gray with brown pattern forming a “)””. Ventral part of limbs and belly yellowish gray.

Variation. Variation in measurements given in [Table 1](#). Females larger than males, SVL 19.2–19.6 mm in females (n = 3) and 16.2–18.5 mm in males (n = 9). Lateral tubercles of males more evident than for females. Individuals relatively uniform in body coloration.

Secondary sexual characters. Male internal single subgular vocal sac with a pair of slit-like vocal sac openings near corners of mouth; nuptial pads covered by small whitish spines present on dorsal side of the first finger and inner side of the second finger.

Comparison. *Liuxalus shiwandashan* sp. nov. differs from *L. hainanus* by having nuptial pads on both 1st and 2nd finger in males (only one nuptial pad in 1st finger for *L. hainanus*) and tibia-tarsal articulation reaching or beyond nostril (exceeds snout in *L. hainanus*). *Liuxalus shiwandashan* sp. nov. differs from *L. calcarius* by the absence of dark color on throat vocal sac (present in *L. calcarius*). *Liuxalus shiwandashan* sp. nov. differs from *L. ocellatus* by the presence of a prominent dermal fringe (absent in *L. ocellatus*) and tibia-tarsal articulation reaching or extending over nostril (reaches anterior eye in *L. ocellatus*). *Liuxalus shiwandashan* sp. nov. differs from *L. romeri* by presence of a prominent dermal fringe (absent in *L. romeri*).

Distribution. This species is currently known only from the type locality.

The results of bioacoustic and osteological analyses, and the habitat were shown at [Figs 4, 5](#) and [6](#).

Table 2. Measurements (in mm) of *Liuxalus shiwandashan* sp. nov. and *Liuxalus jinxiuensis* sp. nov.

	<i>Liuxalus shiwandashan</i> sp. nov.										<i>Liuxalus jinxiuensis</i> sp. nov.					
	holotype					Paratypes					holotype			Paratype		
	Males					females					males			females		
	101052	101053	101055	101056	101061	101057	101058	101059	101050	101051	101054	200804109	200804107	200804108	200804110	101060
SVL	17.14	18.40	17.06	16.20	17.76	18.49	17.30	16.35	19.33	19.62	19.17	17.48	16.58	16.61	15.86	18.84
A-G	7.30	8.15	6.51	7.24	7.90	8.07	6.85	6.78	8.11	8.07	8.61	6.87	4.49	6.72	6.13	6.97
HL	6.78	7.51	7.00	7.63	7.96	7.85	7.36	7.51	7.11	6.92	7.07	5.81	6.41	6.61	6.28	6.63
HW	5.75	5.91	6.04	5.39	5.71	6.02	5.68	5.66	6.44	6.15	6.58	5.62	6.22	6.06	5.68	6.42
HD	3.41	4.82	3.92	3.77	3.99	4.10	4.62	3.71	3.58	3.96	4.50	3.31	3.16	4.11	3.39	4.03
UEW	1.13	1.05	1.25	1.06	1.42	1.26	1.38	1.20	1.44	1.30	1.35	1.09	1.27	1.14	1.22	1.34
IOD	3.37	4.01	3.82	3.34	3.64	4.07	4.67	3.88	4.38	3.67	4.79	2.62	3.17	2.85	2.78	3.30
ED	1.68	1.78	2.33	2.23	2.47	2.05	2.69	2.18	2.08	2.31	2.07	2.23	2.37	2.65	2.48	2.48
TD	1.40	1.09	1.20	1.00	1.93	1.21	1.21	1.34	1.41	1.27	1.27	0.90	0.96	1.09	0.70	1.09
ESL	2.51	3.49	3.03	2.76	2.96	2.88	3.07	2.35	3.04	3.15	2.83	2.81	2.93	2.67	2.76	3.55
TED	0.47	0.46	0.38	0.43	0.50	0.44	0.55	0.40	0.59	0.43	0.50	0.49	0.57	0.49	0.75	0.53
IND	2.06	1.92	2.00	1.78	1.91	1.86	2.02	1.75	2.25	2.31	2.14	2.21	2.19	2.03	2.27	2.71
END	1.28	1.52	1.09	1.37	1.25	1.25	1.24	1.48	1.36	1.52	1.58	1.29	1.43	1.34	1.25	1.47
FLL	8.38	7.38	7.62	6.93	7.35	8.51	6.87	7.53	9.48	8.73	8.05	7.34	7.13	7.29	7.15	7.82
FFL	1.00	1.32	1.16	1.07	1.25	1.13	1.22	1.19	1.77	1.36	1.17	1.50	1.55	1.06	1.31	1.26
TFL	2.93	2.81	2.86	2.81	3.47	3.26	2.98	2.68	3.34	3.09	3.44	1.77	1.70	1.69	1.89	1.89
FTD	0.64	0.60	0.64	0.45	0.49	0.51	0.58	0.63	0.54	0.53	0.72	0.51	0.72	0.57	0.40	0.70
Relative fingers length	1<2<4<3	1<2<4<3	1<2<4<3	1<2<4<3	1<2<4<3	1<2<4<3	1<2<4<3	1<2<4<3	1<2<4<3	1<2<4<3	1<2<4<3	1<2<4<3	1<2<4<3	1<2<4<3	1<2<4<3	1<2<4<3
Relative toes length	1<2<3 = 5<4	1<2<3 = 5<4	1<2<3 = 5<4	1<2<3 = 5<4	1<2<3 = 5<4	1<2<3 = 5<4	1<2<3 = 5<4	1<2<3 = 5<4	1<2<3 = 5<4	1<2<3 = 5<4	1<2<3 = 5<4	1<2<3 = 5<4	1<2<3 = 5<4	1<2<3 = 5<4	1<2<3 = 5<4	1<2<3 = 5<4

