A new species of the Asian music frog genus *Nidirana* (Amphibia, Anura, Ranidae) from Southwestern China

Shize Li[1,2], Gang Wei[3], Ning Xu[1], Jianguo Cui[2], Liang Fei[2], Jianping Jiang[2], Jing Liu[1] and Bin Wang[2]

[1] Department of Food Science and Engineering, Maotai University, Renhuai, China
[2] CAS Key Laboratory of Mountain Ecological Restoration and Bioresource Utilization & Ecological Restoration Biodiversity Conservation Key Laboratory of Sichuan Province, Chengdu Institute of Biology, Chinese Academy of Sciences, Chengdu, China
[3] Biodiversity Conservation Key Laboratory, Guiyang College, Guiyang, China

ABSTRACT

The Asian music frog genus *Nidirana* is widely distributed in East and Southeastern Asia. Systematic profiles of the group remain on debate, and cryptic species are expected especially in the species with wide distributional range. Here, we describe a new species of the genus from Southwestern China. Phylogenetic analyses based on mitochondrial DNA and nuclear DNA supported the new species as an independent clade nested into the *Nidirana* clade and sister to *N. hainanensis*. Morphologically, the new species could be distinguished from its congeners by a combination of the following characters: a large body size in males (SVL > 49 mm); the presence of lateroventral grooves both on fingers and toes; relative finger lengths: II < IV < I < III; tibiotarsal articulation reaching the level between eye and nostril when leg stretched forward; a pair of subgular internal vocal sacs at corners of throat in males; nuptial pad present on the inner side of base of fingers I and II in males in breading season; webbing formula: I 2 –$2^{1/3}$ II 2 –$2^{2/3}$ III $3^{1/2}$ –$3^{2/3}$ IV $3^{2/3}$ –3V. The findings provided a better knowledge on phylogenetic assignments of the genus *Nidirana*, and indicated future deeper investigations necessarily for exploring systematic settings of the group.

INTRODUCTION

The Asian music frog genus *Nidirana Dubois, 1992* is widely distributed in East and Southeast Asia, from Japan, westwards to southern China, southwards to northern Thailand and northern Vietnam and Laos (*Frost, 2019*). Systematic assignments of the genus have been much controversial. *Thompson (1912)* established the genus *Babina*, separating from the genus *Rana Linnaeus, 1758*, and transferred two species (*Rana holsti Boulenger, 1892* and *Rana subaspera Barbour, 1908* to the new genus. *Dubois (1992)* established the subgenus *Nidirana* under the genus *Rana*, which contained six species: *Rana psaltes Kuramoto, 1985*, *Rana adenopleura Boulenger, 1909*, *Rana caldwelli Schmidt, 1925*, *Rana chapaensis Bourret, 1937*, *Rana daunchina Chang, 1933* and *Rana pleuraden Boulenger, 1904*. However, "*Rana psaltes*" was synonymized with "*R. okinavana*" (*Matsui, 2007*), and "*Rana caldwelli*"

Corresponding author
Bin Wang, wangbin@cib.ac.cn

was considered as the subjective junior synonym of "*R. adenopleura*" (*Lyu et al., 2017*). Although *Frost et al. (2006)* merged the "*Nidirana*" group into the genus *Babina*, most systematic studies suggested that "*Nidirana*" group was distinct from the genus *Babina* seusu stricto (e.g., *Chen et al., 2005*; *Chuaynkern et al., 2010*; *Fei et al., 2009*; *Fei, Ye & Jiang, 2010*; *Fei, Ye & Jiang, 2012*). As note, all the previous studies did not include all species of *Babina* seusu lato. Therefore, *Lyu et al. (2017)* performed a most-species-comprehensive analysis based on molecular phylogenetics, morphology and bioacoustics of *Babina* seusu lato, and confirmed that *Nidirana* was a distinct genus containing eight species: *Nidirana okinavana* (*Boettger, 1895*), *Nidirana adenopleura* (*Boulenger, 1909*), *Nidirana hainanensis* (*Fei, Ye & Jiang, 2007*), *Nidirana chapaensis* (*Boulenger, 1909*), *Nidirana daunchina* (*Chang, 1933*), *Nidirana lini* (*Chou, 1999*), *Nidirana pleuraden* (*Boulenger, 1904*) and *Nidirana nankunensis Lyu et al., 2017*.

In the genus *Nidirana*, *N. adenopleura* was reported to have the widest distributional range across the Iriomote and Ishigaki Isands and Yaeyama group of Ryukyu Island in Japan and Taiwan, Guizhou, Anhui, Zhejiang, Jiangxi, Hunan, Fujian, Guangdong and Guangxi provinces of China (*Frost, 2019*; *Fei et al., 2009*; *Fei, Ye & Jiang, 2012*). The species is heavily dependent on the still water environment (*Fei et al., 2009*). It is expected that the wide distributional range and habitat requirements of the species might promote population divergence even speciation in it. Two new species were described in the populations of *N. adenopleura*: *N. hainanensis* found by *Fei, Ye & Jiang (2007)* from Diaoluo Mountain of Hainan Island and *N. nankunensis* found by *Lyu et al. (2017)* from Longmen County, Guangdong Province, China. Noticeably, neither of the two species were found to be the sister clade of *N. adenopleura* (*Lyu et al., 2017*). Even so, distinct populations of *N. adenopleura* were still noted to encompass differences on morphology, breeding habit and calls (*Fei et al., 2009*). For instance, *Wu, Dong & Xu (1986)* found that there were obvious morphological differences between populations from Leigong Mountain and Fanjing Mountain of Guizhou Province and other populations of *N. adenopleura* in China. This indicated that the populations probably represented cryptic species, and thus deeper investigations especially with molecular phylogenetic methods were necessary to evaluate the taxonomic status of them.

In recent years, we carried out a series of biodiversity surveys in Leigong Mountain, Leishan County, Guizhou Province, China, and collected some specimens superficially resembling *N. adenopleura* on morphology. Phylogenetic analyses based on mitochondrial DNA and nuclear DNA, morphological comparisons and bioacoustics comparisons consistently indicated these specimens as a new taxon of *Nidirana*. Herein we describe it as a new species.

METHODS

Sampling

Four adult females and six adult males of the new taxon of *Nidirana* were collected from Leigong Mountain, Leishan County, Guizhou Province, China between June 2015 and July 2017 (for voucher information see Table S1; Fig. 1). Seven tadpoles of the new taxon
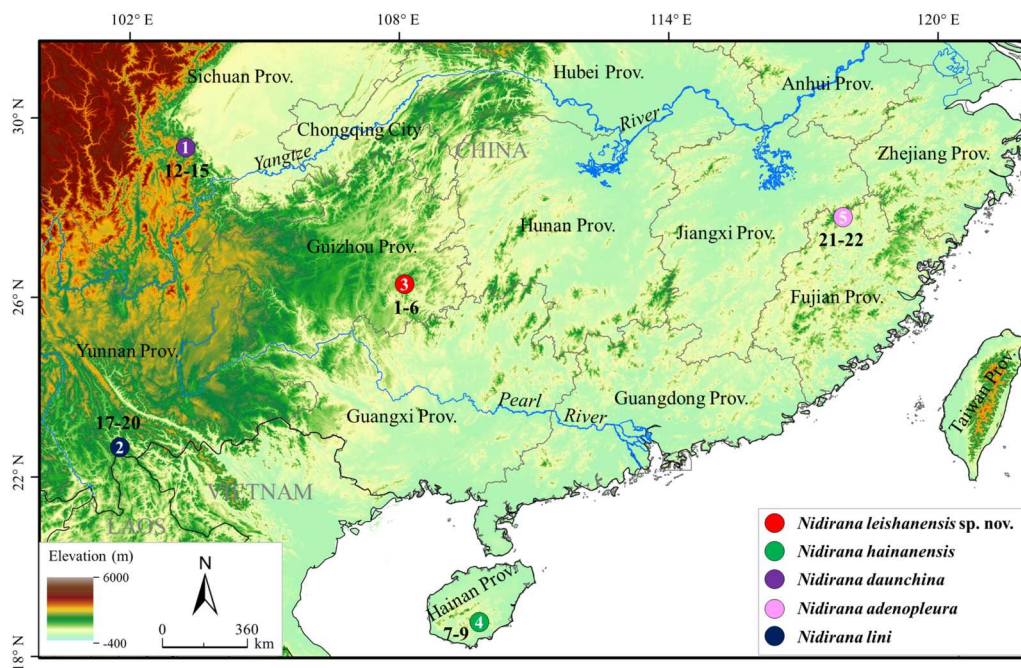
Figure 1 **Sampling localities in this study.** Localities 1–5 were all in China: 1, Emei Mountain, Sichuan Province (Prov.); 2, Jiangcheng County (Co.), Yunnan Prov.; 3, Leigong Mountain, Leishan Co., Guizhou Prov.; 4, Diaoluo Mountain, Lingshui Co., Hainan Prov.; 5, Wuyi Mountain, Fujian Prov. Different species were denoted as different colors. Sample numbers (for information see Table 1) were labeled beside localities.

(for voucher information see Table S2) were also collected at the same site as the adult specimens on 26 August 2018. For tadpole matching, we compare the genetic divergence between the collected tadpoles and adult specimens. The stages of tadpoles were identified following *Gosner (1960)*. After taking photographs, they were euthanized using isoflurane, and then the specimens were fixed in 10% buffered formalin. Tissue samples were taken and preserved separately in 95% ethanol prior to fixation (*Li et al., 2018*). Specimens were deposited in Chengdu Institute of Biology, Chinese Academy of Sciences (CIB, CAS).

Morphological analysis

The terminology and methods were followed *Fei & Ye (2005)*. Measurements were taken with a dial caliper to the nearest 0.1 mm. Seventeen morphometric characters of adult specimens were measured: snout-vent length (SVL; distance from the tip of the snout to the posterior edge of the vent), head length (HDL; distance from the tip of the snout to the articulation of jaw), head width (HDW; greatest width between the left and right articulations of jaw), snout length (SL; distance from the tip of the snout to the anterior corner of the eye), eye diameter (ED; distance from the anterior corner to the posterior corner of the eye), interorbital distance (IOD; minimum distance between the inner edges of the upper eyelids), internasal distance (IND; minimum distance between the inner margins of the external nares), upper eyelid width (UEW; greatest width of the upper eyelid margins measured perpendicular to the anterior-posterior axis), maximal

Li et al. (2019), *PeerJ*, DOI 10.7717/peerj.7157

Table 1 Information of molecular samples used in the phylogenetic analyses in this study. # sequence was downloaded from GenBank.

