Zoosyst. Evol. 101 (1) 2025, 103–117 | DOI 10.3897/zse.101.135586

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Description of a new leaf litter toad of *Leptobrachella* (Anura, Megophryidae) from Hunan, China

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https://zoobank.org/20764ABF-6164-4195-9F49-3F42D019EDD8

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Academic editor: Umilaela Arifin ♦ **Received** 26 August 2024 ♦ **Accepted** 18 December 2024 ♦ **Published** 16 January 2025

Abstract

A new leaf litter toad, *Leptobrachella yongshunensis* **sp. nov.**, is described on the basis of morphological, acoustic, and molecular data in this study. The new species was distributed in Xiaoxi National Nature Reserve, Yongshun County, Xiangxi Tujia and Miao Autonomous Prefecture, Hunan Province, China. Phylogenetical analysis revealed that the new species is sister species of *L. wulingensis* (p-distance 0.019 in 16s rRNA gene, p-distance 0.073 in COI gene). The duration of advertisement call was 194.2 ± 6.7 ms, the mean dominant frequency of the first note was 3.885 ± 0.066 kHz, and the mean dominant frequency of the second note was 3.914 ± 0.052 kHz. The new species can be distinguished from its congers by the following morphological characters: snout-vent length (SVL) 27.2–28.9 in males, SVL 26.2–31.6 in females; black spots on flanks; toes webbing rudimentarily; narrow fringes on toes; creamy white ventral body with indistinct black speckling at margins; dorsal body with sparse large warts, dense little wart grains, and longitudinal ridges; head width greater than head length; tibiotarsal articulation reaching to anterior edge of the eye; brick-red color in the dorsal surface; upper parts of iris bright coppery in life. We still supplemented the molecular data of the COI gene of *L. wulingensis* for further research. The discovery of the new species not only enhances the species diversity of the Wuling Mountains, but also suggests the hidden species diversity in the area.

Key Words

Character, diversity, *Leptobrachella*, sister taxon

Introduction

The *Leptobrachella* Smith, 1925 (Anura, Megophryidae) species, Asian leaf litter toads, exhibit a broad distribution ranging from southern China, west to northeastern India and Myanmar, through mainland Indochina to peninsular Malaysia and the island of Borneo (Chen et al. 2018; Frost, 2024). The genus *Leptobrachella* possesses a convoluted classification history, featuring as many as five synonyms, namely, *Paramegophrys* (Liu, 1964), *Carpophrys* (Sichuan Biological Research Institute, 1977), *Leptolalax* (Dubois, 1980), *Lalax* (Delorme, Dubois, Grosjean & Ohler, 2006), and *Lalos* (Dubois, Grosjean, Ohler, Adler & Zhao, 2010), which was ultimately clarified by Chen et al. (2018).

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So far, this genus contains 106 species (Li et al. 2024; Chen et al. 2024; Ninh et al. 2024; Hoang et al. 2024), among which three are clearly distributed in Hunan, China: *L. wulingensis*, *L. mangshanensis*, and *L. dong* (Qian et al. 2020; Liu et al. 2023). Recently, several specimens of *Leptobrachella* were collected during the field scientific expedition in Hunan Xiaoxi National Nature Reserve, Yongshun County, Xiangxi Autonomous, Hunan Province, China. Morphological comparisons, molecular phylogenetic analyses, and bioacoustic comparisons consistently indicated that this population should be regarded as a new species, and we named it *L. yongshunensis* sp. nov.

Materials and methods

Sampling

In March and July 2024, as well as July 2020, eleven specimens of genus *Leptobrachella* (voucher number: JSUHJ2024001–JSUHJ2024008, JSUWT2020001–JSUWT2020003) were collected from Hunan Xiaoxi National Nature Reserve (Fig. 1). Each specimen was anesthetized with alcohol and fixed in 80% ethanol for two days and finally transferred to 70% ethanol for preservation. Tongue tip muscle tissues of the specimens (JSUHJ2024001–JSUHJ2024008) were sampled for molecular analysis. All holotype and paratype specimens were stored at animal specimen room in Jishou University (JSU).