doi:10.1371/journal.pone.0136134.t002

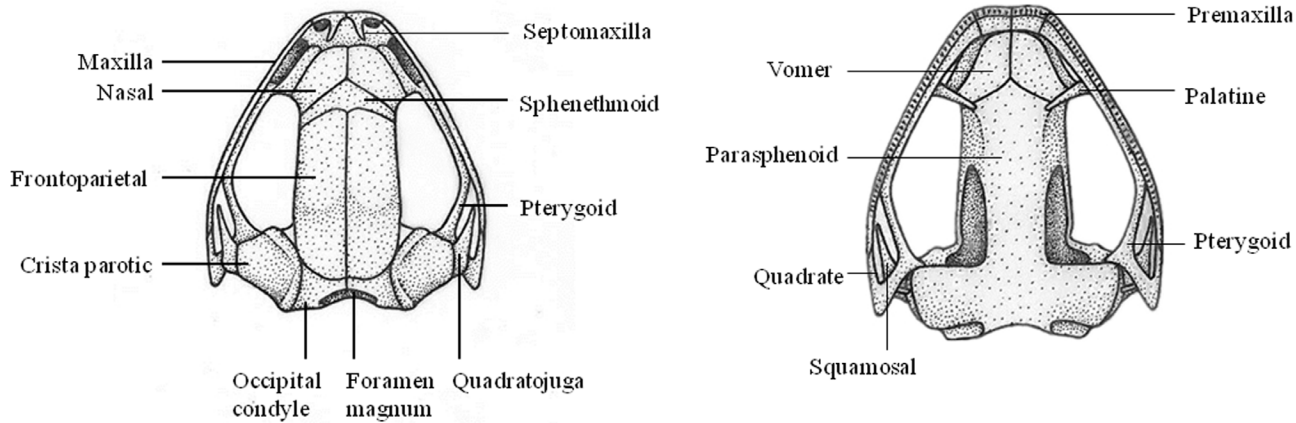


Fig 4. Skull of *Liuixalus shiwandashan* sp. nov. Left, dorsal view; right, ventral view.

doi:10.1371/journal.pone.0136134.g004

Liuixalus jinxiuensis sp. nov.

(Li, Mo, Jiang, Xie & Jiang)

urn:lsid:zoobank.org:act:A0E3794E-1963-4DF2-BEED-2EB33D107688

Holotype. GXNM200804109, an adult male (Figs 7, 8 and 9) from Mt. Dayao, Jinxiu, Guangxi, China (110°14.291'E 24°06.019'N 1163m), collected by Yunming MO and Shichu ZHOU, deposited in Guangxi Zhuang Autonomous Region Museum of Natural History.

Paratype. GXNM2000804107–108, GXNM2000804110, three adult males, collected together with the holotype; CIB101060, adult female from Mt. Dayao, Jinxiu, Guangxi, collected by Jia-Tang Li, deposited in Chengdu Institute of Biology, Chinese Academy of Sciences.