Sample no.	Species	Locality	Voucher number	GenBank accession number				
				12S	16S	COI	Tyr1	CXCR4
1	*Nidirana leishanensis* sp. nov.	*Leigong Mountain, Leishan County, Guizhou Province, China	CIBLS20150627003	MK293792	MK293810	MK293828	MK293859	MK293845
2	*Nidirana leishanensis* sp. nov.	*Leigong Mountain, Leishan County, Guizhou Province, China	CIBLS20150628007	MK293793	MK293811	MK293829	MK293860	MK293846
3	*Nidirana leishanensis* sp. nov.	*Leigong Mountain, Leishan County, Guizhou Province, China	CIBLS20150628002	MK293794	MK293812	MK293830	MK293861	MK293847
4	*Nidirana leishanensis* sp. nov.	*Leigong Mountain, Leishan County, Guizhou Province, China	CIBLS20150628005	MK293795	MK293813	MK293831	MK293862	MK293848
5	*Nidirana leishanensis* sp. nov.	*Leigong Mountain, Leishan County, Guizhou Province, China	CIBLS20180628001	MK293796	MK293814	MK293832	MK293863	MK293849
6	*Nidirana leishanensis* sp. nov.	*Leigong Mountain, Leishan County, Guizhou Province, China	CIBLS20180628002	MK293797	MK293815	MK293833	MK293864	MK293850
7	*Nidirana hainanensis*	*Diaoluo Mountain, Lingshui County, Hainan Province, China	CIB20110629003	MK293789	MK293807	MK293825	/	MK293843
8	*Nidirana hainanensis*	*Diaoluo Mountain, Lingshui County, Hainan Province, China	CIB20110629004	MK293790	MK293808	MK293826	/	/
9	*Nidirana hainanensis*	*Diaoluo Mountain, Lingshui County, Hainan Province, China	CIB20110629005	MK293791	MK293809	MK293827	MK293858	MK293844
10	*Nidirana hainanensis*	*Diaoluo Mountain, Lingshui County, Hainan Province, China	SYS a003741	# MF807899	# MF807821	# MF807860	/	/

Li et al. (2019), *PeerJ*, DOI 10.7717/peerj.7157

Table 1 (*continued*)

Sample no.	Species	Locality	Voucher number	GenBank accession number				
				12S	16S	COI	Tyr1	CXCR4
11	Nidirana daunchina	*Emei Mountain, Sichuan Province, China	SYS a004594	# MF807900	# MF807822	# MF807861	/	/
12	Nidirana daunchina	*Emei Mountain, Sichuan Province, China	CIB2011081601	MK293801	MK293819	MK293837	MK293868	/
13	Nidirana daunchina	*Emei Mountain, Sichuan Province, China	CIB2011081602	MK293802	MK293820	MK293838	MK293869	/
14	Nidirana daunchina	*Emei Mountain, Sichuan Province, China	CIB2011081603	MK293803	MK293821	MK293839	MK293870	MK293854
15	Nidirana daunchina	*Emei Mountain, Sichuan Province, China	CIB20110629001	MK293804	MK293822	MK293840	MK293871	MK293855
16	Nidirana chapaensis	*Sa pa, Lao Cai, Vietnam	T2483/2000.4850	/	# KR827711	# KR087625	/	/
17	Nidirana lini	*Jiangcheng County, Yunnan Province, China	SYS a003967	# MF807896	# MF807818	# MF807857	/	/
18	Nidirana lini	*Jiangcheng County, Yunnan Province, China	CIB20110629011	MK293798	MK293816	MK293834	MK293865	MK293851
19	Nidirana lini	*Jiangcheng County, Yunnan Province, China	CIB20110629010	MK293799	MK293817	MK293835	MK293866	MK293852
20	Nidirana lini	*Jiangcheng County, Yunnan Province, China	CIB20110629008	MK293800	MK293818	MK293836	MK293867	MK293853
21	Nidirana adenopleura	Wuyi Mountain, Fujian Province, China	CIB20180827001	MK293787	MK293805	MK293823	MK293856	MK293841
22	Nidirana adenopleura	Wuyi Mountain, Fujian Province, China	CIB20180827002	MK293788	MK293806	MK293824	MK293857	MK293842
23	Nidirana adenopleura	Yanping District, Nanping City, Fujian Province, China	SYS a005911	# MF807922	# MF807844	# MF807883	/	/
24	Nidirana adenopleura	New Taipei City, Taiwan Province, China	UMMZ 189963	# DQ283117	# DQ283117	/	/	/
25	Nidirana okinavana	*Okinawa, Japan	/	# AB761266	# AB761266	# AB761266	/	/

Table 1 (*continued*)

Sample no.	Species	Locality	Voucher number	GenBank accession number				
				12S	**16S**	**COI**	**Tyr1**	**CXCR4**
26	*Nidirana nankunensis*	*Nankun Mountain, Guangdong Province, China	SYS a003618	# MF807906	# MF807828	# MF807867	/	/
27	*Nidirana pleuraden*	Gaoligong Mountain, Yunnan Province, China	SYS a003775	# MF807894	# MF807816	# MF807855	/	/
28	*Babina subaspera*	*Amami Island, Kagoshima, Japan	/	# AB761265	# AB761265	# AB761265	/	/
29	*Babina holsti*	*Okinawa, Japan	/	# AB761264	# AB761264	# AB761264	/	/
30	*Amolops mantzorum*	Xiling Mountain, Sichuan Province, China	/	# NC024180	# NC024180	# NC024180	/	/
31	*Odorrana margaretae*	China	HNNU1207003	# NC024603	# NC024603	# NC024603	/	/
32	*Nidirana daunchina*	Sichuan Province, China	CIB-WU37990	/	/	/	# DQ360041	/
33	*Nidirana chapaensis*	Xieng Khouang Province, Laos, Vietnam	FMNH 256531	/	/	/	# EU076792	/
34	*Nidirana chapaensis*	Xieng Khouang Province, Laos, Vietnam	FMNH 256531	/	/	/	/	# KR264317
35	*Nidirana pleuraden*	Yunnan Province, China	SCUM0405185CJ	/	/	/	# DQ360042	/

Notes.
* denotes the locality being the type locality of the species.

tympanum diameter (TYD), length of lower arm and hand (LAL; distance from the elbow to the distal end of the Finger IV), lower arm width (LW; maximum width of the lower arm), hand length (HAL; distance from the posterior end of the inner metacarpal tubercle to the distal tip of Finger IV), hindlimb length (HLL; maximum length from the vent to the distal tip of the Toe IV), tibia length (TL; distance from knee to tarsus), maximal tibia width (TW), length of foot and tarsus (TFL; distance from the tibiotarsal articulation to the distal end of the toe IV) and foot length (FL; distance from tarsus to the tip of the fourth toe). Ten morphometric characters of tadpoles were measured: total length (TOL), SVL, maximum body height (BH), maximum body width (BW), SL, snout to spiraculum (SS; distance from spiraculum to the tip of the snout), mouth width (MW; distance between two corners of mouth), maximum width of tail base (TBW), tail length (TAL; distance from base of vent to the tip of tail) and tail height (TAH; maximum height between upper and lower edges of tail).

Molecular data and phylogenetic analyses

Three male specimens, one female specimen and two tadpoles of the new taxon were included in the molecular analyses (for voucher information see Table 1). For phylogenetic analyses, three samples of *N. hainanensis* from Diaoluo Mountain, Hainan Province, China (type locality of the species), four samples of *N. daunchina* from Emei Mountain, Sichuan Province, China (type locality of the species), three samples of *N. lini* from Jiangcheng County, Yunnan Province, China (type locality of the species) and two samples of *N. adenopleura* from Wuyi Mountain, Fujian Province, China were also sequenced (for voucher information see Table 1; Fig. 1).

Total DNA was extracted using a standard phenol-chloroform extraction protocol (*Sambrook, Fritsch & Maniatis, 1989*). Three fragments of the mitochondrial genes 12S rRNA,16S rRNA and cytochrome oxidase subunit I (COI) were amplified using the primers in *Kocher et al. (1989)*, *Simon et al. (1994)* and *Che et al. (2012)*, respectively. They were amplified under the following conditions: an initial denaturing step at 95 °C for 4 min; 36 cycles of denaturing at 95 °C for 30 s, annealing at 52 °C (for 12S and 16S)/47 °C (for COI) for 40 s and extending at 72 °C for 70 s, and a final extending step of 72 °C for 10 min. The nuclear gene fragments C-X-C chemokine receptor type 4 (CXCR4) and Tyrosinase exon 1 (Tyr1) were amplified using the primers and protocols in *Biju & Bossuyt (2003)* and *Bossuyt & Milinkovitch (2000)*, respectively. All primers were presented in the Table S3. PCR products were purified with spin columns and then were sequenced with both forward and reverse primers same as used in PCR. Sequencing was conducted using an ABI3730 automated DNA sequencer in Shanghai DNA BioTechnologies Co., Ltd. (Shanghai, China). All sequences were deposited in GenBank (for GenBank accession numbers see Table 1).

For phylogenetic analyses, the available sequence data for all related species of the genus *Nidirana* were downloaded from GenBank (for GenBank accession numbers of all sequences see Table 1). Corresponding sequences of one *Amolops mantzorum* and one *Odorrana margaretae* were used as outgroups according to *Lyu et al. (2017)*.

Sequences were assembled and aligned using the Clustalw module in BioEdit v. 7.0.9.0 (*Hall, 1999*) with default settings. Alignments were checked by eye and revised manually if necessary. To avoid bias in alignments, GBLOCKS v. 0.91.b (*Castresana, 2000*) with default settings was used to extract regions of defined sequence conservation from the length-variable 12S and 16S gene fragments. Non-sequenced fragments were defined as missing loci. Finally, for phylogenetic analyses, the dataset concatenated with 12S (for 30 samples) + 16S (for 31 samples) + COI (for 30 samples) + Tyr1 (for 16 samples) + CXCR4 (for 15 samples) gene sequences.

Gene trees were reconstructed for the mitochondrial gene concatenated dataset and the concatenated dataset of all five genes, respectively. Phylogenetic analyses were conducted using maximum likelihood (ML) and Bayesian Inference (BI) methods, implemented in PhyML v. 3.0 (*Guindon et al., 2010*) and MrBayes v. 3.12 (*Ronquist & Huelsenbeck, 2003*), respectively. To avoid under- or over-parameterization (*Lemmon & Moriarty, 2004*; *McGuire et al., 2007*), the best partition scheme and the best evolutionary model for each partition were chosen for the phylogenetic analyses using PARTITIONFINDER v. 1.1.1 (*Robert et al., 2012*). In this kind of analyses, 12S, 16S genes and each codon position of each protein-coding gene were defined and Bayesian Inference Criteria was used. ML and BI analyses were based on the PARTITIONFINDER results (Table S4). For the ML tree, branch supports were drawn from 10,000 nonparametric bootstrap replicates. In BI analyses, the parameters for each partition were unlinked, and branch lengths were allowed to vary proportionately across partitions. Two runs each with four Markov chains were simultaneously run for 50 million generations with sampling every 1,000 generations. The first 25% trees were removed as the "burn-in" stage followed by calculations of Bayesian posterior probabilities and the 50% majority-rule consensus of the post burn-in trees sampled at stationarity.

