

A new species of the genus *Batrachuperus* (Urodela: Hynobiidae) from Southwestern China

DEAR EDITOR,

A new stream salamander species, *Batrachuperus daochengensis* sp. nov., from southwestern China, is described herein based on morphological and molecular evidence. Molecular phylogeny derived from the mitochondrial gene together with previous nuclear data revealed that *B. daochengensis* sp. nov. is sister to *B. yenyuanensis*. The new species differs from all other species of the genus by the following combination of characters: brown horny epidermis on tips of fingers and toes absent; tubercles on palms and soles absent; costal grooves 12; dorsal brown, mottled with blackish spots; fingers 2-3-4-1 in order of decreasing length; tips of longest digits of fore- and hindlimbs largely separated by one to two costal spaces when adpressed towards each other along sides of body. The new species is currently known in the central and southern Shaluli Mountains in southwestern China.

Batrachuperus Boulenger, 1878 and three other genera (i.e., *Liuia* Zhao and Hu, 1983, *Pachyhynobius* Fei, Qu and Wu, 1983, and *Pseudohynobius* Fei and Ye, 1983) from the family Hynobiidae Cope, 1859 are endemic to China. *Batrachuperus* is currently comprised of five recognized species, including *B. pinchonii* (David, 1872), *B. tibetanus* Schmidt, 1925, *B. karlschmidti* Liu, 1950, *B. yenyuanensis* Liu, 1950, and *B. londongensis* Liu and Tian, 1978 (Frost, 2020). These aquatic salamanders live in fast-moving streams throughout the year and are primarily distributed in mountainous areas of western China, including the Sichuan, Shanxi, Gansu, Xizang, and Yunnan Provinces (Fei et al., 2006; Fu et al., 2001).

Recent molecular surveys indicate that our understanding of the taxonomic diversity of the genus *Batrachuperus* is still far from complete. Based on allozyme frequency data and mitochondrial gene tree evidence, Fu & Zeng (2008)

supposed seven species within the genus, including the five described species, i.e., *B. pinchonii*, *B. tibetanus*, *B. karlschmidti*, *B. yenyuanensis*, and *B. londongensis*, and two previously unrecognized species, i.e., *B. sp. 1* and *B. sp. 2*. In addition, the validities of two earlier described species, i.e., *B. cochraniae* and *B. taibaiensis*, were not supported by the nuclear data. For unrecognized *B. sp. 2*, specimens for gene analyses were obtained from populations in Daocheng, Xiangcheng, and Litang counties in Sichuan.

From 2002 to 2016, we collected specimens from the above regions in southwestern China, including the Daocheng and Xiangcheng counties in Sichuan and the Ninglang Yi Autonomous and Xianggelila counties in Yunnan. Based on morphological and molecular analysis, we determined that these specimens belong to an independent lineage, which is described herein based on the specimens from the Daocheng and Xiangcheng counties, Sichuan, China.

During our fieldwork, a total of 29 adults were collected from four localities in Sichuan, i.e., Sangdui in Daocheng County, Shagong in Xiangcheng County, and Qincaiping and Longcanggou in Yingjing County, and two localities in Yunnan, i.e., Tangman and Jidi in Xianggelila County (Figure 1A and Supplementary Table S1). Upon arrival to the laboratory, the animals were euthanized via submergence in a buffered MS-222 solution, after which muscle and liver tissue samples were taken and preserved in 95% ethanol for genetic analysis. Specimens were fixed in 10% buffered formalin. Voucher specimens were deposited at the Chengdu Institute of Biology (CIB), Chinese Academy of Sciences (CAS), and Museum of Henan University of Science and Technology (HNUSTM).

A total of 16 specimens from four locations, with samples collected from 2014 to 2016, were used for DNA extraction (Supplementary Table S1). Genomic DNA was extracted from liver and muscle tissues by the standard proteinase K method. A cytochrome *b* (*cyt b*) gene fragment was amplified with primers MVZ15 and MVZ16 (Moritz et al., 1992). The