Etymology. The new species was named after the type locality, Jinxiu.

Diagnosis. *Liuixalus jinxiuensis* sp. nov. is assigned to the genus *Liuixalus* on the basis of the following characters: small body-size; head relatively broad; obtusely pointed snout; no vomerine teeth; tympanum distinct; the right and left heel obviously overlap with each other.

Liuixalus jinxiuensis sp. nov. is distinguished from all other species of *Liuixalus* by the following combination of morphological character: 1) large black plaque on the cephalic edge, under the eye (especially evident in living specimens); 2) sparse flat wart on the dorsal skin; 3) tibia-tarsal articulation reaching anterior eye; 4) light-colored nuptial pad present on the first and second fingers; and 5) internal single subgular vocal sac in males.

Description of the Holotype. Adult male (Figs 7, 8 and 9), body length 16.61 mm. Further measurement provided in Table 1. Body compact; head longer than wide (HL/HW = 1.09);

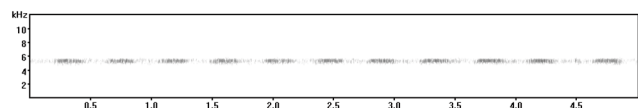


Fig 5. Spectrogram of calls series of *Liuixalus shiwandashan* sp. nov. The recording was high-pass filtered (above 5.5 kHz) to avoid high-frequency noise. Calls for figure spectrogram were created using 22005 Hz sampling frequency. Figure spectrogram was created using Hamming window, FFT-length 512 points, frame 100%, and overlap 75%.

doi:10.1371/journal.pone.0136134.g005



Fig 6. The habitat of *Liuxalus shiwandashan* sp. nov. in Mt. Shiwanda.

doi:10.1371/journal.pone.0136134.g006

snout slightly pointed in dorsal view; canthus rostral rounded and obvious, loreal region slightly concave; eye large, the horizontal of eye is less than eye to nostril distance; tympanum distinctly visible, rounded, less than half width of eye; vomerine teeth absent, tongue large and oval, distinctively notched posteriorly; skin on dorsum smooth with little and sparse tubercles; ventral skin non-uniformly verrucose.

Forelimb 7.29 mm, accounts for 43.89% of SVL. Finger slender, with strongly reduced interdigital web, no dermal fringe; relative finger length $I < II < IV < III$; finger discs rounded and expanded; subarticular tubercles indistinct; inner metatarsal tubercle elliptical and inconspicuous; outer metatarsal tubercle large but flat.

Hindlimbs relatively short, tibiotarsal articulation only reaching anterior eye when adpressed to the body; heels overlap when folded at right angle to body; relative toe length $I < II < III = V < IV$; rounded discs with ventral circummarginal groove; webs between toes rudimentary; dermal fringe missing; inner metatarsal tubercle flat and smaller than exterior metatarsal tubercle.

Coloration of holotype in life. Dorsal and lateral surface dark claybank to sepia, with sparse, dark, carbonarius (')-shaped tubercles; upper surface of forelimb same as dorsum with numerous dark-brown spots; ventral surface densely covered with hoary speckles and yellow asymmetrical spots mainly distributed on thorax-abdominal skin (Fig 9). Bilateral dark colored fusiform markings under eyes. Underjaw decorated with black stripe. Iris is celadon.



Fig 7. *Liuixalus jinxiuensis* sp. nov., dorso-lateral view. In life, from Jinxiu, Mt. Dayao.

doi:10.1371/journal.pone.0136134.g007

Coloration of holotype in preservative. Brightness reduces and tends to grayish-brown color. Bark tubercles fogged.

Variation and measurement of paratypes. Variation given in [Table 1](#). Female SVL (18.84 mm) apparently larger than male (15.86–17.48 mm; n = 4).

Secondary sexual characters. An internal single subgular vocal sac is present in males. A pair of slit-like vocal sac openings occurs near the corners of the mouth. Nuptial pad present on dorsal side of the first finger and inner side of the second finger.