On each nuclear gene, there was no enough number of variable sites for reconstructing reliable phylogenetic relationships between *Nidirana* species. Therefore, we just examine haplotype relationships of the new taxon and its related species based on Tyr1 and CXCR4 gene datasets, respectively, by constructing haplotype networks using the maximum parsimony method in TCS v. 1.21 (*Clement, Posada & Crandall, 2000*). Genetic distance between *Nidirana* species based on uncorrected *p*- distance model was estimated on COI, Tyr1 and CXCR4 genes, respectively, using MEGA v. 6.06 (*Tamura et al., 2011*).

Morphological comparisons

All ten adult specimens (for voucher information see Table S1) and seven tadpole specimens (for voucher information see Table S2) of the new taxon were measured. For comparisons, measurements of the holotype and three paratypes of *N. hainanensis* (for voucher information see Table S1) were retrieved from *Fei, Ye & Jiang (2007)*.

In order to reduce the impact of allometry, the correct value from the ratio of each character to SVL was calculated and then was log-transformed for the following morphometric analyses. One-way analysis of variance (ANOVA) was used to test the significance of differences on morphometric characters between males and females and between different species. The significance level was set at 0.05. To show the spatial

distribution of each species on the morphometric characters, principal component analyses (PCA) were performed. These analyses were carried out in R (*R Development Core Team, 2008*).

The new species was also morphologically compared with all other *Nidirana* species. Comparative morphological data were obtained from literature for *N. adenopleura* (*Boulenger, 1909*), *N. chapaensis* (*Bourret, 1937*), *N. daunchina* (*Chang & Hsu, 1932*), *N. hainanensis* (*Fei, Ye & Jiang, 2007*), *N. lini* (*Chou, 1999*), *N. nankunensis* (*Lyu et al., 2017*), *N. okinavana* (*Boettger, 1895*) and *N. pleuraden* (*Boulenger, 1904*).

Bioacoustics analyses

The advertisement call of the new species from Leigong Mountain, Leishan County, Guizhou Province, China was recorded from the specimen CIBLS20170727002 at ambient air temperature of 23 °C and air humidity of 87% in the ridge of paddy field on 27 June 2017. For comparisons, the advertisement call of *N. hainanensis* from Diaoluo Mountain, Lingshui County, Hainan Province, China were recorded from the specimen CIB20110629003 at ambient air temperature of 24 °C and air humidity of 85% in the ridge of paddy field on 10 May 2012. SONY PCM-D50 digital sound recorder was used to record within 20 cm of the calling individual. The sound files in wave format were resampled at 48 kHz with sampling depth 24 bits. Calls were recoded and examined as described by *Wijayathilaka & Meegaskumbura (2016)*. Call recordings were visualized and edited with SoundRuler 0.9.6.0 (Gridi-Papp 2003–2007) and Raven Pro 1.5 software (Cornell Laboratory of Ornithology, Ithaca, NY, USA). Ambient temperature of the type locality was taken by a digital hygrothermograph.

Skull scanning

Skulls of three male specimens (voucher numbers: CIBLS20170727002, CIBLS20150628001 and CIBLS20150628002) and two female specimens (voucher numbers: CIBLS20150627001 and CIBLS20150627002) of the new taxon were scanned in the high-resolution X-ray scanner (Quantum GX micro-CT Imaging System; PerkinElmer, Boston, MA, USA). The specimens were scanned along the coronal axis at an image resolution of 2,000 × 2,000. Segmentation and three-dimensional reconstruction of the CT images were made using VG57 Studio Max 2.2 (Volume Graphics, Heidelberg, Germany). Raw data of skull from CT scanning were deposited in the database figshare with doi: 10.6084/m9.figshare.7980158.

The Animal Care and Use Committee of Chengdu Institute of Biology, CAS provided full approval for this purely observational research (Number: CIB2011032201). Field work was approved by the Management Office of the Leigong Mountain National Nature Reserve (project number: LGS-201304006).

The electronic version of this article in Portable Document Format (PDF) will represent a published work according to the International Commission on Zoological Nomenclature (ICZN), and hence the new names contained in the electronic version are effectively published under that Code from the electronic edition alone. 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
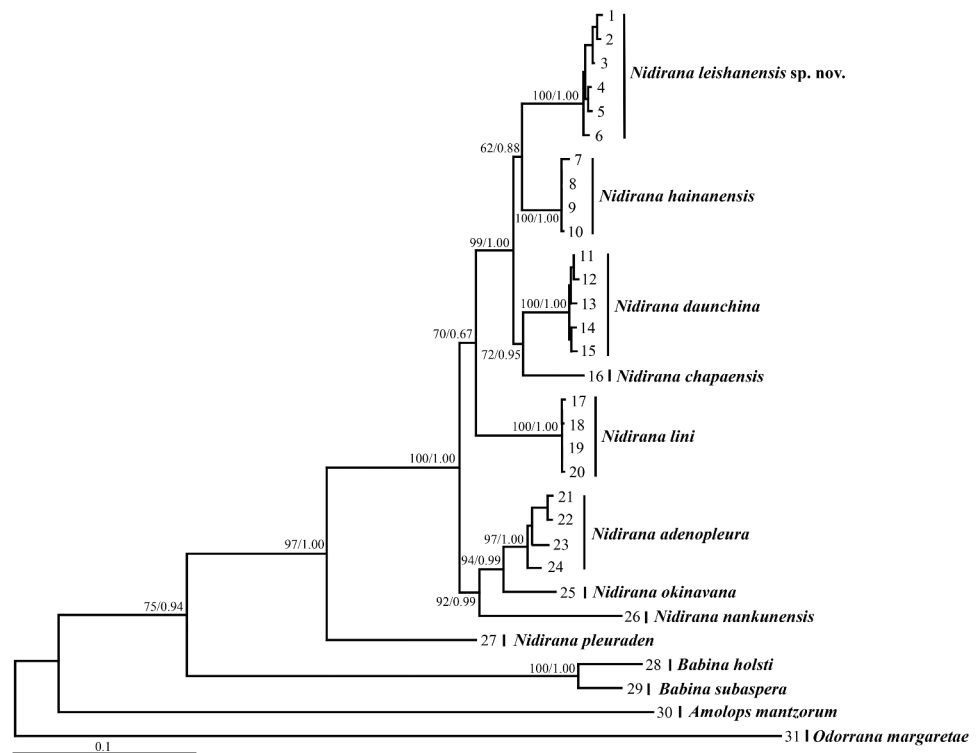
Figure 2 Maximum likelihood (ML) tree of *Nidirana leishanensis* sp. nov. and the related species reconstructed based on the concatenated sequences of the mitochondrial 12S, 16S and COI genes and the nuclear Tyr1 and CXCR4 genes. Bootstrap supports from ML analyses/Bayesian posterior probabilities from BI analyses were labeled beside nodes. See information of samples 1–31 in Table 1.

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:08105A58-4826-475D-BF07-A0C5C16D6BDD. The online version of this work is archived and available from the following digital repositories: PeerJ, PubMed Central and CLOCKSS.

RESULTS

Phylogenetic analyses and genetic divergence

Aligned sequence matrix of 12S + 16S + COI, Tyr1and CXCR4 genes contained 1,488 bp, 519 bp and 642 bp, respectively. ML and BI analyses based on the 12S + 16S + COI matrix and all-five-genes matrix resulted in essentially identical topologies (Fig. 2 and Fig. S1). All samples of the new taxon occurring from Leishan Mountain of Guizhou Province, China were strongly clustered into a monophyly, which was nested into the *Nidirana* clade and sister to the *N. hainanensis* clade. Two haplotypes were found for all samples of the new taxon in Tyr1 gene and six haplotypes in CXCR4 gene, respectively, and there was no common haplotype between the new taxon and its related species (Fig. 3). The genetic distance base on COI gene between the new taxon and its sister species *N. hainanensis*
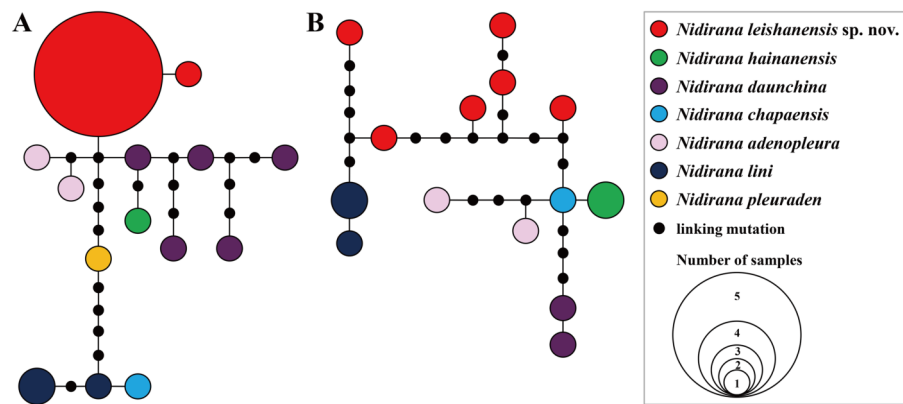
Figure 3 Haplotype networks of *Nidirana leishanensis* sp. nov. and its related species constructed based on the nuclear gene sequences. (A) Tyr1 gene. (B) CXCR4 gene. Different species were denoted as different colors.

Table 2 Genetic distance with uncorrected *p*-distance model between the *Nidirana* and *Babina* species based on the COI gene sequences.

Species	1	2	3	4	5	6	7	8	9	10
1. *Nidirana leishanensis* sp. nov.										
2. *Nidirana hainanensis*	0.057									
3. *Nidirana daunchina*	0.063	0.054								
4. *Nidirana chapaensis*	0.057	0.043	0.048							
5. *Nidirana lini*	0.110	0.095	0.092	0.085						
6. *Nidirana adenopleura*	0.105	0.092	0.095	0.087	0.102					
7. *Nidirana okinavana*	0.107	0.095	0.095	0.085	0.103	0.049				
8. *Nidirana nankunensis*	0.098	0.105	0.109	0.096	0.093	0.075	0.071			
9. *Nidirana pleuraden*	0.133	0.118	0.112	0.110	0.114	0.113	0.114	0.125		
10. *Babina subaspera*	0.186	0.180	0.179	0.185	0.157	0.166	0.173	0.165	0.162	
11. *Babina holsti*	0.190	0.177	0.176	0.185	0.164	0.163	0.169	0.169	0.167	0.043

was mean 0.057 (range 0.055–0.060; Table 2) higher than the interspecific genetic distance between other sister species, i.e., that between *N. hainanensis* and *N. daunchina* (0.054), *N. daunchina* and *N. chapaensis* (0.048) and *N. adenopleura* and *N. okinavana* (0.049). The mean genetic distance on Tyr1 gene between the new taxon and its congeners ranged from 0.009 to 0.024, and that on CXCR4 gene ranged from 0.005 to 0.009.