For molecular phylogenetic analyses, another six tissue samples (fingertip) of *Leptobrachella wulingensis* (voucher number: JSUJWS2024422, JSUJWS2024424, JSUJWS2024426, JSUJWS2024428, JSUJWS20221121, JSUJWS20221122) from Zhangjiajie Froest National Park were collected (Fig. 1).

The permissions for field surveys for scientific purposes were approved by the local Bureau of the National Nature Reserve, and the sample collections and experiment protocols were approved by the Biomedical Ethics Committee of JSU (NO: JSDX-2024-0083), adhering to the relevant laws and guidelines of China.

Molecular phylogenetic analyses

A total of 14 tissue samples were collected for DNA extraction, which was performed by using the Eaxp Sexual Animal Genome DNA Extraction Kit (Shenggong Biotechnology Co. Ltd., Shanghai). The primers used for cytochrome c oxidase subunit 1 (COI) gene and 16s rRNA gene sequence amplification were Chmf4 (5′-TYCWACWAAYCAYAAAGAYATCGG-3′), Chmr4 (5′-ACYTCRGGRTGRCCRAARAATCA-3′) (Che et al. 2012) and P7 (5′-CGCCTGTTTACCAAAAACAT-3′), P8 (5′-CCGGTCTGAACTCAGATCACGT-3′) (Simon et al. 1994), respectively. The PCR reaction system was 25 μL, including 12.5 μL of PCR Master mix (Beijing Kangwei Century), 1 μL each of upstream and downstream primers, 1 μL of template DNA, and 9.5 μL of ddH_2O. Amplifications were performed in a 25 reaction volume under the following cycling conditions: an initial denatured step at 94 °C for 5 min, 35 cycles of denatured step at 94 °C for 45 s, annealing at 46 °C (for COI)/52 °C (for 16s rRNA) for 30 s, extending at 72 °C for 1 min, and a final extending step of 72 °C for 10 min. The PCR products were detected by 1% agarose gel electrophoresis and then sent to Shenggong Biotechnology (Shanghai) Co., Ltd. for sequencing. All new sequences were deposited in GenBank (Suppl. material 1: table S1).

All sequences were assembled in DNAMAN v10.3.6.158 software. In addition, 111 16s rRNA gene sequences and 18 COI gene sequences were downloaded from the Genbank for analysis (including two 16s rRNA gene sequences and two COI gene sequences that belong to *Megophrys glandulosa* and *Leptobrachium huashen* as outgroups; Suppl. material 1: table S1). In PhyloSuite v1.2.3 (Zhang et al. 2020), based on the combined sequences of 16s rRNA gene and COI gene, we constructed a Bayesian phylogenies using MRBAYES v3.2.7a (Ronquist et al. 2012) and a Maximum likelihood phylogenies using IQ-TREE v2.2.0 (Nguyen et al. 2015). Maximum likelihood phylogenies were inferred under the GTR+R5+F model selected by MODELFINDER v2.2.0 (Kalyaanamoorthy et al. 2017) according to AIC criterion for 50000 ultrafast (Minh et al. 2013) bootstraps. Bayesian Inference phylogenies were inferred under GTR+I+G+F model (2 parallel runs, 2×10^6 generations) selected by MODELFINDER v2.2.0 (Kalyaanamoorthy et al. 2017) according to BIC criterion, in which the initial 25% of sampled data were discarded as burn-in. Additionally, uncorrected p-distance of COI and 16s rRNA gene were conducted in MEGA v.11.0.13 (Tamura et al. 2021).

Bioacoustics analysis

Two types of calls were recorded using a SONY PCM-A10 recorder, and the temperature was measured using a digital hygrothermograph. ADOBE AUDITION cc v11.1.1.3 was used to analyze the call recordings. All acoustic parameters were defined in accordance with Köhler et al. (2017).

