ZOOLOGICAL RESEARCH

A new species of the genus *Raorchestes* (Anura: Rhacophoridae) from Yunnan Province, China

DEAR EDITOR,

A new bush frog species, *Raorchestes cangyuanensis* **sp. nov.**, from Cangyuan, Yunnan Province, China, is described based on morphological and molecular analyses. It differs from all known congeners by a combination of the following characters: body size small, adult snout-vent length (SVL) 16.1−20.0 mm in males (*n*=3); tympanum indistinct; tips of all fingers and toes expanded into discs with circummarginal grooves; rudimentary webbing between toes; fingers and toes with lateral dermal fringes; inner and outer metacarpal tubercles present; heels meeting when limbs held at right angles to body; crotch with a distinct black patch; discs of fingers and toes orange; male with external single subgular vocal sac and reddish nuptial pad at the base of first finger.

The genus *Raorchestes* Biju, Shouche, Dubois, Dutta, and Bossuyt, 2010 ranks in the top two most speciose genera of Rhacophoridae. *Raorchestes* is characterized by adult snout-vent lengths between 15.0 mm and 45.0 mm, no vomerine teeth, transparent/translucent vocal sac while calling, direct development without free-swimming tadpoles and nocturnal lifestyles (Biju et al., 2010; Vijayakumar et al., 2016). *Raorchestes* currently contains 62 species, ranging from the southern tip of the Indian Peninsula to northeastern India, Indo-China, and southwestern China (Frost, 2019). Thirty-two new species have been described in the last decade, entirely in India (Biju et al., 2010; Biju & Bossuyt, 2009; Padhye et al., 2013; Seshadri et al., 2012; Vijayakumar et al., 2014; Zachariah et al., 2011). Southeast Asia and southern China (SEA-SC) contain only four known species which are *R. gryllus*, *R. parvulus*, *R. menglaensis*, and *R. longchuanensis*, with no new species records for *Raorchestes* in SEA-SC in recent years. The high density of recently described species in other regions suggests that cryptic lineages may exist in more depauperate areas of *Raorchestes* distribution that have received less investigative attention.

Southwestern China is a global biodiversity hotspot that harbor a high diversity of amphibian species (AmphibiaChina, 2019; Myers et al., 2000), owed largely to its complicated topography (altitude ranges from <2 000 m in some valleys to 7 558 m a.s.l. at the summit of Gongga Mountain) and variety of habitats and climates. The amphibian fauna in southwestern China is rich in terms of species count and endemism (AmphibiaChina, 2019; Frost, 2019). In recent years, several cryptic and new species of amphibians have been described (Chen et al., 2017, 2018; Li et al., 2018; Lyu et al., 2019; Wang et al., 2019; Yang et al., 2016; Yang & Chan, 2018; Yu et al., 2019; Yuan et al., 2018). These results suggest that the rich amphibian diversity in the region still remains underestimated. During fieldwork in Cangyuan, Yunnan Province, southwestern China, we collected specimens that superficially resembled *R. menglaensis*, *R. longchuanensis*, and *R. parvulus*, which potentially occur in this region. We evaluated these individuals using molecular and morphological phylogenetic analyses. Based on an integrative taxonomic approach, we identified a distinct evolutionary lineage and describe it as a new species of the

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genus *Raorchestes*.

A total of three specimens were collected at night by locating calling males (Figure 1A). The frogs were euthanized using benzocaine, and liver tissues taken and preserved in 95% ethanol. The specimens were then fixed with 10% formalin for 24 hours and subsequently transferred to 70% ethanol for permanent preservation. Voucher specimens were deposited in the herpetological collection of the Museum of the Kunming Institute of Zoology (KIZ), Chinese Academy of Sciences (CAS).

Total genomic DNA was extracted, and a partial fragment of the mitochondrial 16S rRNA gene (16S) was amplified and sequenced. DNA extraction, primers, and PCR cycle protocols are shown in the Supplementary Methods. To study the historical relationships among *Raorchestes* species, matrilineal genealogies (phylogenetic trees) were reconstructed based on the 16S fragment. Homologous sequences of *Raorchestes* and representative outgroups (*Kurixalus idiootocus*, *K. eiffingeri*, *Polypedates cruciger*, and *Rhacophorus malabaricus*) were downloaded from GenBank (Supplementary Table S1). Phylogenetic relationships were inferred using maximum likelihood (ML) and Bayesian inference (BI) methods (Supplementary Methods). We also calculated pairwise sequence divergence using uncorrected *P*-distances implemented in MEGA v6.0.6 (Tamura et al., 2013).