Comparison. Compared with all other congeners, *Liuixalus jinxiuensis* sp. nov. differs from *L. hainanus* by the nuptial pad occurring on both 1st and 2nd finger of males (only on 1st finger for *L. hainanus*) and tibia-tarsal articulation reaching anterior eye (exceeds snout in *L. hainanus*). *Liuixalus jinxiuensis* sp. nov. differs from *L. calcarius* by the absence of dark color on throat vocal sac (present in *L. calcarius*). *Liuixalus jinxiuensis* sp. nov. differs from *L. ocellatus* by the bilateral dark colored fusiform markings lying under eyes (small black dollar-spot on occiput of *L. ocellatus*), small, sparse tubercles on dorsal skin (large, dense tubercles in *L.*



Fig 8. *Liuixalus jinxiuensis* sp. nov., dorsal view. In life, from Jinxiu, Mt. Dayao.

doi:10.1371/journal.pone.0136134.g008

ocellatus), and ventral skin not uniformly verrucose (well-proportioned fine point on ventral skin in *L. ocellatus*). *Liuixalus jinxiuensis* sp. nov. differs from *L. romeri* by its large black plaque on the cephalic edge, under the eye. *Liuixalus jinxiuensis* sp. nov. differs from *Liuixalus shiwandashan* sp. nov. by absence of dermal fringe.

Distribution. This species is currently known only from the type locality.

The result of bioacoustic analysis was shown at [Fig 10](#).

Discussion

We described *Liuixalus shiwandashan* sp. nov. and *Liuixalus jinxiuensis* sp. nov. using morphological, genealogical, bioacoustic and osteological analyses. Morphologically, both new species can be reliably distinguished from their congeners. Although the present genetic analyses



Fig 9. *Liuxalus jinxiuensis* sp. nov., ventral view. In life, from Jinxiu, Mt. Dayao.

doi:10.1371/journal.pone.0136134.g009

were based on only one mitochondrial gene, the genetic difference between the two new species was of a comparative magnitude as other known *Liuxalus* species. We treated them as separate species based on the “biological species concept” [27–28].

Our matrilineal genealogy of *Liuxalus* depicted relationships among members of the group based on comprehensive sampling. Although we resolved six matrilineal relationships, relationships *intra se* did not always enjoy strong support.

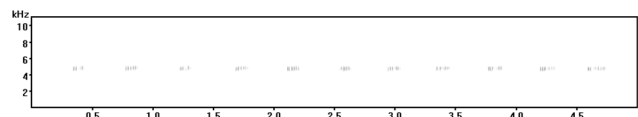


Fig 10. Spectrogram of calls series of *Liuxalus jinxiuensis* sp. nov. The recording was high-pass filtered (above 5.5 kHz) to avoid high-frequency noise. Calls for figure spectrogram were created using 22005 Hz sampling frequency. Figure spectrogram was created using Hamming window, FFT-length 512 points, frame 100%, and overlap 75%.