Morphological comparisons

We examined the characters of unidentified specimens using a vernier caliper (to the nearest 0.1 mm). Abbreviations of character used in this study are as follows: eye diameter (ED, distance from the anterior corner to the posterior corner of the eye); foot length (FL, distance from tarsus to the tip of the fourth toe); 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); hind-limb length (HLL, distance from tip of fourth toe to vent); internasal distance (IND, minimum distance between the inner margins of the external nares); interorbital

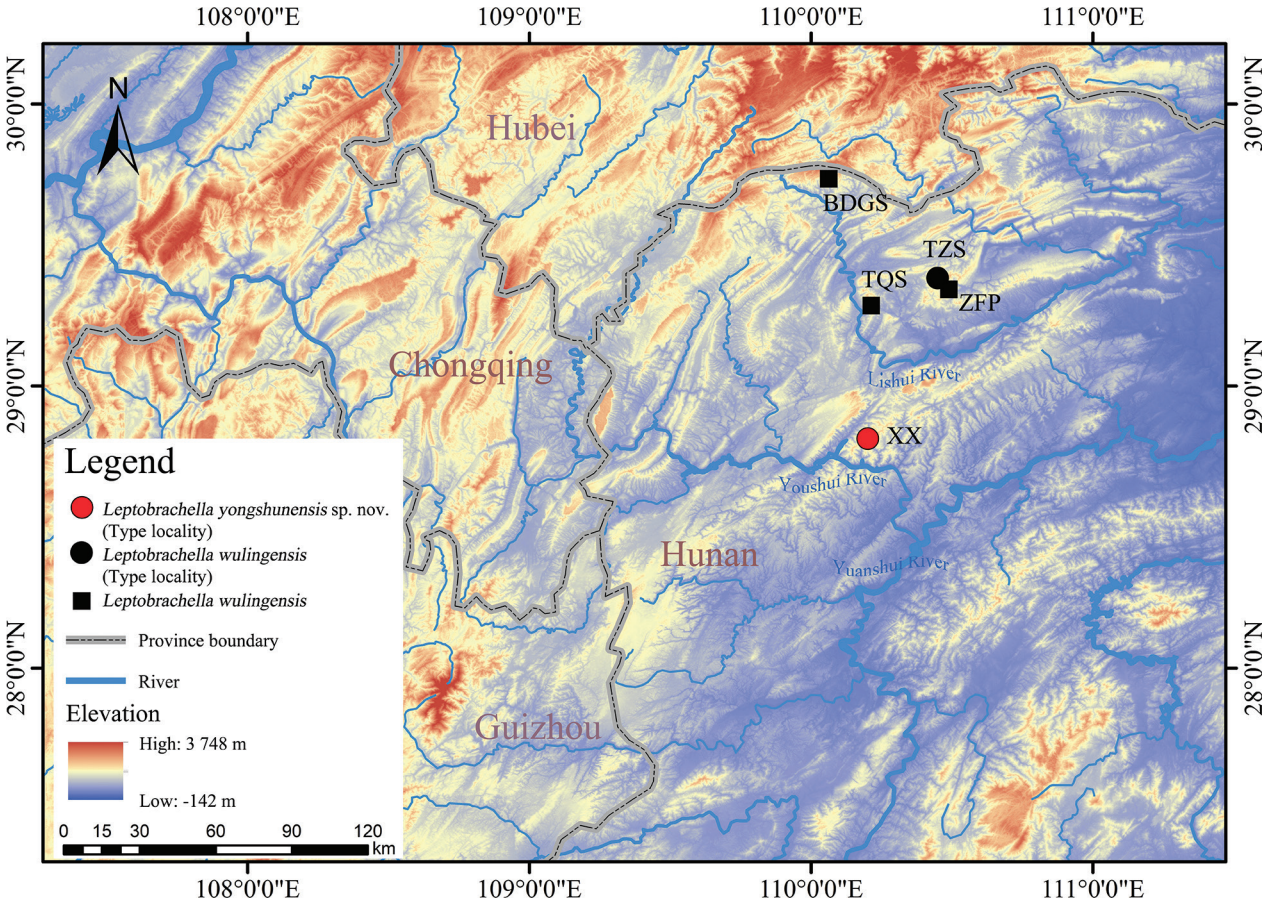

Figure 1. Distribution of the new species and its sister taxon. Characters in the map: XX, Hunan Xiaoxi National Nature Reserve; ZFP, Zhangjiajie Froest National Park; TQS, Hunan Tianquanshan National Forest Park; TZS, Tianzishan Provincial Nature Reserve; BDGS, Badagongshan National Nature Reserve (including Tianping mountains refers from Qian et al. 2023); Lishui River, "澧水" in Chinese; Youshui River, "酉水" in Chinese; Yuanshui River, "沅水" in Chinese. (Distribution of *L. wulingensis* refers to Qian et al. 2020; Qian et al. 2023)