The three preserved adult specimens were measured with digital calipers to the nearest 0.1 mm. Measurements followed Fei et al. (2009) and Poyarkov et al. (2017) (Supplementary Methods). Comparative data on the morphology and taxonomy of *Raorchestes* were obtained from previous publications (Bossuyt & Dubois, 2001; Fei et al., 2009; Fei et al., 2012; Kou, 1990; Kuramoto & Joshy, 2003; Padhye et al., 2013; Smith, 1924; Vijayakumar et al., 2014; Yang et al., 1978; Yu et al., 2019).

The topologies recovered by both ML and BI analyses were essentially identical, with relatively robust support for most terminal clades (Figure 1B). The monophyly of *Raorchestes* was strongly supported and in agreement with the results of Biju et al. (2010) and Vijayakumar et al. (2016). Among other members of the genus *Raorchestes*, the Cangyuan population represented a distinct phylogenetic lineage with strong support (Bayesian posterior probabilities=1; bootstrap support=100; Figure 1B). The genetic distance between the Cangyuan population and other described species of the genus ranged from 5.4% (with *R. gryllus*) to 14.1% (with *R. archeos*) (Supplementary Table S2). A 3% *P*-distance value for 16S rRNA is considered a useful indicator for new candidate species in frogs (Vieites et al., 2009). Thus, genetic divergence between the Cangyuan population and its congeners exceeds the proposed threshold for species-level differentiation in frogs. Morphologically, the newly identified matriline differed from all named species. Thus, we describe the new species of the genus *Raorchestes* below.

Taxonomic account

Raorchestes cangyuanensis **sp. nov.** Wu, Suwannapoom, Xu, Murphy et Che (Figure 1; Table 1)

Holotype: Adult male (KIZ015856) from Cangyuan County, Yunnan Province, China (N23.22542°, E99.22509°, 1 272 m a. s.l.), collected by Da-Hu Zou and Kai Xu on 25 May 2016.

Paratypes: Two males KIZ015855 and KIZ015857, collected by Da-Hu Zou and Kai Xu. Location and date are the same as those of the holotype.

Diagnosis: *Raorchestes cangyuanensis* **sp. nov.** is diagnosed as a member of the genus *Raorchestes* by the following morphological characters: small body size; vomerine teeth absent; nocturnally active; tips of all fingers and toes expanded into discs with circummarginal grooves. The new species is distinguished from geographically and molecularly relevant congeners by the following combination of characters: (1) body size small, adult SVL 16.1–20.0 mm in males (*n*=3); (2) tympanum indistinct; (3) tongue pyriform, with a deep notch at the posterior tip; (4) tips of all fingers and toes expanded into discs with circummarginal grooves; (5) no webbing between fingers; (6) rudimentary webbing between toes; (7) fingers and toes with lateral dermal fringes; (8) inner and outer metacarpal tubercles present; (9) inner metatarsal tubercle oval, outer metatarsal tubercle absent; (10) heels meeting when limbs held at right angles to body; (11) tibiotarsal articulation reaching anterior of eye when hindlimb is stretched along the side of the body; (12) dark brown interorbital triangle between eyes; (13) crotch with a distinct black patch; (14) discs of fingers and toes orange; (15) dorsal surface brown with a dark ") (" -shaped marking; (16) supratympanic fold distinct, from posterior corner of eye to above insertion of arm; (17) iris golden brown; (18) male with external single subgular vocal sac; and (19) reddish nuptial pad at the base of first finger.