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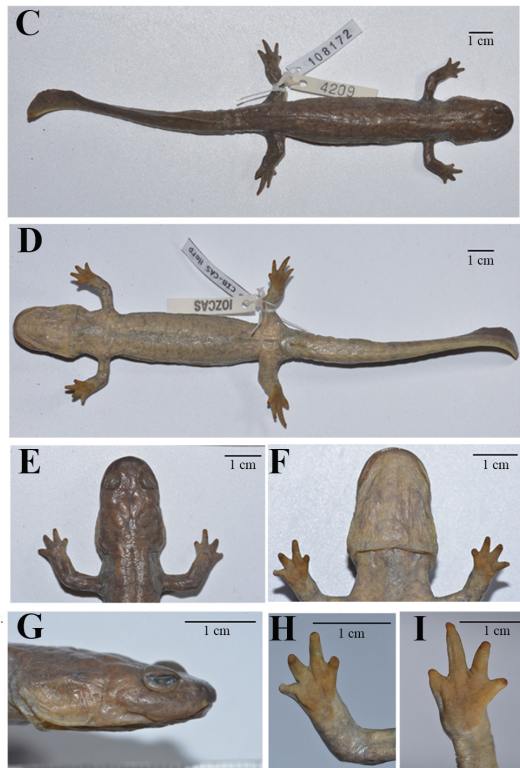
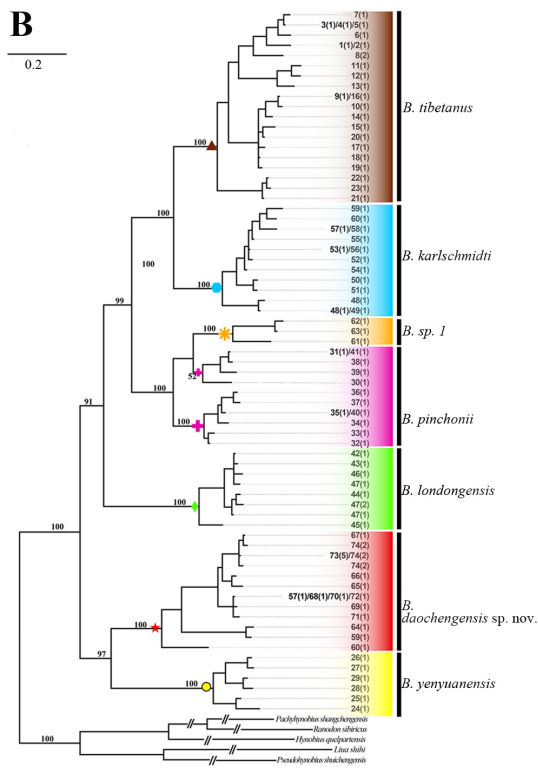
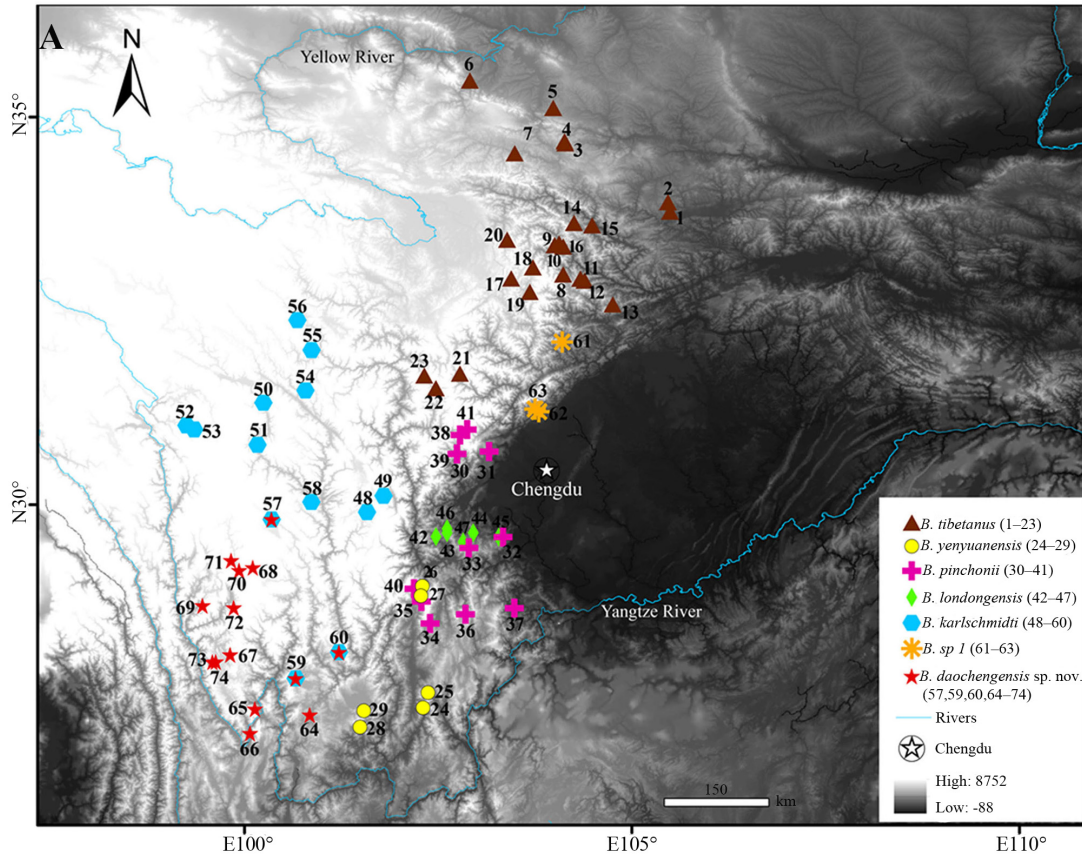