distance (IOD, minimum distance between the inner edges of the upper eyelids); length of lower arm and hand (LAL, distance from the elbow to the distal end of finger IV); snout length (SL, distance from the tip of the snout to the anterior corner of the eye); snout-vent length (SVL, distance from the tip of the snout to the vent); maximal tympanum diameter (TD); tibia length (TL, distance from knee to tarsus); tibia width (TW, the widest length of the tibia); upper eyelid width (UEW, greatest width of the upper eyelid margins measured perpendicular to the anterior-posterior axis); distance from the anterior edge of tympanum to posterior corner of eye (TEY); manus length from the tip of third digit to the base of inner palmar tubercle (ML). Sex was determined by the presence/absence of a vocal sac. All other species comparison data come from reliable literatures (Table 1).

The value from the ratio of each measurement to SVL was calculated for the following morphometric analyses to reduce the impact of allometry (detailed comparative data presented in Suppl. material 1: table S2). Mann–Whitney U analysis was used to test the significance of differences on morphometric characters between the new species and its sister taxon. The significance level was set at 0.05. Furthermore, principal component analyses (PCA) were conducted to show the spatial distribution of different species on the morphometric characters, using R software (R Development Core Team).

Results

Phylogenetic analyses

The molecular analyses of combined sequences of 16s rRNA and COI gene conducted using Bayesian and maximum likelihood methods produced similar topologies, as depicted in Fig. 2. Phylogenetic tree indicated that the *Leptobrachella* population from Hunan Xiaoxi National Nature Reserve form a monophyletic clade (ML = 98, BI = 1), and is the sister group of *L. wulingensis* (ML = 98, BI = 0.99).

Furthermore, the mean genetic divergence of the 16s rRNA gene fragments between the *Leptobrachella* population from Hunan Xiaoxi National Nature Reserve and all other available homologous sequences of *Leptobrachella* species was 0.019–0.216, and for the COI gene, it was 0.073–0.202. (details in Suppl. material 1: tables S3, S4).

Table 1. Data source of the currently known species of the genus *Leptobrachella*.