Description of holotype (all measurements in mm; see Table 1): KIZ015856, adult male (Figure 1). Body size small (SVL= 20.0); head large, width of head larger than head length (maximum head width (HDW) =7.2; head length (HDL) =6.6); top of head relatively flat; snout rounded in profile, projecting beyond lower jaw; snout length almost equal to diameter of eye (eye diameter (ED) =2.6; snout length (SNT) =2.4); canthus rostralis rounded, loreal region slightly concave; tympanum indistinct; interorbital distance wider than upper eyelid width and internasal distance (interorbital distance (IOD) =2.4; width of upper eyelid (UEW) =1.6; internarial distance (IND) =2.0); interorbital distance between posterior margins of eyes 1.9 times that of anterior margins (the distance between anterior orbital borders (IFE) =3.5; the distance between posterior orbital borders (IBE) =6.8); nostril slightly closer to tip of snout than to anterior corner of eyes (snout-nostril distance (SN) =1.3; distance from nostril to eye (DNE) =1.5); tongue pyriform, with a deep notch at posterior tip; vomerine teeth absent; pineal ocellus absent; eyes moderately large (eye diameter (ED) =2.6) and protruding, pupil horizontal; supratympanic fold distinct, from posterior corner of eye to above insertion of arm.

Forelimbs fairly robust; forelimb length shorter than hand

Table 1 Measurements (mm) of *Raorchestes cangyuanensis* sp. nov.

Catalog No.	KIZ015855	KIZ015856*	KIZ015857
Sex	Male	Male	Male
SVL	19.3	20.0	16.1
HDL	6.4	6.6	5.5
HDW	7.2	7.2	6.2
SNT	2.5	2.4	2.3
DNE	1.4	1.5	1.1
IND	1.9	2.0	1.9
IOD	2.5	2.4	2.2
UEW	1.8	1.6	1.7
ED	2.8	2.6	2.3
SN	1.1	1.3	0.9
IFE	3.8	3.5	2.6
IBE	6.7	6.8	4.7
FAL	4.2	4.5	3.9
HL	5.3	5.4	4.9
THL	9.0	8.9	8.2
TL	9.0	9.1	7.7
FL	7.3	7.5	6.2
FLI	1.6	1.8	1.0
FLII	2.1	2.1	1.9
FLIII	3.3	3.7	3.5
FLIV	2.4	2.7	2.1
TLI	1.2	1.2	1.2
TLII	1.9	1.7	1.7
TLIII	3.1	2.9	2.0
TLIV	3.4	4.2	3.3
TLV	2.1	2.5	2.0

For abbreviations, see text and Supplementary Methods. Asterisk (*) indicates holotype.

length (forearm length (FAL) =4.5; hand length (HL) =5.4); relative finger lengths: I<II<IV<III (finger length (FL)I=1.8; FLII= 2.1; FLIII=3.7; FLIV=2.7); tips of all four fingers expanded into discs with circummarginal grooves; all fingers with lateral dermal fringes on both sides; subarticular tubercles distinct, rounded, formula: 1, 1, 1, 1; supernumerary tubercles absent; no webbing between fingers; inner and outer metacarpal tubercles present; male with reddish nuptial pad at the base of first finger (Figure 1H).

Hindlimbs long and relatively robust, thigh length (thigh length (THL)=8.9) shorter than tibia length (tibia length (TL)= 9.1), but greater than foot length (FL=7.5); tibiotarsal articulation reaching anterior of eye when hindlimb is stretched along the side of the body; heels meeting when limbs held at right angles to body; relative toe lengths: I<II<V< III<IV (toe length (TL) I=1.2; TLII=1.7, TLIII=2.9; TLIV=4.2; TLV=2.5); tips of toes with discs having circummarginal grooves, fewer than discs on fingers; all toes with lateral dermal fringes on both sides; subarticular tubercles distinct, rounded, formula: 1, 1, 1, 2, 1; supernumerary tubercles absent; rudimentary webbing between toes; inner metatarsal tubercle rounded, outer metatarsal tubercle absent (Figure 1G).

Dorsal surfaces rough with small granules, flank of body, dorsal part of forelimbs, thighs, and tibia relatively smooth and scattered with sparse granules; upper eyelid with several small granules; throat, chest, and ventral surfaces of forelimbs smooth; abdomen, underside of thigh, and around vent with granules; dorsolateral folds absent; surfaces of hands and feet with numerous granules (Figure 1).

Color of holotype in life: For color of holotype in life see Figure 1. Dorsal surface brown, with golden brown band between eyes; dorsal surface with a dark") ("-shaped marking; dark brown interorbital triangle between eyes; upper and lower lips with white and black dots; supratympanic fold dark brown; iris golden brown; dorsal parts of limbs with dark brown crossbars; crotch with a distinct black patch bordering large creamy white plaque below the black patch near the groin; dorsal thigh orange with two black crossbars; ventral surface body and limbs brown, with small black and white spots; discs of fingers and toes orange (Figure 1).