Morphological comparisons

The results of one-way ANOVA showed that in the new taxon, the male group was significantly different from the female group on the ratio of IND to SVL (p-value < 0.05; Table 3). Therefore, morphometric analyses between the new taxon and *N. hainanensis* were conducted only in males. In PCA for males, the total variation of the first two principal components was 69.3%. In males on the two-dimensional plots of PC1 vs. PC2, the new taxon could be distinctly separated from *N. hainanensis* (Fig. 4). The results of one-way ANOVA indicated that in males, the new taxon was significantly different from

Table 3 Morphometric comparisons between *Nidirana leishanensis* sp. nov. (*NL*) and *N. hainanensis* (*NH*).

| | Male | | | | Female | | *p*-value from ANOVA | |
| | *NL* (*n* = 6) | | *NH* (*n* = 4) | | *NL* (*n* = 4) | | *NL* vs. *NH* in male | Male vs. Female in *NL* |
	Ranging	Mean ± SD	Ranging	Mean ± SD	Ranging	Mean ± SD		
SVL	49.5–56.4	53.3 ± 3.2	32.8–33.5	33.3 ± 0.3	43.7–55.3	50.2 ± 5.3	0.000	0.343
HDL	18.4–23.7	20.5 ± 2.0	12.7–12.9	12.8 ± 0.1	17.0–19.3	18.1 ± 1.1	0.971	0.254
HDW	17.5–22.2	19.5 ± 1.7	11.5–12.5	12.0 ± 0.4	16.5–19.3	17.6 ± 1.2	0.710	0.490
SL	7.0–8.5	7.7 ± 0.6	5.3–5.7	5.5 ± 0.2	6.7–8.4	7.5 ± 0.7	0.001	0.398
IND	5.6–6.7	6.2 ± 0.4	3.5–4.4	4.1 ± 0.4	5.6–6.9	6.3 ± 0.6	0.341	0.001
IOD	4.0–4.7	4.4 ± 0.2	3.4–3.7	3.6 ± 0.1	3.8–5.2	4.5 ± 0.7	0.000	0.214
UEW	3.7–4.5	4.2 ± 0.3	3.1–3.1	3.1 ± 0.0	3.2–4.4	3.8 ± 0.5	0.001	0.638
ED	6.0–7.0	6.4 ± 0.4	4.4–5.0	4.6 ± 0.3	5.2–6.2	5.8 ± 0.4	0.003	0.181
TYD	4.7–5.6	5.0 ± 0.4	3.6–4.3	3.9 ± 0.3	4.0–5.6	4.7 ± 0.7	0.002	0.975
LAL	20.6–25.3	23.4 ± 1.9	14.8–15.1	15.0 ± 0.2	19.8–23.7	22. 0 ± 1.6	0.132	0.279
LW	4.4–5.9	4.9 ± 0.6	2.0–2.9	2.5 ± 0.4	4.0–4.4	4.2 ± 0.2	0.021	0.639
HAL	12.5–15.2	13.8 ± 1.3	9.0–9.7	9.2 ± 0.3	12.0–14.9	13.7 ± 1.2	0.028	0.162
HLL	82.2–97.4	89.3 ± 6.0	58.0–61.0	59.0 ± 1.4	79.0–92.7	86.2 ± 5.7	0.003	0.462
TL	26.1–31.5	29.2 ± 2.1	17.6–19.4	18.6 ± 0.8	25.2–31.2	27.9 ± 2.5	0.260	0.719
TW	7.3–8.6	8.0 ± 0.7	4.5–5.4	4.9 ± 0.4	6.3–8.3	7.2 ± 0.8	0.397	0.299
TEL	37.0–46.5	42.1 ± 3.7	26.1–27.7	26.8 ± 0.7	37.3–42.4	39.9 ± 2.2	0.341	0.457
FL	27.3–34.6	30.7 ± 2.7	18.5–18.8	18.7 ± 0.1	26.3–32.4	29.4 ± 2.5	0.327	0.444

Notes.
Unit: mm. ANOVA, one-way analyses of variance. Significant level: *p* <0.05. See abbreviations for the measurements in 'Methods'.

N. hainanensis on many morphometric characters (all *p*-values < 0.05; Table 3). More detailed descriptions of results from morphological comparisons between the new taxon and its congeners were presented in the following sections for describing the new species.

Bioacoustics comparisons

There were obvious differences on sonograms and waveforms of calls between the new taxon (Figs. 5A and 5B) and *N. hainanensis* (Figs. 5C and 5D). Firstly, the call of the new taxon had only one strophe with one syllable, but the call of *N. hainanensis* had one strophe containing two syllables. Secondly, the new taxon had a longer time of syllable duration (0.33–0.43 s) than *N. hainanensis* (0.20–0.25 s). Thirdly, the new taxon had a longer time of interval between syllables duration (8.85–15.77 s) than *N. hainanensis* (0.13–0.15 s). Finally, the new taxon had a broader frequency range (480–20,640 Hz) than *N. hainanensis* (440–7,170 Hz).

In all, molecular phylogenetic analyses, morphological comparisons and bioacoustics comparisons consistently indicated that our specimens from the Leigong Mountain of Guizhou Province, China represented a new taxon of the genus *Nidirana*. It is described as a new species in the following sections:

Nidirana leishanensis sp. nov.
urn:lsid:zoobank.org:act:72C795CB-8FCF-40B7-9C0F-6F9D04B47C5E
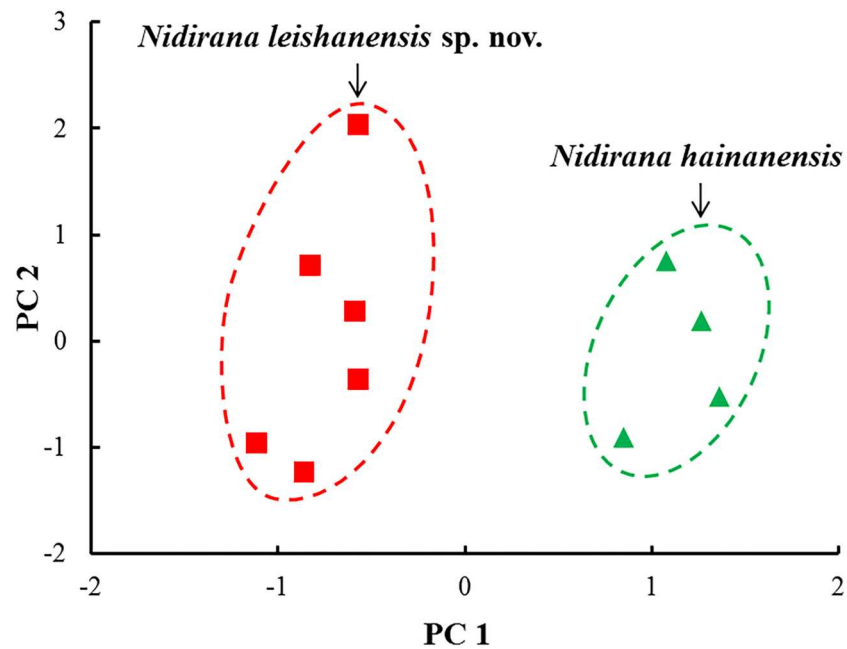
Figure 4 **Plots of principal component analyses of *Nidirana leishanensis* sp. nov. and *N. hainanensis.***
PC1, the first principal component; PC2, the second principal component. Different species were denoted as different colors and shapes.
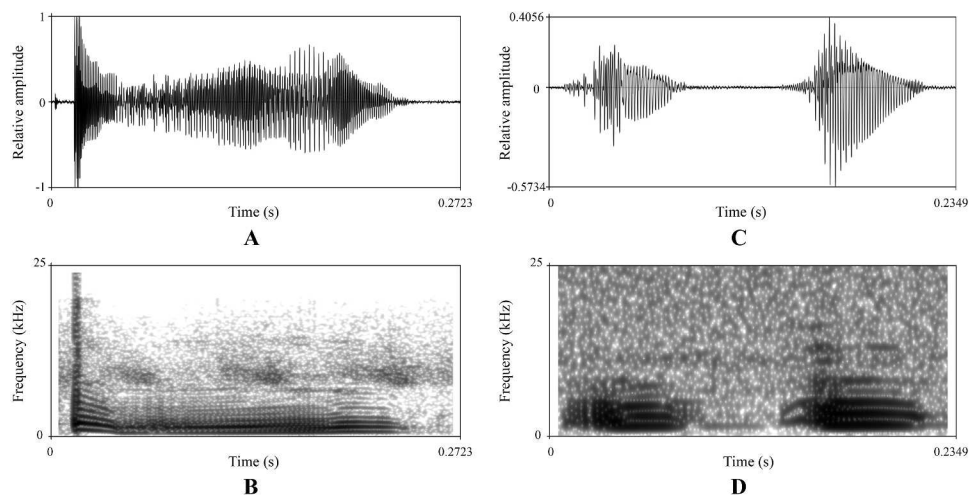
Figure 5 **Advertisement calls of a male *Nidirana leishanensis* sp. nov. (the holotype CIBLS20170727002) and a male *N. hainanensis* (specimen CIB20110629003).** (A) and (B) waveform and sonogram showing a strophe of *Nidirana leishanensis* sp. nov. respectively. (C) and (D) waveform and sonogram showing a strophe of *N. hainanensis* respectively.
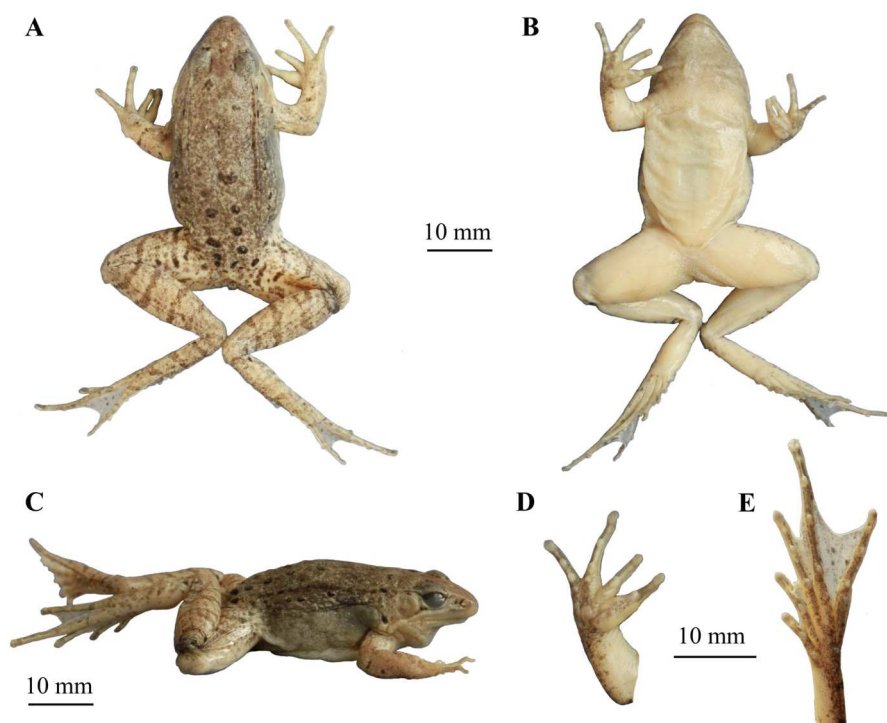
Figure 6 **The holotype specimen (CIBLS20170727002) of *Nidirana leishanensis* sp. nov.** (A) Dorsal view. (B) Ventral view. (C) Lateral view. (D) Ventral view of hand. (E) Ventral view of foot. Photographed by Shize Li.