ID	Species	References
1	*L. aerea* (Rowley, Stuart, Richards, Phimmachak & Sivongxay, 2010)	Rowley et al. 2010a
2	*L. alpina* (Fei, Ye & Li, 1990)	Fei et al. 1990
3	*L. applebyi* (Rowley & Cao, 2009)	Rowley and Cao 2009
4	*L. arayai* (Matsui, 1997)	Matsui 1997
5	*L. ardens* (Rowley, Tran, Le, Dau, Peloso, Nguyen, Hoang, Nguyen & Ziegler, 2016)	Rowley et al. 2016
6	*L. aspera* Wang, Lyu, Qi & Wang, 2020	Wang et al. 2020
7	*L. baluensis* Smith, 1931	Smith 1931
8	*L. bashaensis* Lyu, Dai, Wei, He, Yuan, Shi, Zhou, Ran, Kuang, Guo, Wei & Yuan, 2020	Lyu et al. 2020
9	*L. bidoupensis* (Rowley, Le, Tran & Hoang, 2011)	Rowley et al. 2011
10	*L. bijie* Wang, Li, Li, Chen & Wang, 2019	Wang et al. 2019
11	*L. bondangensis* Eto, Matsui, Hamidy, Munir & Iskar, 2018	Eto et al. 2018
12	*L. botsfordi* (Rowley, Dau & Nguyen, 2013)	Rowley et al. 2013
13	*L. bourreti* (Dubois, 1983)	Dubois 1983
14	*L. brevicrus* Dring, 1983	Dring 1983; Eto et al. 2015
15	*L. chishuiensis* Li, Liu, Wei & Wang, 2020	Li et al. 2020
16	*L. crocea* (Rowley, Hoang, Le, Dau & Cao, 2010)	Rowley et al. 2010b
17	*L. damingshanensis* Chen, Yu, Cheng, Meng, Wei, Zhou, Lu, 2021	Chen et al. 2021c
18	*L. dong* Liu, Shi, Li, Zhang, Xiang, Wei & Wang, 2023	Liu et al. 2023
19	*L. dorsospina* Wang, Lyu, Qi & Wang, 2020	Wang et al. 2020
20	*L. dushanensis* Li, Li, Cheng, Liu, Wei, Wang, 2024	Li et al. 2024
21	*L. dringi* (Dubois, 1987)	Dubois1987
22	*L. eos* (Ohler, Wollenberg, Grosjean, Hendrix, Vences, Ziegler & Dubois, 2011)	Ohler et al. 2011
23	*L. feii* Chen, Yuan & Che, 2020	Chen et al. 2020
24	*L. firthi* (Rowley, Hoang, Dau, Le & Cao, 2012)	Rowley et al. 2012
25	*L. flaviglandulosa* Chen, Wang & Che, 2020	Chen et al. 2020
26	*L. fritinniens* (Dehling & Matsui, 2013)	Dehling and Matsui2013
27	*L. fuliginosa* (Matsui, 2006)	Matsui2006
28	*L. fusca* Eto, Matsui, Hamidy, Munir & Isk & ar, 2018	Eto et al. 2018
29	*L. gracilis* (Günther, 1872)	Dehling2012a
30	*L. graminiCOla* Nguyen, Tapley, Nguyen, Luong & Rowley, 2021	Nguyen et al. 2021