Color of holotype in preservative: Dorsum faded to dark brown; golden brown band between eyes still clear; a dark ") (" -shaped marking faintly present on dorsum; black patch present at crotch still distinct; large creamy white plaque below the black patch still clear; cross bands present on dorsal side of forelimbs and hind limb still clear; discs of fingers and toes fades to brown; throat, chest, abdomen and ventral surface of limbs dark brown, mottled with white dots (Supplementary Figure S1).

Male secondary sexual characteristics: Adult males possess nuptial pads covering the dorsal surface of the base of FI; external single subgular vocal sac; and slit-like opening at posterior of jaw.

Variation: Morphometric measurements of holotype and two paratypes are given in Table 1. Paratypes generally agree with the holotype morphologically, with the following exceptions: smaller body length for one of the three adult male specimens (KIZ015857); body length of KIZ015857, KIZ015855, and KIZ015856 16.1 mm, 19.3 mm, and 20.0 mm, respectively. KIZ015855 and KIZ015856 have light brown band between eyes, which is absent in paratype KIZ015857.

Distribution and habitat: *Raorchestes cangyuanensis* **sp. nov.** is known only from a single locality, Cangyuan County, Yunnan, China (N23.22542° , E99.22509°). The new species was found at an elevation of 1 272 m a.s.l. in shrubbery near streams.

Comparisons: Based on morphology, we compared *Raorchestes cangyuanensis* **sp. nov.** with morphologically,

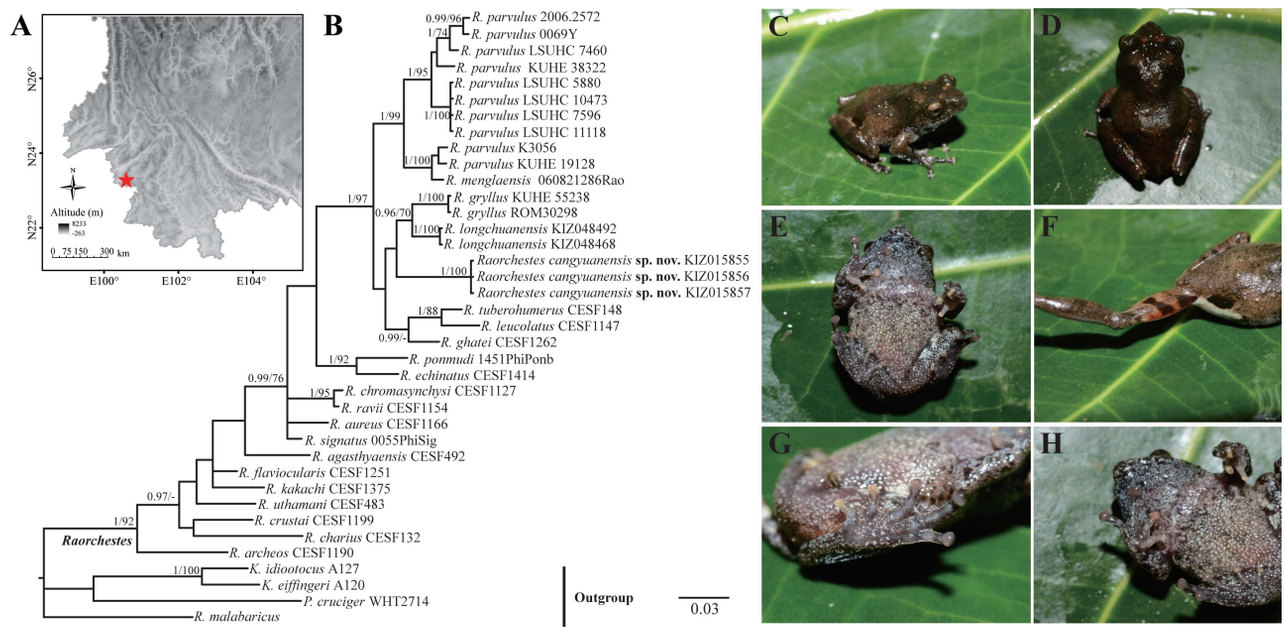

Figure 1 Distribution, Bayesian inference tree and holotype of *Raorchestes cangyuanensis* sp. nov.