Holotype

CIBLS20170727002, adult male (Figs. 6A–6C, 7A, 7B and 7F), collected by S. Z. Li in the Leigong Mountain National Nature Reserve (26.37533°N, 108.16107°E; elevation 1,298 m a.s.l.), Leishan County, Guizhou Province, China on 27 July 2017.

Paratypes

A total of nine adult specimens (five males and four females) collected by S. Z. Li from Leigong Mountain National Nature Reserve in Leishan County, Guizhou Province, China. Five male specimens: CIBLS20150628001, CIBLS20150628002, CIBLS20150628005, CIBLS20150628006 and CIBLS20150628007 collected on 28 June 2015; four female specimens: CIBLS20150627001, CIBLS20150627002 and CIBLS20150627003 collected on June 27, 2015 and CIBLS20170727001 on 27 July 2017. Seven tadpoles (CIBLS20180826001–CIBLS20180826007) collected by S. Z. Li on 6 August 2018 in the same site.

Diagnosis

Nidirana leishanensis sp. nov. is assigned to the genus *Nidirana* based on molecular data and the following morphological characters: disks of digits dilated, pointed; lateroventral grooves present on digits; feet full webbed or half webbed; dorsolateral folds distinct; the presence of large suprabrachial gland in males; nuptial pad present at the base of first finger in males; vocal sacs present in males.
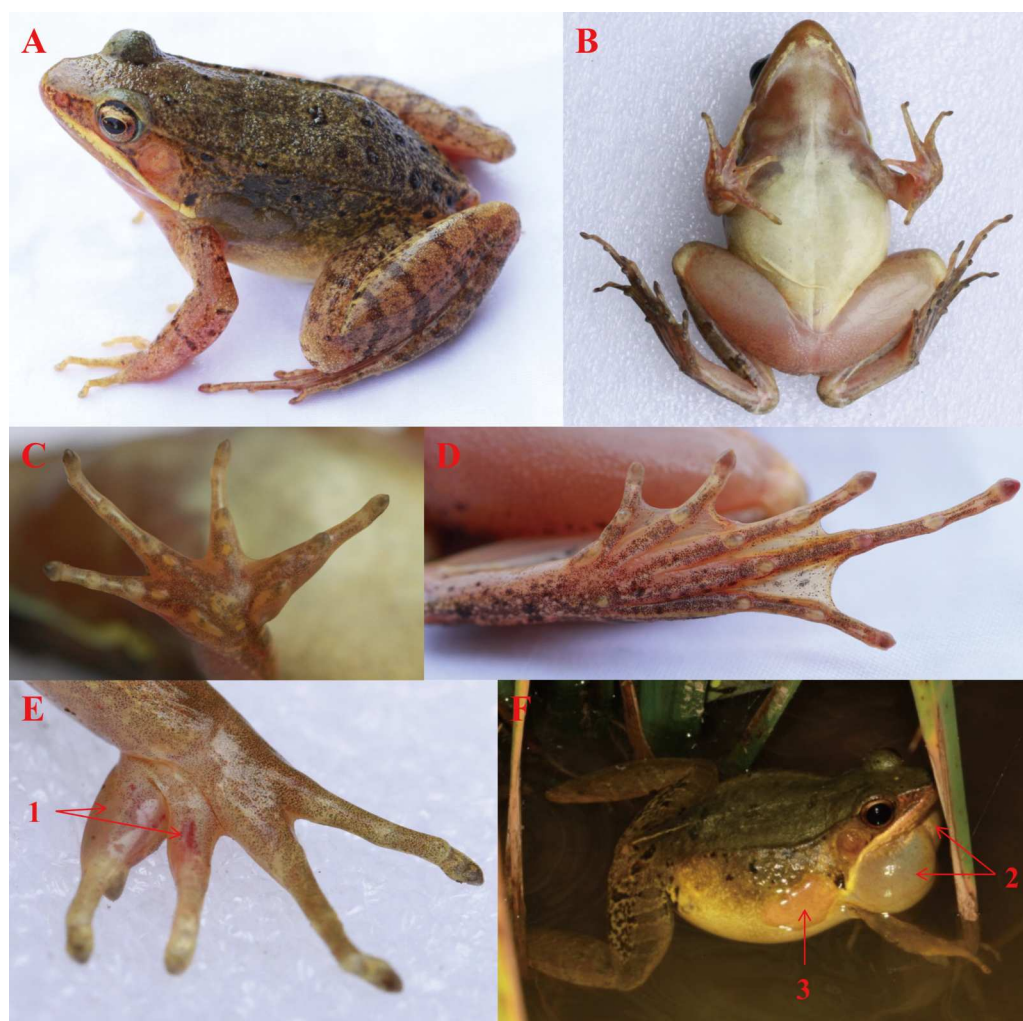
Figure 7 **Photos of the holotype CIBLS20170727002 of *Nidirana leishanensis* sp. nov. in life.** (A) Dorsal view. (B) Ventral view. (C) Ventral view of hand. (D) Ventral view of foot. (E) Nuptial pads on the first and second fingers. (F) Lateral view showing subgular external vocal sac. 1, nuptial pad on inner side of base of fingers I and II; 2, a pair of subgular internal vocal sac at corners of throat; 3, suprabrachial gland. Photographed by Shize Li.

Nidirana leishanensis sp. nov. could be distinguished from its congeners by a combination of the following characters: (1) a large body size in males (SVL > 49 mm); (2) the presence of lateroventral grooves both on fingers and toes; (3) relative finger lengths: II < IV < I < III; (4) tibiotarsal articulation reaching to the level between eye and nostril when leg stretched forward; (5) a pair of subgular internal vocal sacs at corners of throat present in males; (6) the presense of nuptial pad on the inner side of base of fingers I and II in males; (7) webbing formula: I 2 −2 $\frac{1}{3}$ II 2 −2 $\frac{2}{3}$ III 3 $\frac{1}{2}$ −3 $\frac{2}{3}$ IV 3 $\frac{2}{3}$ −3V.

Description of holotype

Body size large, SVL 55.8 mm; head longer than wide (HDL/HDW = 1.08), flat above; snout round, slightly projecting beyond lower jaw in ventral view; snout and tympanum

to axial region, forming a maxillary gland in posterior corner of mouth, behind the gland a soya bean size gland present; supratympanic fold absent; interorbital space narrower than internarial distance (IND/IOD = 1.4); eye large and convex, ED 0.78 times of SL; tympanum round, distinct, and close to eye; vomerine teeth on well-developed ridges; tongue deeply notched posteriorly; a pair of subgular internal vocal sacs at corners of throat.

Forelimbs moderately robust (LW/SVL = 0.11); lower arm and hand less than a half of body length (LAL/SVL = 0.44); relative finger lengths: II < IV < I < III; tip of fingers weakly dilated, forming elongated and pointed disks; lateroventral grooves on the disks of finger; fingers free of webbing, with lateral fringes; subarticular tubercles and supernumerary tubercles below the base of finger present, and subarticular tubercles prominent; palmar tubercles three, elliptic, distinct.

Hindlimbs relatively robust, tibia 54% of SVL; heels overlapping when hindlimbs flexed at right angles to axis of body; tibiotarsal articulation reaching the level between eye to nostril and closer to eye when leg stretched forward; toes long and thin, relative toe lengths: I < II < V < III < IV; tip of toes dilated, forming significantly elongated disks; distinct lateroventral grooves on toes; webbing moderate, webbing formula: I $2 -2 \frac{1}{3}$ II $2 -2 \frac{2}{3}$ III $3 \frac{1}{2} -3 \frac{2}{3}$ IV $3 \frac{2}{3} -3$V; toes with lateral fringes; subarticular tubercles oval, prominent; inner metatarsal tubercle elliptic, prominent; outer metatarsal tubercle absent.

Dorsal skin of head and anterior part of body smooth, posterior part and flanks with several tubercles, on some tubercles with black spot; a large suprabrachial gland behind base of forelimb; dorsolateral fold extending from posterior margin of upper eyelid to above groin; several granules on the dorsal surfaces of thigh, tibia and tarsus; ventral surface of head, body and limbs smooth, several flattened tubercles densely arranged on the rear of thigh and around vent.

Coloration in life

Dorsal surface and suprabrachial gland olive; flank light yellow; several black spots on flank and dorsum; a light yellow streak from posterior head to cloacae discontinuously; dorsal forelimbs reddish brown and one brown stripe in front of the base of forelimb; dorsal hindlimb reddish brown, two brown bands on the thigh, four on the tibia and three on the tarsus; tympanum and temporal region light yellow; maxillary gland white; ventral surface smooth, throat and ventral of thigh and forelimbs incarnadine, belly and chest light yellow; lips incarnadine and lower jaw brownish white (Fig. 7).

Color in preservative

Dorsal surface faded to brown; black spots on dorsum and flank more distinct; limbs faded light brown and the crossbars becoming clearer; ventral surface faded to pale cream (Fig. 6).

Variation

Measurements and basic statistics of adult specimens were presented in Table S1 and Table S4, respectively. All specimens were similar in morphology but some individuals different from the holotype in color pattern. In some adult males, the dorsal coloration was pale brown (Fig. 8A); in some adult males, the dorsal coloration was olive-grey without black spots on the posterior of dorsum, and the gland was smaller and obvious (Fig. 8B); in
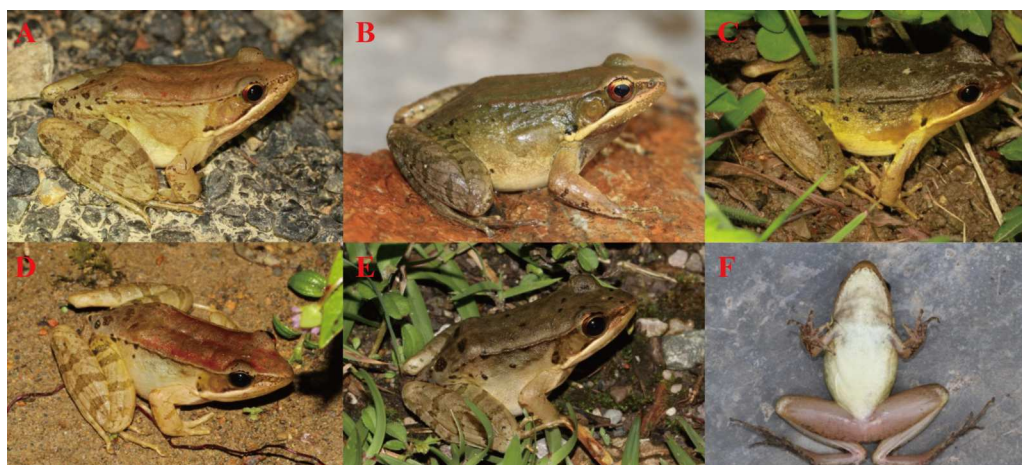
Figure 8 **Color variations in *Nidirana leishanensis* sp. nov. in life.** (A), (B) and (C) dorsolateral view of the male specimens CIBLS20150628001, CIBLS20150628005 and CIBLS20150628007, respectively. (D) and (E) dorsolateral view of the female specimens CIBLS20150627002 and CIBLS20150627003, respectively. (F) ventral view of the female specimen CIBLS20150627002. Photographed by Shize Li.

some adult males, the dorsolateral folds not continuous and split from the middle dorsum (Fig. 8C); in some adult females, dorsal coloration was brown with brownish red (Fig. 8D); in some adult females, dorsal coloration was olive-grey and the black spots on dorsum was more intensive (Fig. 8E); in some adult females, the ventral surface was creamy white (Fig. 8F).