Figure 1 Distribution, phylogenetic tree, and holotype of *Batrachuperus daochengensis* sp. nov.

A: Map of southwestern China and sampling sites of the genus *Batrachuperus*. Chengdu city is presented as a reference point. B: Phylogeny of the genus *Batrachuperus* derived from partial *cyt b* sequences. Terminal taxa are mtDNA haplotypes. Haplotypes are named by location numbers. Haplotypes shared in multiple locations are labelled by all location numbers separated by '/'. Numbers in parentheses are numbers of specimens that share haplotype. Numbers above branches are Bayesian posterior probabilities from Bayesian analysis. Dorsal (C) and ventral (D) views of holotype of *Batrachuperus daochengensis* sp. nov. (adult female, CIB108172). Dorsal (E), ventral (F), and lateral (G) views of head, and ventral views of right forelimb (H) and right hindlimb (I) of holotype of *Batrachuperus daochengensis* sp. nov. Photos by Jin-Long Ren.

polymerase chain reaction (PCR) program included 3 min for initial denaturation, 30 cycles at 94 °C/50 °C/72 °C for 30 s/30 s/1 min, followed by a 10 min extension at 72 °C. The PCR products were sequenced at the Sangon Sequencing Center (Shanghai, China). Sequences were deposited in GenBank under accession Nos. MT513951–513966 (Supplementary Table S1).

A total of 96 specimens were sampled for molecular phylogenetic analysis, including 91 individuals within *Batrachuperus* from 74 collection sites covering the whole geographic distribution of the genus in China, and five individuals belonging to five species closely related to *Batrachuperus* as outgroups (Supplementary Table S1). In addition to the 16 sequences from the present study, 80 previously published sequences were downloaded from GenBank. The *cyt b* gene fragment was 783 bp in length. Sequence alignment was conducted with ClustalX v2 (Larkin et al., 2007). Bayesian analysis was conducted on these datasets. For Bayesian analysis, the best substitution models were identified by PartitionFinder v2.1.1 (Lanfear et al., 2017) as follows: SYM+G for the first *cyt b* codon partition, HKY+G for the second *cyt b* codon partition, and GTR+G for the third *cyt b* codon partition. Bayesian analysis was conducted using MrBayes v3.2.22 (Ronquist et al., 2012). Four Markov chains were used, and the data were run for 20 000 000 generations to allow adequate time for convergence, with samples taken every 1 000 generations and the first 25% discarded as burn in.

Morphological data were recorded from 13 preserved adult

specimens. The sexes were identified based on vent characteristics (Fei et al., 2006; Liu, 1950). Measurements of all individuals were taken using digital calipers to the nearest 0.1 mm. The following measurements were obtained (Table 1): snout-vent length (SVL, from tip of snout to posterior angle of cloaca); head length (HL, from tip of snout to gular fold); head width (HW, width of head at widest point); tail length (TL, from posterior angle of cloaca to tip of tail); tail height (TH, maximum height of tail). The descriptions of morphological characteristics followed Liu (1950) and Fei et al. (2006). Vomerine tooth rows were examined with a Zeiss Stemi 2000-C dissecting microscope (Germany). Comparative morphological data of *Batrachuperus* were taken from previous publications (Fei et al., 2006; Liu, 1950).

All applicable international, national, and/or institutional guidelines for the care and use of animals were strictly followed. All animal sample collection protocols complied with the current laws of China.