doi:10.1371/journal.pone.0136134.g010

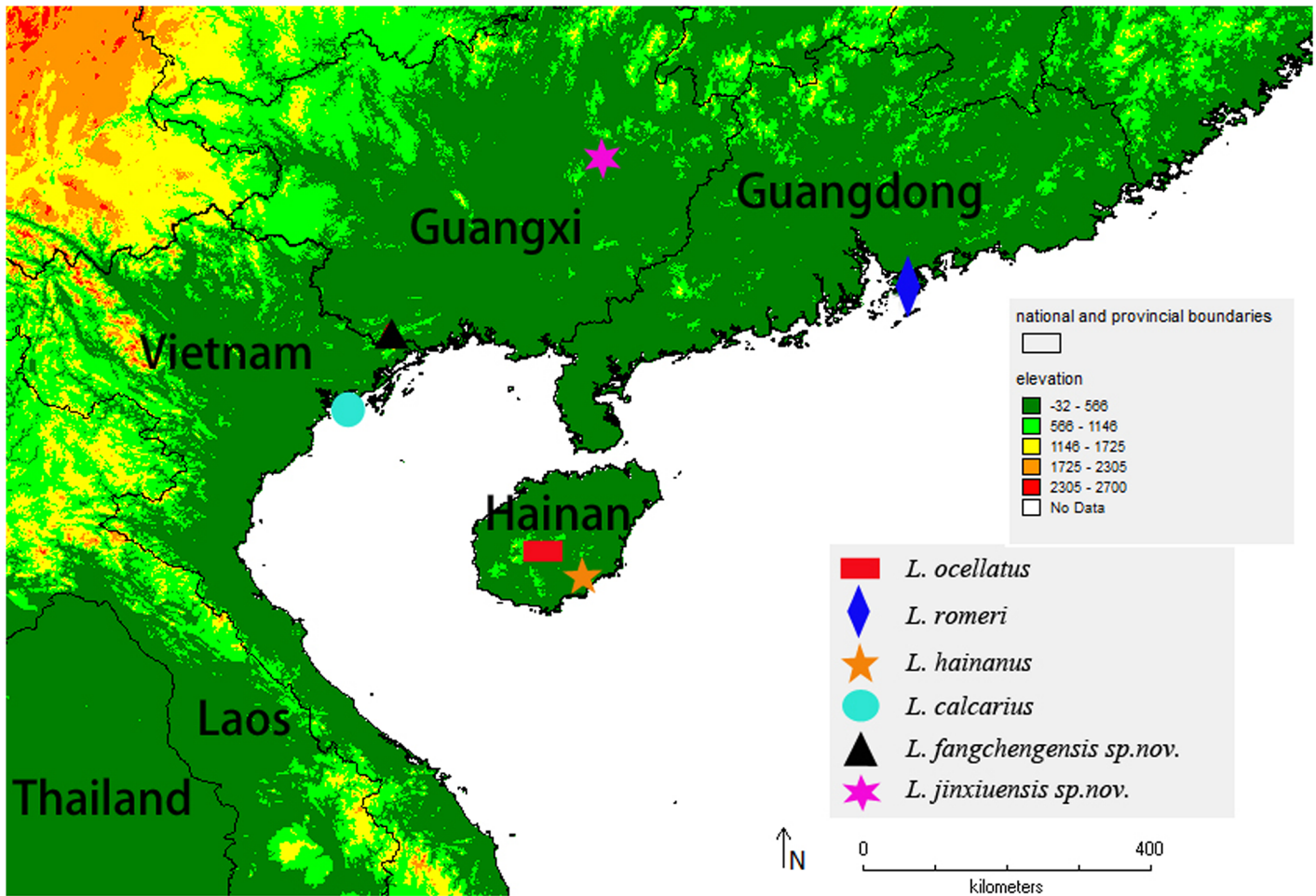


Fig 11. Collection sites of the species in this study.

doi:10.1371/journal.pone.0136134.g011

Lineage A—*L. ocellatus*: Liu *et al.* [7] investigated the amphibians of Hainan Island and described *Philautus ocellatus* as a new species based on morphological characters. At 2009, Fei *et al.* assigned it to *Aquixalus* in *Fauna Sinica*. Based on molecular investigation, Li *et al.* [1] suggested the genus *Aquixalus* as a junior synonym of *Kurixalus*. Later, Li *et al.* [2] assigned *P. ocellatus* into *Liuixalus* based on molecular analysis.

Lineage B—*Liuixalus jinxiuensis sp.nov.*: In this study, molecular analysis showed a distinct clade related to this species. We suggested it to be a new species using extensive sampling and various analyses. Description was given above.

Lineage C—*L. calcarius*: Milto *et al.*, 2013 and Nguyen *et al.*, 2014 described a new species of *Liuixalus* from Cat Ba Island, Vietnam named *L. calcarius* and *L. catbaensis*, respectively. We suggested that *L. catbaensis* was a junior synonym of *L. calcarius* by priority of date of publication, which was consistent with the suggestion of Frost (2014). Regardless, the occurrence of the species demarked the first record of *Liuixalus* outside China.

Lineage D—*L. hainanus*: Liu *et al.* (2004) described *Philautus hainanus* as a new species based on morphological evidence: tibiotarsal articulation over the tip of snout, 2~3 dark cross-bands on back of lower arm, beige ellipse speckle on the middle part of the body back in life, and yellow-white on abdomen. Then this species was then assigned to *Liuixalus* by Li *et al.* [2].

Lineage E—*Liuixalus shiwandashan* **sp. nov.**: Molecular analysis showed a distinct clade related to this species. Using extensive sampling and various analyses, we suggested it to be a new species. Description was given above.