Skull

The skull morphology of the five scanned specimens were almost identical, and thus, only one representative (voucher number: CIBLS20170727002) was presented (Fig. 9) and described as following: skull flat, maxillary teeth well developed, vomerine teeth present; mandible without teeth; a pair of premaxillas, separated from each other and connects with nasal; nasals large, separated from each other, and connects with the sphenethmoid; sphenethmoid tubular, forms the anterolateral walls of the braincase; frontoparietal roof the braincase and wider anteriorly than posteriorly; prootic large and separated from the exoccipital; a pair of exoccipitals situated the end of the brain; palatines arcuate and long, behind the prevomers; a pair of prevomers obliquely lie anterior to the palatines, vomerine teeth on the prevomers distinct; parasphenoid in sword shape, supports the braincase ventrally, connects with palatines; a pair of squamosals on the dorsolateral side of the prootic, each squamosal consists of three rami: the zygomatic ramus, the otic ramus, and the ventral ramus; zygomatic ramus pointing to the orbit, otic ramus shorted, ventral ramus outboard and covered the posterior of pterygoid; a pair of pterygoids, outside of the ventral surface of the squamosal, anterior ramus is the longest, center is inward and leading edge extends to the orbit, medial ramus shorted and attached to the anterior lateral part of the prootic, posterior ramus edge-on and extend to maxillary; a pair of columellaes situated ventral to the crista parotica (Fig. 9).
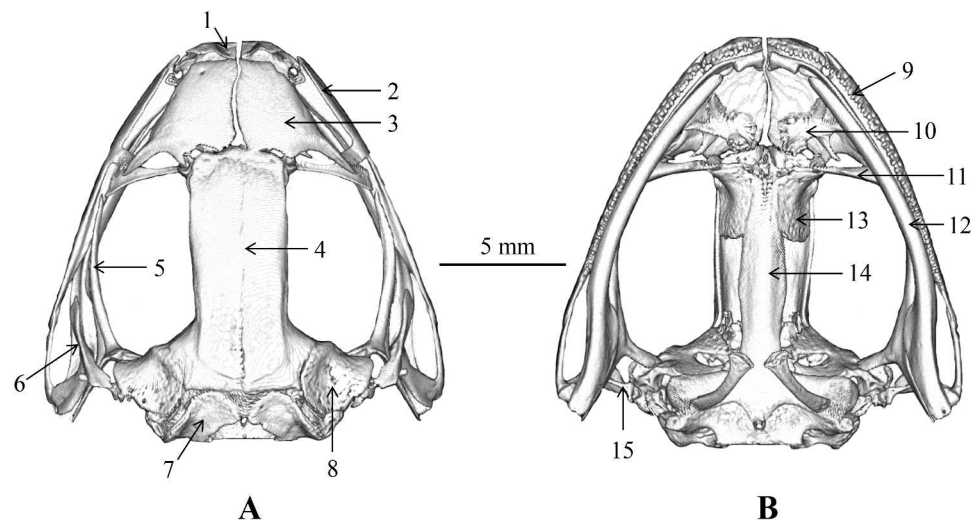
Figure 9 Skull of *Nidirana leishanensis*. sp. nov., the holotype CIBLS20170727002. (A) Dorsal view. (B) Ventral view. 1, premaxilla; 2, maxillary; 3, nasal; 4, frontoparietal; 5, pterygoid; 6, squamosal; 7, ex-occipital; 8, prootic; 9, maxillary teeth; 10, prevomer; 11, palatine;12, mandible; 13, sphenethmoid; 14, parasphenoid; 15, columella. Photographed by Shize Li.

Figure 10 Tadpole of *Nidirana leishanensis*. sp. nov., the specimen CIBLS20180826007, in life. (A) Dorsal view. (B) Lateral view. (C) Ventral view. (D) Mouth structure. 1, Spiracle; 2, lower keratodonts; 3, upper keratodonts; 4, labial papillae on lower lips; 5, labial papillae on upper lips; 6, additional tubercles at the angles of mouth. Photographed by Shize Li.

Tadpole description

Measurements of the seven tadpole specimens were presented in Table S2. All specimens of tadpole were similar in morphology and color pattern, the following tadpole description is based on a single specimen (CIBLS20180826007) at Stage 31 (Fig. 10), which was confirmed as *Nidirana leishanensis* sp. nov. by the molecular analyses (Figs. 2 and 3).

Body oval, body and tail yellowish-brown, flattened above; several brown spots on dorsum; eyes lateral, nostril near snout; spiracle on left side of body, directed

dorsoposteriorly; keratodont formula I: 1–2/II: 1–1; ventral of body oval, creamy white; both upper and lower lips with labial papillae; some additional tubercles at the angles of the mouth, usually with small keratodonts; TOL 38.3 mm; tail fusiform, approximately 2.0 times as long as snout-vent length, tail height 21.3% of tail length; dorsal fin arising behind the origin of the tail; maximum depth near mid-length, and larger than body depth; body width longer than body height (BW/ BH = 1.5); several brown spots on the dorsal of body (Fig. 10).

Vocalization

The sonograms and waveforms are shown in Figs. 5A and 5B respectively. The call has only one strophe with one syllable. The syllable has a duration of 0.33–0.43 s (mean $\pm$ SD: 0.37 $\pm$ 0.053 s, $N = 10$). The interval between syllables has a duration of 8.85–15.77 s (mean $\pm$ SD: 12.31 $\pm$ 4.89 s, $N = 9$). Amplitude modulation within strophe is apparent, beginning with highest energy pulses, decreasing slightly to a minimum then increase to mid strophe approximately, subsequently increasing to a peak then decreasing rapidly and then increasing to another peak and subsequently decreasing towards the end of each strophe. Calls have a broad frequency range of 480–20,640 Hz.

Secondary sexual characteristics

A pair of subgular internal vocal sacs, a pair of slit-like openings at posterior of jaw (Fig. 7F); linea musculina present on dorsum; incarnadine nuptial pad on the inner side of base of fingers I and II (Fig. 7E); nuptial spinules invisible; suprabrachial gland present.

Comparisons

Nidirana leishanensis sp. nov. differs from *N. chapaensis*, *N. nankunensis* and *N. okinavana* by having larger body size in males (SVL > 49 mm in the new species vs. SVL < 43 mm in the latter).

Nidirana leishanensis sp. nov. differs from *N. pleuraden* by the presence of lateroventral grooves both on fingers and toes (vs. absent in the latter).

Nidirana leishanensis sp. nov. differs from *N. chapaensis*, *N. daunchina*, *N. lini*, *N. nankunensis*, *N. okinavana* and *N. pleuraden* by the relative finger lengths II < IV < I < III (vs. II < I < IV < III or II < I = IV < III in the latter).

Nidirana leishanensis sp. nov. differs from *N. chapaensis*, *N. daunchina*, *N. lini* and *N. nankunensis* by tibiotarsal articulation reaching the level between eye to nostril when leg stretched forward (vs. reaching nostril or beyond snout in the latter).

Nidirana leishanensis sp. nov. differs from *N. okinavana* by having a pair of subgular internal vocal sacs in males (vs. gular vocal sacs absent in the latter).

Nidirana leishanensis sp. nov. differs from *N. daunchina*, *N. lini*, *N. nankunensis*, *N. okinavana* and *N. pleuraden* by having nuptial pad on the inner side of base of fingers I and II in males in breeding season (vs. nuptial pad only on the finger I in males in the latter).

Nidirana leishanensis sp. nov. differs from *N. daunchina*, *N. lini* and *N. nankunensis* by the webbing formula I 2 –2 $\frac{1}{3}$ II 2 –2 $\frac{2}{3}$ III 3 $\frac{1}{2}$ –3 $\frac{2}{3}$ IV 3 $\frac{2}{3}$ –3V (vs. I 2 $\frac{1}{2}$ –2 $\frac{1}{3}$ II 2 $\frac{1}{2}$ –III 2 $\frac{2}{3}$ –3 IV 3 –2V in *N. daunchina*, I 2 $\frac{1}{2}$ –II 2 $\frac{1}{2}$ –2 III 3 $\frac{1}{2}$ –3 $\frac{1}{3}$ IV 3 $\frac{1}{3}$ –3 $\frac{1}{2}$ V in *N. lini* and I 2 –2 $\frac{1}{2}$ II 1 $\frac{12}{3}$ –3 III 2 $\frac{1}{3}$ –3 $\frac{1}{2}$ IV 3 $\frac{1}{2}$ –2 V in *N. nankunensis*).

Nidirana leishanensis sp. nov. is superficially resembling *N. adenopleura* on morphology but can be distinguished from the latter by a series of morphological characters as follows: the relative finger lengths II < IV < I < III (vs. II < I < IV < III); having nuptial pad on the inner side of base of fingers I and II in males in breeding season (vs. nuptial pad only on the finger I in males); tibiotarsal articulation reaching the level between eye to nostril when leg stretched forward (vs. reaching the eye); webbing formula: I $2-2\frac{1}{3}$ II $2-2\frac{2}{3}$ III $3\frac{1}{2}-3\frac{2}{3}$ IV $3\frac{2}{3}-3$V (vs. I $2-2\frac{1}{3}$ II $2\frac{2}{3}-3\frac{1}{3}$III $3-4$ IV $4-3$V).

Nidirana leishanensis sp. nov. is genetically most closer to *N. hainanensis*. It differs from *N. hainanensis* by the following characters: having larger body size in males (SVL 49.5–56.4 mm in the new species vs. SVL 32.8–33.5 mm in *N. hainanensis*); having nuptial pad on the inner side of base of fingers I and II in males in breeding season (vs. nuptial pad absent in breeding season); tibiotarsal articulation reaching the level between eye to nostril when leg stretched forward (vs. reaching nostril); the webbing formula: I $2-2\frac{1}{3}$ II $2-2\frac{2}{3}$ III $3\frac{1}{2}-3\frac{2}{3}$ IV $3\frac{2}{3}-3$V (vs. I $2\frac{1}{2}-$II $2\frac{1}{2}-2\frac{1}{2}$ III $3\frac{1}{2}-3\frac{1}{3}$ IV $3\frac{1}{3}-3$V); having significantly lower ratios of SL, IOD, UEW, ED, TYD, HAL and HLL to SVL in males and having significantly higher ratios of LW to SVL in males (all *p*-values < 0.05; Table 3).