The *cyt b* dataset contained 69 haplotypes (Supplementary Table S1). The haplotypes were clearly grouped into six major clades, i.e., *B. tibetanus*, *B. karlshmidtii*, *B. pinchonii* + *B. sp. 1*, *B. londongensis*, *B. yenyuanensis*, and samples from the Shaluli Mountains, with good nodal support (Bayesian posterior probability of 100), relatively long branches uniting the clades, and relatively short branches within the clades (Figure 1B). Haplotypes from most localities were phylogenetically closely related. The three exceptions were the Litang population (57), Yongning population (59), and Muli population (60). Two specimens from each population,

Table 1 Morphological measurements (mm) of adult *Batrachuperus daochengensis* sp. nov. individuals

No.	Status	Sex	Snout-vent	Head length	Head width	Tail length	Tail height
CIB108172	Holotype	F	106.1	26.0	18.1	78.7	10.1
CIB83584	Paratype	F	110.4	24.7	19.5	80.0	13.0
CIB83588	Paratype	F	88.5	22.1	16.8	76.5	11.1
CIB83589	Paratype	M	115.7	29.2	21.8	100	16.1
CIB83590	Paratype	M	108.2	27.4	18.9	104.7	13.3
CIB83591	Paratype	M	100.1	24.7	19.9	84.2	11.6
CIB99969	Paratype	F	84.8	21.8	16.3	73.0	11.8
CIB99971	Paratype	F	96.2	24.2	16.9	88.7	12.8
CIB99979	Paratype	M	110.3	28.5	20.4	112.7	15.4
CIB99980	Paratype	M	93.2	23.5	16.6	88.7	12.6
CIB99993	Paratype	F	90.0	25.7	17.4	81.9	12.0
CIB99994	Paratype	F	97.6	24.4	16.8	83.1	11.2
CIB108180	Paratype	M	92.9	26.1	21.6	90.1	15.8

M: Male; F: Female.

sequenced separately from Litang, Yongning, and Muli, were grouped into the *B. karlshmidti* clade and *B. daochengensis* **sp. nov.** clade, respectively. In addition, the mitochondrial gene data clearly indicated that the specimens distributed along the Shaluli Mountains, including the Daocheng and Xiangcheng areas, represented an independent evolutionary lineage. These specimens also possessed a combination of morphological characters different from those of all known congeners. Therefore, we describe them as a new species of the genus *Batrachuperus* below.

Taxonomic account

Batrachuperus daochengensis **sp. nov.** (Figure 1C–I)

Holotype: CIB108172 (Figure 1C–I), adult female, collected on 06 June 2006 by Zhi-Jun Liu from a mountain stream (N29°11.34', E100°06.61', 4 000 m a.s.l.), 2 km from Sangdui, Daocheng County, Ganzi Tibetan Autonomous Prefecture, Sichuan Province, China.

Paratypes: Twelve specimens, six adult females (CIB83584, CIB83588, CIB99969, CIB99971, CIB99993, and CIB99994) and six adult males (CIB83589, CIB83590, CIB83591, CIB99979, CIB99980, and CIB108180). Of these, two individuals (CIB83584 and CIB83588) were collected at the same locality as the holotype by Zhi-Jun Liu, Yu-Chi Zheng, and Bo Wang on 06 June 2006; four individuals (CIB83590, CIB83591, CIB99980, and CIB99994) were collected near the type locality (N29°11.341', E100°06.618', 4 100 m a.s.l.) by Zhi-Jun Liu on 08 May 2002; six individuals (CIB83589, CIB99969, CIB99979, CIB99971, CIB99993, and CIB108180) were collected from Shagong, Xiangcheng County, Ganzi Tibetan Autonomous Prefecture, Sichuan Province, China (N29°09.182', E99°56.028', 4 000 m a.s.l.) by Zhi-Jun Liu on 09 May 2002.

Diagnosis: The new species of *Batrachuperus* differs from all other species of the genus by the following combination of characters: brown horny epidermis on tips of fingers and toes absent; tubercles on each palm and sole absent; costal grooves 12; dorsal brown, mottled with blackish spots; fingers 2-3-4-1 in order of decreasing length; tips of longest digits of fore- and hindlimbs largely separated by one to two costal spaces when adpressed towards each other along sides of body.

Description of holotype: Medium-sized specimen (SVL 106.1 mm) in a good state of preservation (Figure 1C, D).