31	*L. guinanensis* Chen, Li, Peng, Liu & Huang, 2024	Chen et al. 2024
32	*L. hamidi* (Matsui, 1997)	Matsui1997
33	*L. heteropus* (Boulenger, 1900)	Boulenger1900
34	*L. isos* (Rowley, Stuart, Neang, Hoang, Dau, Nguyen & Emmett, 2015)	Rowley et al. 2015
35	*L. itiokai* Eto, Matsui & Nishikawa, 2016	Eto et al. 2016
36	*L. jinshaensis* Cheng, Shi, Li, Liu, Li & Wang, 2021	Cheng et al. 2021
37	*L. jinyunensis* Shi, Shen, Wang, Jiang & Wang, 2023	Shi et al. 2023
38	*L. juliandringi* Eto, Matsui & Nishikawa, 2015	Eto et al. 2015
39	*L. kajangensis* (Grismer, Grismer & Youmans, 2004)	Grismer et al. 2004
40	*L. kalonensis* (Rowley, Tran, Le, Dau, Peloso, Nguyen, Hoang, Nguyen & Ziegler, 2016)	Rowley et al. 2016
41	*L. kecil* (Matsui, Belabut, Ahmad & Yong, 2009)	Matsui et al. 2009
42	*L. khasiorum* (Das, Tron, Rangad & Hooroo, 2010)	Das et al. 2010
43	*L. korifi* Matsui, Panha & Eto, 2023	Matsui et al. 2023
44	*L. lateralis* (Anderson, 1871)	Anderson 1871; Humtsoe et al. 2008
45	*L. laui* (Sung, Yang & Wang, 2014)	Sung et al. 2014
46	*L. liui* (Fei & Ye, 1990)	Fei et al. 1990; Sung et al. 2014
47	*L. macrops* (Duong, Do, Ngo, Nguyen & Poyarkov, 2018)	Duong et al. 2018
48	*L. maculosa* (Rowley, Tran, Le, Dau, Peloso, Nguyen, Hoang, Nguyen & Ziegler, 2016)	Rowley et al. 2016
49	*L. mangshanensis* (Hou, Zhang, Hu, Li, Shi, Chen, Mo & Wang, 2018)	Hou et al. 2018
50	*L. maoershanensis* (Yuan, Sun, Chen, Rowley & Che, 2017)	Yuan et al. 2017
51	*L. marmorata* (Matsui, Zainudin & Nishikawa, 2014)	Matsui et al. 2014b
52	*L. maura* (Inger, Lakim, Biun & Yambun, 1997)	Inger et al. 1997
53	*L. melanoleuca* (Matsui, 2006)	Matsui2006
54	*L. melica* (Rowley, Stuart, Neang & Emmett, 2010)	Rowley et al. 2010a
55	*L. minima* (Taylor, 1962)	Ohler et al. 2011; Taylor1962
56	*L. mjobergi* Smith, 1925	Smith 1925
57	*L. murphyi* Chen, Suwannapoom, Wu, Poyarkov, Xu, Pawangkhanant & Che, 2021	Chen et al. 2021a
58	*L. nahangensis* (Lathrop, Murphy, Orlov & Ho, 1998)	Lathrop et al. 1998