Ecology

Nidirana leishanensis sp. nov. is currently known only from Leigong Mountain (26.25°N–26.53°N, 108.08°E–108.41°E) in Leishan County, Guizhou Province, China (Fig. 1). *Nidirana leishanensis* sp. nov. inhabited the paddy field or nearby the artificial trench where the water flows very slowly (Fig. 11A), at elevations from 650 to 1,300 m a. s. l., and the individuals could be found on the ridge of paddy field or in the paddy field (Figs. 11A–11C). The tadpoles of the species could be found in the water of ponding in the paddy (Fig. 11D). The species could eat some small mollusks for food (Fig. 11C). Two sympatric amphibian species, *Microhyla fissipes* (*Boulenger, 1884*) and *Polypedates megacephalus* (*Hallowell, 1861* "1860") were found in the type locality.

Etymology

This specific epithet "leishanensis" is a Latinize toponymic adjective that refers to Leigong Mountains, Leishan County, Guizhou Province, China, where the new species was collected. For the common name, we suggest Leishan Music Frog (English) and Lei Shan Qin Wa (Chinese).

DISCUSSION

There has been a lot of controversy on systematic profiles of the genus *Nidirana*. At first, some populations in this genus had been recognized as separated species, such as "*psaltes*" and "*caldwelli*" which were suggested to be the subjective junior synonym of "*okinavana*" (*Matsui, 2007*) and "*adenopleura*" (*Lyu et al., 2017*), respectively. Next, species assignments of some populations were suggested to be misguided. For example, several populations in the southwestern corner of Sichuan Province and Chongqing City and northwestern part of Guizhou Province of China, previously ever recognized as *N. adenopleura*, were later identified as *N. daunchina* (*Fei, Ye & Huang, 1990*; *Fei et al., 2009*; *Fei & Ye, 2005*); and the recently described species (*N. nankunensis*) had also been
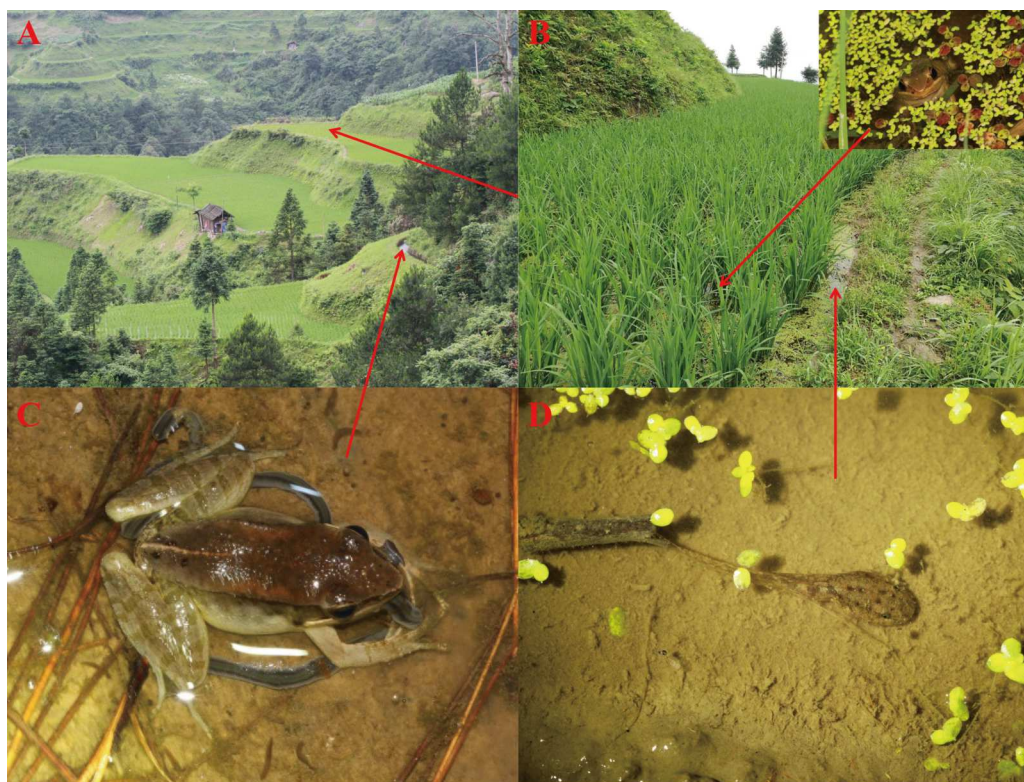
Figure 11 **Habitats of *Nidirana leishanensis.* sp. nov. in the type locality, Leigong Mountain, Leishan County, Guizhou Province, China.** (A) Landscape. (B) A paddy field with a *Nidirana leishanensis* sp. nov. in the water (*insert*). (C) A frog eating a small mollusk for food. (D) A tadpole swimming in the paddy field. Photographed by Shize Li.

assigned to *N. adenopleura*, although molecular phylogenetic analyses indicated that the two species were paraphyly. Similarly, the new species, *Nidirana leishanensis* sp. nov., were misidentified as *N. adenopleura* though the population had been found for many decades (*Fei et al., 2009*; *Wu, Dong & Xu, 1986*). It is assumed that morphological similarity of the related species in the genus much probably led to the misclassifications. Thus, integrative taxonomy with multiple evidences especially molecular phylogenetic reconstructions that have become the main trend and has been proved to be quite effective (e.g., *Lyu et al., 2017*; *Li et al., 2018*) is expected to be applied to resolve the taxonomic confusions of the genus *Nidirana*. In this study, molecular phylogenetics based on mitochondrial DNA and nuclear DNA indicated that *Nidirana leishanensis* sp. nov. was an independent clade from its congeners, and the genetic distance between the new species and its sister species (*N. hainanensis*; mean 5.7% on COI gene) was higher than those between several pairs of sister species of *Nidirana* and also match the level about interspecific divergences in amphibians (3.1–28.2% on COI gene; *Che et al., 2012*). Moreover, the distinct genetic separations between the new species and its related species on two nuclear locus implicated very restricted gene flow among these species. Finally, morphological comparisons and bioacoustics comparisons also supported the validity of the new species. As noted, we

found that the species are seriously threatened by local villagers and pesticides use in the paddy fields. Hence, it is urgent to make further investigations to evaluate its distributional range and population status of the new species.

Based on currently available information, it could presume that species diversity of the genus *Nidirana* is still underestimated, requiring future more studies. On one hand, the populations distinctly distributed far from the type locality of its temperately-ranked species should be carefully investigated especially using sophisticated molecular phylogenetic approaches. For examples, the populations previously recognized as *N. adenopleura* in Fanjing Mountain of Guizhou Province and Dayao Mountain of Guangxi Province in China are expected to be different species because their morphology was supposed to be different from *N. adenopleura* (*Wu, Dong & Xu, 1986*; *Fei et al., 2009*) while the localities were far from the type locality of *N. adenopleura* (Taiwan Island); and the populations previously assigned to *N. lini* in northern Thailand and that identified as *N. chapaensis* in the southern Laos (*Chuaynkern et al., 2010*) may be undescribed species because of their far and probably isolated distributional areas. On the other hand, further field surveys should be carried out in the poorly studied regions, such as west part of Guizhou-west part of Guangxi provinces of China, Yunnan Province of China and tropical regions along the northeastern Indo-China Peninsula, which are the biodiversity hotspots and are expected to bear much underestimated species diversity (*Myers et al., 2000*).

CONCLUSION

We described a new species of the Asian music frog genus *Nidirana* (Amphibia, Anura, Ranidae) from Southwestern China, and confirmed its phylogenetic placements. *Nidirana leishanensis* sp. nov. is currently only known from Leigong Mountain, Guizhou Province of China, and inhabits still waters in the mountains at mid and low elevations similar to most music frogs. Although there were still a mass of unresolved systematic assignments and assumed underestimated diversity in the genus *Nidirana*, the findings here give a better knowledge on species diversity and phylogenetic assignments of the group. Future works are expected mainly on re-examination of the doubtful classifications of proposed-misidentified populations using credible molecular phylogenetic approaches and painstaking field surveys especially in the poorly investigated regions that harboring high species richness.

ACKNOWLEDGEMENTS

We thank C. Li and J.C. Lv on helping collection of samples. We are much indebted to the editor and referees for their helpful comments.

Funding

This work was supported by the National Key Research and Development Program of China (No. 2017YFC0505202), National Natural Sciences Foundation of China (NSFC-31201702), Biodiversity Conservation Key Laboratory of Guizhou province Education Department, Guiyang College, the laboratory on biodiversity conservation and applied ecology of Guiyang college, Ocean Park Conservation Foundation of Hong Kong (No. PR 1030001252), Biodiversity Conservation Program of Ministry of Ecology and Environment of China (#2110404), Guizhou Provincial Department of Education Youth Science and Technology Talents Growth Project (Nos. KY[2018]455, KY[2018]468 and KY[2018]453), The Specialty Food Resource Utilization Talent Base of Moutai University. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Grant Disclosures

The following grant information was disclosed by the authors:
National Key Research and Development Program of China: 2017YFC0505202.
National Natural Sciences Foundation of China: NSFC-31201702.
Biodiversity Conservation Key Laboratory of Guizhou province Education Department, Guiyang College.
The laboratory on biodiversity conservation and applied ecology of Guiyang college, Ocean Park Conservation Foundation of Hong Kong: PR 1030001252.
Biodiversity Conservation Program of Ministry of Ecology and Environment of China: # 2110404.
Guizhou Provincial Department of Education Youth Science and Technology Talents Growth Project: KY[2018]455, KY[2018]468, KY[2018]453.
The Specialty Food Resource Utilization Talent Base of Moutai University.

Competing Interests

The authors declare there are no competing interests.

Author Contributions

- Shize Li and Bin Wang conceived and designed the experiments, performed the experiments, analyzed the data, contributed reagents/materials/analysis tools, prepared figures and/or tables, authored or reviewed drafts of the paper, approved the final draft.
- Gang Wei and Ning Xu performed the experiments, authored or reviewed drafts of the paper, approved the final draft.
- Jianguo Cui performed the experiments, analyzed the data, approved the final draft.
- Liang Fei performed the experiments, approved the final draft.
- Jianping Jiang and Jing Liu analyzed the data, approved the final draft.

Animal Ethics

The following information was supplied relating to ethical approvals (i.e., approving body and any reference numbers):

The Animal Care and Use Committee of Chengdu Institute of Biology, CAS provided full approval for this research (Number: CIB2011032201).

Field Study Permissions

The following information was supplied relating to field study approvals (i.e., approving body and any reference numbers):

Field work was approved by the Management Office of the Leigong Mountain Nature Reserve (project number: LGS-201304006).

Data Availability

The sequences of Nidirana are accessible at GenBank: MK293787–MK293871.

The raw data of skull from CT scanning is available at figshare: Li, Shize (2019): *Nidirana leishanensis* sp. nov. .avi. figshare. Media. https://doi.org/10.6084/m9.figshare.7980158.v1.

New Species Registration

The following information was supplied regarding the registration of a newly described species:

Publication LSID: urn:lsid:zoobank.org:pub:08105A58-4826-475D-BF07-A0C5C16D6BDD

Nidirana LSID: urn:lsid:zoobank.org:act:F6AEACB6-7C39-483E-A412-DF0037729323

Nidirana leishanensis sp. nov. LSID: urn:lsid:zoobank.org:act:72C795CB-8FCF-40B7-9C0F-6F9D04B47C5E.