Head flattened (Figure 1E, F), 24.5% of SVL and longer than wide (HW/HL 69.62%); skin on throat very loose, with distinct gular fold, extending to dorsolateral sides of neck (Figure 1F, G); labial folds well developed, upper labial fold covering back of lower fold, also with fold but less well developed; angle of mouth reaching beyond posterior corner of eye (Figure 1G); shallow groove behind eye; both maxilla and mandible with tiny teeth; tongue small and elongate, with narrow free edges; vomerine tooth rows located on posterior portion of vomer in two slightly arched series between choanae and separated from each other by space twice as long as each vomerine tooth row, anterior portion of tooth

rows extends beyond anterior edge of internal naris, five teeth in left series and four teeth in right.

Body cylindrical and rather stout, with 12 costal grooves.

Limbs short, flattened as far as ends of digits; tips of longest digits of fore- and hindlimbs largely separated by two costal spaces when adpressed towards each other along sides of body; fingers four, 2-3-4-1 (Figure 1H); toes five, 3-2-4-1 (Figure 1I) in order of decreasing length; brown horny epidermis on tips of fingers and toes as well as palms and soles absent; no obvious palmar or tarsal tubercles (Figure 1H, I).

Tail strong, shorter than body, cylindrical at base, flattened gradually towards tip, obtuse at tip; tail with dorsal and ventral fins; dorsal fin moderately high, originating nearly at base, gradually increasing; ventral fin weak, originating nearly at end of tail (Figure 1D).

Color of holotype: In life, dorsal brown, mottled with blackish spots; ventral generally pale. In preservative, dorsal generally grayish or olive brown; ventral gray (Figure 1C, D).

Variation: Vomerine tooth rows vary in number and position of teeth. Number of teeth vary among individuals, e.g., usual number of vomerine tooth in each row three or four, but varies from two to eight in some specimens (Supplementary Figure S1A–C). Anterior portion of tooth rows does not extend beyond anterior edge of internal naris in one individual (CIB83589, Supplementary Figure S1C). Distance between limbs varies from one to two costal grooves when tips of longest digits of fore- and hindlimbs are adpressed towards each other along sides of body.

Distribution and habitat: *Batrachuperus daochengensis* **sp. nov.** is currently known from the central and southern Shaluli Mountains (Figure 1A). The new species is an aquatic salamander adapted to cold water environments. The holotype and paratypes were found under stones of small mountain streams at elevations ranging from 4 000 m to 4 100 m a.s.l. The breeding habits of this species are not well known.

Etymology: The name *daochengensis* is derived from the name of the county where the holotype was collected. We recommend “Daocheng Salamander” as its English common name and “稻城山溪鲵” as its Chinese common name.

Comparisons: *Batrachuperus daochengensis* **sp. nov.** can be distinguished from all known congeners (i.e., *B. pinchonii*, *B. tibetanus*, *B. karlshmidti*, *B. yenyuanensis*, and *B. londongensis*) in its overall morphology. It differs from *B. pinchonii* by a combination of the following: brown horny epidermis on tips of fingers and toes as well as palms and soles absent (vs. present); tubercles on each palm and sole absent (vs. two small tubercles present); The new species differs from *B. tibetanus* by: costal grooves 12 (vs. 13); space between tips of longest digits of fore- and hindlimbs (one to two costal spaces) when adpressed wider (vs. slightly overlapping or meeting); brown horny epidermis on tips of fingers and toes absent (vs. present). *Batrachuperus daochengensis* **sp. nov.** can be distinguished from *B. karlshmidti* by the following: blackish spots on dorsal present (vs. spots and marbling absent); space between vomerine

tooth rows wider (twice length of one vomerine tooth row vs. two-thirds to full length of one tooth row); costal grooves 12 (vs. 13). It differs from *B. yenyuanensis* by: SVL>TL (vs. SVL<TL); tubercles on each palm and sole absent (vs. two tubercles present); fingers 2-3-4-1 (vs. 3-2-4-1) in order of decreasing length. The new species can be distinguished from *B. londongensis* by: brown horny epidermis on tips of fingers and toes absent (vs. thick horny epidermis present); fingers 2-3-4-1 (vs. 3-2-4-1) in order of decreasing length.