Lineage F—*L. romeri*: *Liuixalus romeri* was firstly described as *Philautus romeri* by Smith in 1953 from Lamma Island, Hong Kong, China. Subsequently, this species was transferred among several genera [29–30][1]. Due to the presence of a tadpole, *Philautus romeri* was tentatively assigned to *Chirixalus* [29]. Wilkinson *et al.* [14] suggested that *P. romeri* may be a putative member of *Kurixalus*, pending further study of the type specimens and specimens in the field. Frost *et al.* [30] moved this species to the genus *Chiromantis*, pending resolution of its phylogenetic position. Li *et al.* [1] assigned it to *Liuixalus* based on both morphological and molecular evidence.

The localities of these species were marked in the map (Fig 11), and they were well separated geographically. The close relationships among *Liuixalus* suggested an ancient link between Hainan, Guangxi, Hong Kong and Vietnam. According to the geological evidence, the separation between Hainan Island and mainland China occurred at Early Cretaceous Epoch [31–32]. The separation accelerated the speciation within the genus *Liuixalus*.

Acknowledgments

We are grateful to Prof. Liang Fei and Changyuan Ye for her advice, Yueying Chen for helping to collect specimens. This study was supported by the National Natural Science Foundation of China (31372173, 31411130190, 31101618, 31471964, 31372174), Youth Innovation Promotion Association CAS, and grants from the Ministry of Science and Technology of China (MOST Grant 2011FY120200, Grant 2005DKA21402).

Author Contributions

Conceived and designed the experiments: JTL YW. Performed the experiments: SQ KJ BC. Analyzed the data: SQ KJ. Contributed reagents/materials/analysis tools: YM FX JJ. Wrote the paper: SQ JTL RWM.

References

1. Li JT, Che J, Bain RH, Zhao EM, Zhang YP (2008) Molecular phylogeny of Rhacophoridae (Anura): A framework of taxonomic reassignment of species within the genera *Aquixalus*, *Chiromantis*, *Rhacophorus*, and *Philautus*. *Mol Phylogenet Evol* 48: 302–312. doi: [10.1016/j.ympev.2008.03.023](https://doi.org/10.1016/j.ympev.2008.03.023) PMID: [18442928](https://pubmed.ncbi.nlm.nih.gov/18442928/)
2. Li JT, Che J, Murphy RW, Zhao H, Zhao EM, Rao DQ, et al. (2009) New insights to the molecular phylogenetics and generic assessment in the Rhacophoridae (Amphibia: Anura) based on five nuclear and three mitochondrial genes, with comments on the evolution of reproduction. *Mol Phylogenet Evol* 53: 509–522. doi: [10.1016/j.ympev.2009.06.023](https://doi.org/10.1016/j.ympev.2009.06.023) PMID: [19616637](https://pubmed.ncbi.nlm.nih.gov/19616637/)
3. Yu G, Zhang M, Yang J (2010). Generic allocation of Indian and Sri Lankan *Philautus* (Anura: Rhacophoridae) inferred from 12S and 16S rRNA genes. *Biochem Syst Ecol* 38: 402–409.
4. Pyron RA, Wiens JJ (2011) A large-scale phylogeny of Amphibia including over 2800 species, and a revised classification of advanced frogs, salamanders, and caecilians. *Mol Phylogenet Evol* 61: 543–583. doi: [10.1016/j.ympev.2011.06.012](https://doi.org/10.1016/j.ympev.2011.06.012) PMID: [21723399](https://pubmed.ncbi.nlm.nih.gov/21723399/)
5. Fei L, Hu SQ, Ye CY, Huang YZ (2009) *Fauna Sinica. Amphibia. Volume 2. Anura.* Beijing: Chinese Academy of Science. Science Press. (In Chinese)
6. Smith M (1953) Description of a new species of frog of the genus *Philautus*. *J Nat Hist* 6: 477–478.
7. Liu CC, Hu SQ, Fei L, Huang CC (1973) On collections of amphibians from Hainan Island. *Acta Zoologica Sinica* 19: 385–404. (In Chinese)
8. Liu SL, Wang LJ, Lü SQ, Zhao H, Che J, Wu GF. (2004) A new species of the genus *Philautus* (Anura: Rhacophoridae) and winter herpetological exploration in Hainan Province, China. *Sichuan J Zool* 123: 202–20. (In Chinese)