ID	Species	References
59	*L. namdongensis* Hoang, Nguyen, Luu, Nguyen & Jiang, 2019	Hoang et al. 2019
60	*L. natunae* (Günther, 1895)	Günther 1895
61	*L. neangi* Stuart & Rowley, 2020	Stuart and Rowley 2020
62	*L. niveimontis* Chen, Poyarkov, Yuan & Che, 2020	Chen et al. 2020
63	*L. nokrekensis* (Mathew & Sen, 2010)	Mathew and Sen 2010
64	*L. nyx* (Ohler, Wollenberg, Grosjean, Hendrix, Vences, Ziegler & Dubois, 2011)	Ohler et al. 2011
65	*L. oshanensis* (Liu, 1950)	Liu1950; Shi et al. 2023
66	*L. pallida* (Rowley, Tran, Le, Dau, Peloso, Nguyen, Hoang & Nguyen, 2016)	Rowley et al. 2016
67	*L. palmata* Inger & Stuebing, 1992	Inger and Stuebing 1992
68	*L. parva* Dring, 1983	Dring 1983
69	*L. pelodytoides* (Boulenger, 1893)	Boulenger 1893; Ohler et al. 2011
70	*L. petrops* (Rowley, Dau, Hoang, Le, Cutajar & Nguyen, 2017)	Rowley et al. 2017b
71	*L. phiadenensis* Luong, Hoang, Pham, Ziegler, & Nguyen, 2023	Luong et al. 2023
72	*L. phiaoacensis* Luong, Hoang, Pham, Ziegler, & Nguyen, 2023	Luong et al. 2023
73	*L. picta* (Malkmus, 1992)	Malkmus 1992
74	*L. pingbianensis* (Rao, Hui, Zhu & Ma, 2020)	Zhu and Rao 2020
75	*L. platycephala* (Dehling, 2012)	Dehling 2012b
76	*L. pluvialis* (Ohler, Marquis, Swan & Grosjean, 2000)	Ohler et al. 2000
77	*L. puhoatensis* (Rowley, Dau & Cao, 2017)	Rowley et al. 2017a
78	*L. purpuraventra* Wang, Li, Li, Chen & Wang, 2019	Wang et al. 2019
79	*L. purpurus* (Yang, Zeng & Wang, 2018)	Yang et al. 2018
80	*L. pyrrhops* (Poyarkov, Rowley, Gogoleva, Vassilieva, Galoyan & Orlov, 2015)	Poyarkov et al. 2015
81	*L. rowleyae* (Nguyen, Poyarkov, Le, Vo, Ninh, Duong, Murphy & Sang, 2018)	Nguyen et al. 2018
82	*L. sabahmontana* (Matsui, Nishikawa & Yambun, 2014)	Matsui et al. 2014a
83	*L. serasanae* Dring, 1983	Dring 1983
84	*L. shangsiensis* Chen, Liao, Zhou & Mo, 2019	Chen et al. 2019
85	*L. shimentaina* Wang, Lyu & Wang, 2022	Wang et al. 2022
86	*L. shiwandashanensis* Chen, Peng, Pan, Liao, Liu & Huang, 2021	Chen et al. 2021b
87	*L. sinorensis* Matsui, Panha & Eto, 2023	Matsui et al. 2023
88	*L. sola* (Matsui, 2006)	Matsui 2006
89	*L. suiyangensis* Luo, Xiao, Gao & Zhou, 2020	Luo et al. 2020
90	*L. sungi* (Lathrop, Murphy, Orlov & Ho, 1998)	Lathrop et al. 1998
91	*L. tadungensis* (Rowley, Tran, Le, Dau, Peloso, Nguyen, Hoang, Nguyen & Ziegler, 2016)	Rowley et al. 2016
92	*L. tamdil* (Sengupta, Sailo, Lalremsanga, Das & Das, 2010)	Sengupta et al. 2010
93	*L. tengchongensis* (Yang, Wang, Chen & Rao, 2016)	Yang et al. 2018
94	*L. tuberosa* (Inger, Orlov & Darevsky, 1999)	Inger et al. 1999
95	*L. ventripunctata* (Fei, Ye & Li, 1990)	Fei et al. 1990
96	*L. verrucosa* Wang, Zeng, Lin & Li, 2022	Lin et al. 2022
97	*L. wuhuangmontis* Wang, Yang & Wang, 2018	Wang et al. 2018
98	*L. wulingensis* Qian, Xiao, Cao, Xiao & Yang, 2020	Qian et al. 2020
99	*L. wumingensis* Chen, Peng, Li & Yu, 2023	Chen et al. 2023
100	*L. yeae* Shi, Hou, Song, Jiang & Wang, 2021	Shi et al. 2021
101	*L. yingjiangensis* (Yang, Zeng & Wang, 2018)	Yang et al. 2018
102	*L. yunkaiensis* Wang, Li, Lyu & Wang, 2018	Wang et al. 2018
103	*L. yunyangensis* Luo, Deng & Zhou, 2022	Luo et al. 2022
104	*L. zhangyapingi* (Jiang, Yan, Suwannapoom, Chomdej & Che, 2013)	Jiang et al. 2013
105	*L. huynhi* Hoang, Luong, Nguyen, Nguyen, Ninh, Le, Ziegler & Pham, 2024	Hoang et al. 2024
106	*L. aurantirosea* Ninh, Nguyen, Le, Nguyen, Quoc, Orlov, Bezman-Moseyko, Le, Nguyen & Ziegler, 2024	Ninh et al. 2024