Supplemental Information

Supplemental information for this article can be found online at http://dx.doi.org/10.7717/peerj.7157#supplemental-information.

REFERENCES

Barbour T. 1908. Some new Amphibia Salientia. *Proceedings of the Biological Society of Washington* **21**:189–190.

Biju SD, Bossuyt F. 2003. New frog family from India reveals an ancient biogeographical link with the Seychelles. *Nature* **425**:711–714 DOI 10.1038/nature02019.

Boettger O. 1895. Neue Frösche und Schlangen von den Liukiu-Inseln. *Zoologischer Anzeiger* **18**:266–270.

Bossuyt F, Milinkovitch MC. 2000. Convergent adaptive radiations in Madagascan and Asian ranid frogs reveal covariation between larval and adult traits. *Proceedings of the National Academy of Sciences of the United States of America* **12(97)**:6585–6590.

Boulenger GA. 1884. Descriptions of new species of reptiles and batrachians in the British Museum—part II. *Annals and Magazine of Natural History* **13(5)**:396–398 DOI 10.1080/00222938409459259.

Boulenger GA. 1892. Descriptions of new reptiles and batrachians from the Loo Choo Islands. *Annals and Magazine of Natural History* **10(6)**:302–304 DOI 10.1080/00222939208677414.

Boulenger GA. 1904. Descriptions of new frogs and snakes from Yunnan. *Annals and Magazine of Natural History* **13(7)**:130–135 DOI 10.1080/00222930408562447.

Boulenger GA. 1909. Descriptions of four new frogs and a new snake discovered by Mr. H. Sauter in Formosa. *Annals and Magazine of Natural History* **4(8)**:492–495 DOI 10.1080/00222930908692704.

Bourret R. 1937. Notes herpétologiques sur l'Indochine française. XIV. Les batraciens de la collection du Laboratoire des Sciences Naturelles de l'Université. Descriptions de quinze especes ou variétés nouvelles. *Annexe au Bulletin Général de l'Instruction Publique Hanoi* **1937**:5–56.

Castresana J. 2000. Selection of conserved blocks from multiple alignments for their use in phylogenetic analysis. *Molecular Biology and Evolution* **17(4)**:540–552 DOI 10.1093/oxfordjournals.molbev.a026334.

Chang MLY. 1933. A preoccupied name in Rana [Abstract 209]. *China Journal of Science & Arts. Shanghai* **18**.

Chang MLY, Hsu HF. 1932. Study of some amphibians from Szechwan. *Control Biology Laboratory Science Society* **8(5)**:137–181.

Che J, Chen HM, Yang JX, Jin JQ, Jiang K, Yuan ZY, Murphy RW, Zhang YP. 2012. Universal COI primers for DNA barcoding amphibians. *Molecular Ecology Resources* **12(2)**:247–258 DOI 10.1111/j.1755-0998.2011.03090.x.

Chen L, Murphy RW, Lathrop A, Ngo A, Orlov NL, Ho CT, Somorjai IL. 2005. Taxonomic chaos in Asian ranid frogs: an initial phylogenetic resolution. *Herpetological Journal* **15(4)**:231–243.

Chou WH. 1999. A new frog of the genus Rana (Anura: Ranidae) from China. *Herpetologica* **55(3)**:389–400.

Chuaynkern Y, Ohler A, Inthara C, Duengkae P, Makchai S, Salangsingha N. 2010. A revision of species in the subgenus Nidirana Dubois, 1992, with special attention to the identity of specimens allocated to Rana adenopleura Boulenger, 1909, and Rana chapaensis (Bourret, 1937) (Amphibia: Anura: Ranidae) from Thailand and Laos. *Raffles Bulletin of Zoology* **58**:291–310.

Clement M, Posada D, Crandall KA. 2000. TCS: a computer program to estimate gene genealogies. *Molecular Ecology* **9(10)**:1657–1659 DOI 10.1046/j.1365-294x.2000.01020.x.

Dubois A. 1992. Notes sur la classification des Ranidae (Amphibiens anoures). *Bulletin Mensuel de la Société Linnéenne de Lyon* **61**:305–352 DOI 10.3406/linly.1992.11011.

Fei L, Hu SQ, Ye CY, Huang YZ. 2009. *Fauna Sinica, Amphibia 2, Anura Ranidae.* Beijing: Science Press.

Fei L, Ye CY. 2005. *The key and illustration of Chinese.* Chongqing: Sichuan Publishing House of Science and Technology.

Fei L, Ye CY, Huang YZ. 1990. *Key to Chinese Amphibians.* Chongqing: Publishing House for Scientific and Technological.

Fei L, Ye CY, Jiang JP. 2007. A new species of Ranidae *Hylarana* (*Nidirana*) *hainanensis* from China (Amphibia: Anura). *Herpetologica Sinica* **11**:1–4.

Fei L, Ye CY, Jiang JP. 2010. Phylogenetic systematics of Ranidae. *Herpetologica Sinica* **12**:1–43.

Fei L, Ye CY, Jiang JP. 2012. *Colored atlas of Chinese amphibians and their distributions.* Chengdu: Sichuan Publishing House of Science and Technology.

Frost DR. 2019. Amphibian species of the world. Version 6.0. New York: American Museum of Natural History. *Available at* http://research.amnh.org/vz/herpetology/amphibia/ (accessed on 2019 April 11).

Frost DR, Grant T, Faivovich J, Bain RH, Haas A, Haddad CF, De Sa RO, Channing A, Wilkinson M, Donnellan SC, Raxworthy CJ, Campbell JA, Blotto BL, Moler P, Drewes RC, Nussbaum RA, Lynch JD, Green DM, Wheeler WC. 2006. The amphibian tree of life. *Bulletin of the American Museum of Natural History* **297**:1–291 DOI 10.1206/0003-0090(2006)297[0001:TATOL]2.0.CO;2.

Gosner KL. 1960. A simplified table for staging anuran embryos and larvae with notes on identification. *Herpetologica* **16(3)**:183–190.

Guindon S, Dufayard JF, Lefort V, Anisimova M, Hordijk W, Gascuel O. 2010. New algorithms and methods to estimate maximum-likelihood phylogenies: assessing the performance of PhyML 3.0. *Systematic Biology* **59(3)**:307–321 DOI 10.1093/sysbio/syq010.

Hall TA. 1999. BIOEDIT: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series* **41(41)**:95–98.

Hallowell E. 1861, 1860. Report upon the Reptilia of the North Pacific exploring expedition, under command of Capt. John Rogers, U.S. N. *Proceedings of the Academy of Natural Sciences of Philadelphia* **12**:480–510.

Kocher TD, Thomas WK, Meyer A, Edwards SV, Paabo S, Villablanca FX, Wilson AC. 1989. Dynamics of mitochondrial DNA evolution in mammals: amplification and sequencing with conserved primers. *Proceedings of the National Academy of Sciences of the United States of America* **86(16)**:6196–6200 DOI 10.1073/pnas.86.16.6196.

Kuramoto M. 1985. A new frog (genus *Rana*) from the Yaeyama Group of the Ryukyu Islands. *Herpetologica* **41**:150–158.

Lemmon AR, Moriarty EC. 2004. The importance of proper model assumption in Bayesian phylogenetics. *Systematic Biology* **53(2)**:265–277 DOI 10.1080/10635150490423520.

Li SZ, Xu N, Lv JC, Jiang JP, Wei G, Wang B. 2018. A new species of the odorous frog genus *Odorrana* (Amphibia, Anura, Ranidae) from southwestern China. *PeerJ* **6**:e5695 DOI 10.7717/peerj.5695.

Linnaeus C. 1758. *Systema naturae per regna tria naturae, secundum classes, ordines, genera, species, cum characteribus, differentiis, synonymis, locis.* 10th Edition. Volume 1. Stockholm: Laurentii Salvii.

Lyu ZT, Zeng ZC, Wang J, Lin CY, Liu ZY, Wang YY. 2017. Resurrection of genus Nidirana (Anura: Ranidae) and synonymizing *N. caldwelli* with *N. adenopleura*, with description of a new species from China. *Amphibia-Reptilia* **38**:483–502 DOI 10.1163/15685381-00003130.
Matsui M. 2007. Unmasking *Rana okinavana* Boettger, 1895 from the Ryukyus, Japan (Amphibia: Anura: Ranidae). *Zoological Science* **24**:199–204 DOI 10.2108/zsj.24.199.

McGuire JA, Witt CC, Altshuler DL, Remsen JV. 2007. Phylogenetic systematics and biogeography of hummingbirds: Bayesian and maximum likelihood analyses of partitioned data and selection of an appropriate partitioning strategy. *Systematic Biology* **56(5)**:837–856 DOI 10.1080/10635150701656360.

Myers N, Mittermeier RA, Mittermeier CG, Da Fonseca GAB, Kent J. 2000. Biodiversity hotspots for conservation priorities. *Nature* **403**:853–858 DOI 10.1038/35002501.

R Development Core Team. 2008. R: a language and environment for statistical computing. Vienna: R Foundation for Statistical Computing. *Available at http://www.R-project.org*.

Robert L, Brett C, Simon YWH, Stephane G. 2012. PartitionFinder: combined selection of partitioning schemes and substitution models for phylogenetic analyses. *Molecular Phylogenetics and Evolution* **29(6)**:1695–1701.

Ronquist FR, Huelsenbeck JP. 2003. MrBayes3: Bayesian phylogenetic inference under mixedmodels. *Bioinformatics* **19(12)**:1572–1574 DOI 10.1093/bioinformatics/btg180.

Sambrook J, Fritsch EF, Maniatis T. 1989. *Molecular cloning: a laboratory manual.* New York: Cold Spring Harbor Laboratory Press.

Schmidt KP. 1925. New Chinese amphibians and reptiles. *American Museum Novites* **175**:1–3.

Simon C, Frati F, Beckenbach A, Crespi B, Liu H, Flook P. 1994. Evolution, weighting and phylogenetic utility of mitochondrial gene sequences and a compilation of conserved polymerase chain reaction primers. *Annals of the Entomological Society of America* **87(6)**:651–701 DOI 10.1093/aesa/87.6.651.

Tamura K, Stecher G, Peterson D, Fiipski A, Kumar S. 2011. MEGA6: molecular evolutionary genetics analysis using evolutionary distance. *Molecular Biology and Evolution* **28**:2725–2729.

Thompson JC. 1912. Prodrome of a description of a new genus of Ranidae from the Loo Choo islands. *Herpetological Notices* **1**:1–3.

Wijayathilaka N, Meegaskumbura M. 2016. An acoustic analysis of the genus *Microhyla* (Anura: Microhylidae) of Sri Lanka. *PLOS ONE* **11(7)**:e0159003 DOI 10.1371/journal.pone.0159003.

Wu L, Dong Q, Xu RH. 1986. *Amphibians of Guizhou province.* Guiyang: Guizhou People Press.