9. Milto KD, Poyarkov NA, Orlov NL, and Nguyen TT (2013) Two new rhacophorid frogs from Cat Ba Island, Gulf of Tonkin, Vietnam. *Russ J Herpetol* 20: 287–300.
10. Nguyen TT, Matsui M, Yoshikawa N (2014) First record of the tree-frog genus *Liuixalus* from Vietnam with the description of a new species (Amphibia: Rhacophoridae). *Curr Herpetol* 33: 29–37.
11. You Q, Deng DJ (2007) Research Advancement of Animal Diversity in Guangxi. *Guangxi For Sci* 3: 010. (In Chinese)
12. Chomczynski P, Sacchi N (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* 162: 156–159. PMID: [2440339](#)
13. Wilkinson JA, Drewes RC (2000). Character assessment, genus level boundaries, and phylogenetic analyses of the family Rhacophoridae: a review and present day status. *Cont Herpetol* 2000, 2.
14. Wilkinson JA, Drewes RC, Tatum OL (2002) A molecular phylogenetic analysis of the family Rhacophoridae with an emphasis on the Asian and African genera. *Mol Phylogenet Evol* 24: 265–273. PMID: [12144761](#)
15. Altschul SF, Madden TL, Schäffer AA, Zhang J, Zhang Z, Miller W, et al. (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res* 25: 3389–3402. PMID: [9254694](#)
16. Jeanmougin F, Thompson JD, Gouy M, Higgins DG, Gibson TJ (1998) Multiple sequence alignment with Clustal X. *Trends Biochem Sci* 23: 403–405. PMID: [9810230](#)
17. Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG (1997) The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res* 25: 4876–4882. PMID: [9396791](#)
18. Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigan PA, McWilliam H, et al. (2007) Clustal W and Clustal X version 2.0. *J Bioinform* 23: 2947–2948.
19. Ronquist FR, Huelsenbeck JP (2003) MrBayes 3: Bayesian phylogenetic inference under mixed models. *J Bioinform* 19: 1572–1574.
20. Wassersug RJ (1976) A procedure for differential staining of cartilage and bone in whole formalin-fixed vertebrates. *Biotech Histochem* 51: 131–134.
21. Ponsa ML, Heyer WR (2007). Osteological characterization of four putative species of the genus *Adeomera* (Anura: Leptodactylidae), with comments on intra-and interspecific variation. *Zootaxa* 1403: 37–54.
22. Trueb L (1973) Bones, frogs and evolution. In: Vial JL, ed. *Evolutionary biology of the anurans: contemporary research on major problems*. Missouri Press: 65–132.
23. Trueb L (1993) Patterns of cranial diversity among the hissamphibia. In: Hanken J, Hall BK eds. *The skull*. The University of Chicago Press: 255–343.
24. Heyer WR (1975) A preliminary analysis of the intergeneric relationships of the frogs family Leptodactylidae. *Smithson Contrib Zool* 199: 1–55.
25. Lynch JD (1971) Evolutionary relationships, osteology, and zoogeography of leptodactyloid frogs. *Misc Publ Mus Nat Hist Univ Kansas* 53: 1–238.
26. Trueb L, Pu' gener LA, Maglia AM (2000) Ontogeny of the bizarre: an osteological description of *Pipa pipa* (Anura, pipidae), with an account of skeletal development in the species. *J Morphol* 243: 75–104. PMID: [10629097](#)
27. Donoghue MJ (1985). A critique of the biological species concept and recommendations for a phylogenetic alternative. *Bryologist* 88: 172–181.
28. Sokal RR, Crovello TJ (1970). The biological species concept: a critical evaluation. *Am Nat* 104: 127–153.
29. Bossuyt F, Dubois A (2001) A review of the frog genus *Philautus* Gistel, 1848 (Amphibia, Anura, Ranidae, Rhacophorinae). *Zeylanica Colombo* 6: 1–112
30. Frost DR, Grant T, Faivovich J, Bain RH, Haas A, Haddad CFB, et al. (2006) The amphibian tree of life. *Bull Am Mus Nat Hist* 297: 1–370
31. Menzies MA, Fan W, Zhang M (1993) Palaeozoic and Cenozoic lithoprobes and the loss of > 120 km of Archaean lithosphere, Sino-Korean craton, China. *Geol Soc Spec Publ* 76: 71–81.
32. Metcalfe I (1996) Gondwanaland dispersion, Asian accretion and evolution of eastern Tethys. *AGSO J Aust Geol Geophys* 43: 605–623.