A: Known distribution of *R. cangyuanensis* **sp. nov.** from Cangyuan, Yunnan Province, China. Star shows type locality of the new species. B: Phylogram of *Raorchestes* resulting from analyses of a fragment of the mitochondrial 16S rRNA gene. Nodal support values with Bayesian posterior probabilities (BPP)>95/bootstrap support (BS)>70 are shown near node. A "–" denotes bootstrap support<70. Node values with Bayesian posterior probabilities (BPP)<95 and bootstrap support (BS)<70 are not shown. Lateral (C), dorsal (D), ventral (E), crotch (F), ventral view of foot (G) and hand (H) of male specimen (KIZ015856) of *Raorchestes cangyuanensis* **sp. nov.** Photo by Da-Hu Zou.

geographically, and molecularly similar species.

Raorchestes cangyuanensis **sp. nov.** differs from *R. longchuanensis* in the following characters: tympanum indistinct in males (vs. distinct); lateral dermal fringes on all fingers and toes (vs. only on first and second fingers with lateral dermal fringes, lateral dermal fringes of toes absent); rudimentary webbing between toes (vs. 1/4 webbing); iris golden brown (vs. reddish brown). *Raorchestes cangyuanensis* **sp. nov.** differs from *R. menglaensis* by the following combination of characters: male with external single subgular vocal sac (vs. internal single subgular vocal sac); all fingers and toes with lateral dermal fringes (vs. absent); outer metatarsal tubercle absent (vs. present); discs of fingers and toes orange (vs. not orange). *Raorchestes cangyuanensis* **sp. nov.** differs from *R. gryllus* by the following combination of characters: SVL of adult male 16.1–20.0 mm (vs. 25.0–27.0 mm); rudimentary webbing between toes (vs. little more than half webbed); outer metatarsal tubercle absent (vs. present). *Raorchestes cangyuanensis* **sp. nov.** differs from *R. parvulus* in the following characters: tympanum indistinct (vs. distinct); toes with lateral dermal fringes (vs. lateral dermal fringes of fifth toe indistinct); SVL of adult male 16.1–20.0 mm (vs. 20.1–23.2 mm); supernumerary tubercles absent (vs. present on third finger); relative toe lengths: I<II<V<III<IV (vs. relative toe lengths: I<II<III<V<IV). *Raorchestes cangyuanensis* **sp. nov.** differs from *R. ghatei* in the following characters: reddish nuptial pad at the base of first finger (vs. absent); relative finger lengths: I<II<IV<III (vs. relative finger lengths: I<IV<II<III); inner and outer metatarsal tubercles present (vs. single metatarsal tubercle present); all toes with lateral dermal fringes on both sides (vs. absent); dorsal parts of limbs with dark brown crossbars (vs. without crossbars). *Raorchestes cangyuanensis* **sp. nov.** differs from *R. tuberohumerus* in the following characters: inner and outer metatarsal tubercles present (vs. inner metatarsal tubercle moderate, no outer metatarsal tubercle); relative finger lengths: I<II<IV<III (vs. relative finger lengths: I<IV≤II<III); crotch with a distinct black patch bordering large creamy white plaque below the black patch near the groin (vs. large yellow or reddish yellow markings); discs of fingers and toes orange (vs. grey to brown). *Raorchestes cangyuanensis* **sp. nov.** differs from *R. leucolatus* in the following characters: crotch with a distinct black patch bordering large creamy white plaque below the black patch near the groin (vs. groin region with white blotches); loreal region slightly concave (vs. loreal region flat); relative toe lengths: I<II<V<III<IV (vs. relative toe lengths: I<II<III<V<IV).

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

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SCIENTIFIC FIELD SURVEY PERMISSION INFORMATION

Permission for field surveys in Yunnan 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. C., C. S., and Y. H. W. designed the study. J. M. C. and K. X. collected specimens in the field. J.Q.J and H.M.C. performed molecular experiments. Y.H.W. performed data analyses, and wrote the manuscript. J.C., R.W.M., and C. S. revised the manuscript. All authors read and approved the final version of the manuscript.

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