Operational key to species of the genus *Batrachuperus*

- 1) Two tubercles on each palm and sole.....2
No tubercles on palms and soles.....3
- 2) Snout-vent length shorter than tail length.....
.....*B. yenyuanensis*
Snout-vent length longer than tail length.....
.....*B. pinchonii*
- 3) Blackish spots on dorsal.....4
No spots or marbling.....*B. karlschmidti*
- 4) Brown horny epidermis on tips of fingers and toes present.....5
Brown horny epidermis on tips of fingers and toes absent.....
.....*B. daochengensis* **sp. nov.**
- 5) Brown horny epidermis on palms and soles present.....
.....*B. londongensis*
Brown horny epidermis on palms and soles absent.....
.....*B. tibetanus*

Comments

The genus *Batrachuperus* once included species distributed on both sides of the Tibetan Plateau (Fu et al., 2001); however, phylogenetic study revealed that species distributed on the western side of the Tibetan Plateau (in Iran and Afghanistan) belonged to a different lineage and were therefore assigned to the genus *Paradactylodon* Risch, 1984 (Zhang et al., 2006; Zheng et al., 2011). Thus, the genus *Batrachuperus* only includes species distributed along the eastern slopes of the Tibetan Plateau (Fu et al., 2001), with the known distribution of the genus limited to western China.

The new species *B. daochengensis* **sp. nov.** was long considered to be *B. pinchonii* (Fei et al., 2006; Yang & Rao, 2008), but was identified as an independent lineage by Fu & Zeng (2008) based on partial *cyt b* sequences and allozyme data. Connecting samples from the four additional sites to previously published sequence data (Supplementary Table S1), we re-built the phylogeny of the genus *Batrachuperus* derived from partial *cyt b* sequences. Our Bayesian analysis results revealed a similar topology to previous studies on *Batrachuperus* sequences (Fu & Zeng, 2008; Lu et al., 2012). These molecular data, as well as morphological evidence, clearly indicate that *B. daochengensis* **sp. nov.** represents an independent evolutionary lineage, distinct from the remaining members of the genus. In the phylogenetic tree, *B. daochengensis* **sp. nov.** was most closely related to *B. yenyuanensis*. Thus, with the new description of *B. daochengensis* **sp. nov.**, six species are currently recognized in the genus *Batrachuperus*.

Batrachuperus salamanders are distributed along the montane eastern edge of the Tibetan Plateau, and each species occupies a relatively distinct geographical range (Figure 1A). *Batrachuperus tibetanus* is widely distributed along the Min Mountains to the western Qinling Mountains, thereby occupying the northern distribution of the genus. In the northwest, *B. karlschmidti* covers the upstream area of the Yalong River as well as the northern Shaluli Mountains. In the southwest, *B. daochengensis* **sp. nov.** is located in the central and southern Shaluli Mountains. *Batrachuperus yenyuanensis* ranges along the Daliang, Xiaoxiangling, and Jinping mountains of the downstream area of the Yalong River, thereby occupying the southern distribution of the genus. In the east, *B. pinchonii* is found along the Qionglai, Daxiangling, Xiaoxiangling, and Daliang mountains. *Batrachuperus londongensis* is also limited to the Daxiangling Mountains in the east. It is worth noting that some overlap of the species distribution ranges occurs in the Daxiangling Mountains between *B. londongensis* and *B. pinchonii*, Xiaoxiangling Mountains between *B. yenyuanensis* and *B. pinchonii*, and the species boundary between *B. karlschmidti* and *B. daochengensis* **sp. nov.**

Previous studies (Fu et al., 2001; Fu & Zeng, 2008) have also indicated that the populations in Penxian County and nearby areas represent another undescribed species (*B. sp.* 1). Thus, future studies should examine the morphological characteristics of specimens from this region to confirm their taxonomic identities.

NOMENCLATURAL ACTS REGISTRATION

The electronic version of this article in portable document format represents 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 (see Articles 8.5–8.6 of the Code). 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 can be viewed through any standard web browser by appending the LSID to the prefix <http://zoobank.org/>.

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SCIENTIFIC FIELD SURVEY PERMISSION INFORMATION

Permission for field surveys in Sichuan Province was granted by the Forestry Department and National Reserves of China.

SUPPLEMENTARY DATA

Supplementary data to this article can be found online.

COMPETING INTERESTS

The authors declare that they have no competing interests.

AUTHORS' CONTRIBUTIONS

J.L.X. and X.M.Z. designed the study. J.L.X., W.L., and X.M.Z. collected specimens in the field. W.L. performed molecular experiments and data analyses. J.L.X. and X.M.Z. performed morphological experiments. J.L.X. and W.L. wrote the manuscript. X.M.Z. revised the manuscript. All authors read and approved the final version of the manuscript.

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