Bioacoustics

Two types of calls of the new species were documented during field investigations when the spring temperature was 12.3 °C, with each call being recorded for more than 30 seconds (Type A and Type B; Fig. 3). As Type A is the predominant call during the breeding season survey in March, this constitutes an advertisement call with taxonomic significance (Köhler et al. 2017).

For Type A (advertisement call), the first note contained two similar pulses with a mean duration of 40.4 ± 12.1 ms (range = 28.4–58.6 ms), and the second note contained three (rarely four) decreasing pulses with a longer mean duration of 75.2 ± 4.1 ms (range = 70.4–79.3 ms). The call intervals between the first note and the second ranged from 78.6 ± 7.8 ms (range = 68.9–86.9 ms), and the call intervals between the

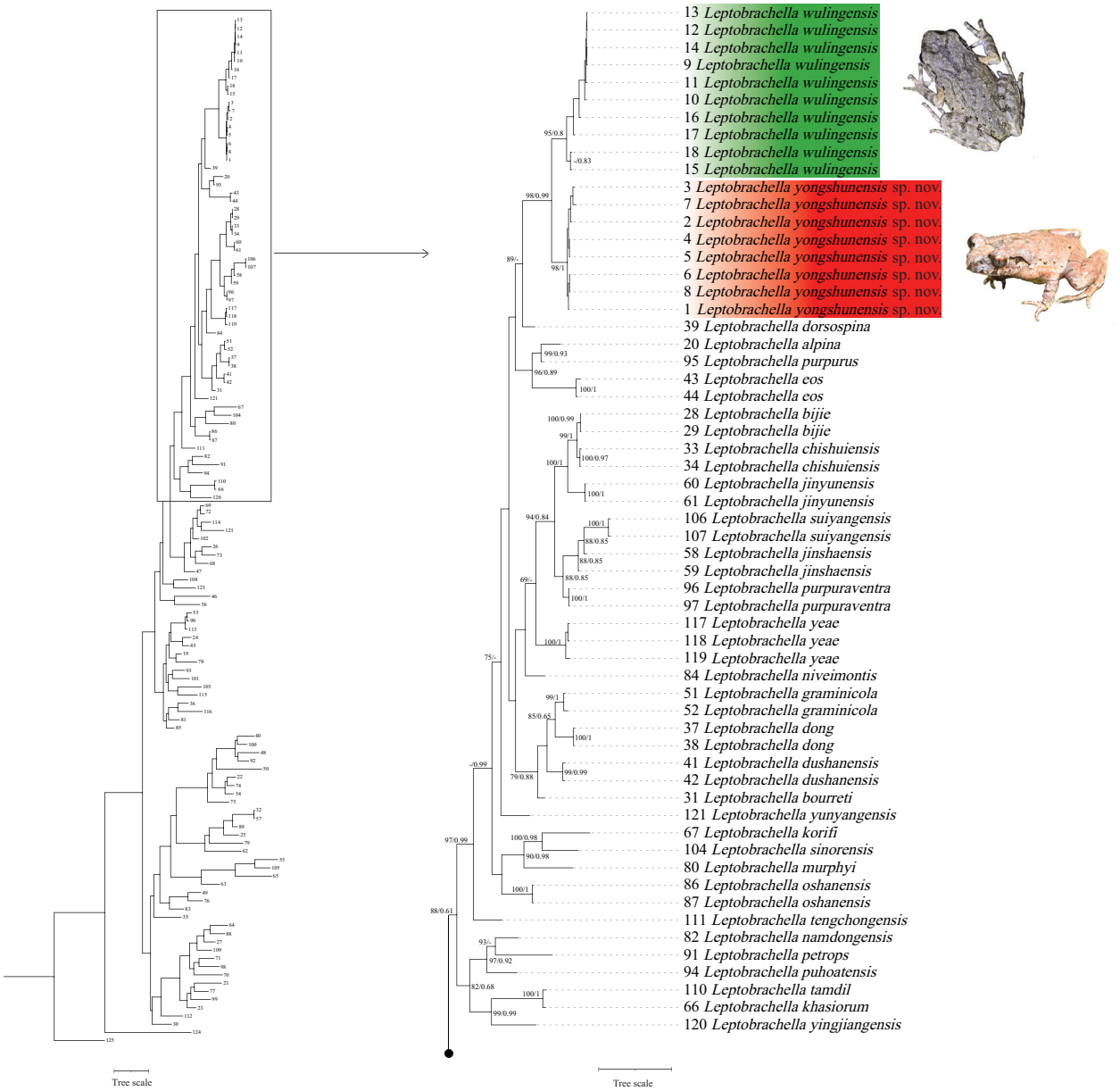

Figure 2. BI tree based on the 16s rRNA gene (538 bps) and COI gene (583 bps) combined sequences. Node support is indicated on branches as Maximum likelihood support (ML) and Bayesian posterior probabilities (BI), the ones lower than 60 or 0.6 are displayed as "-" or not displayed. Photos by Wan-Sheng Jiang and Tao Wu.

second note and next chirp ranged from 151.7 ± 6.8 ms (range = 145.2–158.7 ms). The mean dominant frequency of the first note was 3.885 ± 0.066 kHz (range = 3.811–3.963 kHz), and the mean dominant frequency of the second note was 3.914 ± 0.052 kHz (range = 3.886–3.985 kHz). The duration of Type A call was 194.2 ± 6.7 ms (range = 190.1–203.6 ms). For Type B, the call durations had an average of 212.9 ± 6.1 ms (range = 205.7–221.1 ms), and the call intervals had an average of 359.9 ± 138.7 ms (range = 262.2–565.5 ms). The mean dominant frequency of Type B call was 3.979 ± 0.063 kHz (range = 3.916–4.042 kHz).

Morphological analyses

All new species specimens have been measured, refer to Suppl. material 1: table S5 for details. The results of Mann–Whitney U tests revealed significant differences between new species and its sister species, *L. wulingensis*, in various measurements in all males, females and subadults (details in Suppl. material 1: table S2). In males, there are significant differences between the two in ED/SVL and IOD/SVL. In females, significant differences are exhibited between the two in ED/SVL, HDL/SVL, IOD/SVL, TD/SVL and TEY/SVL. In subadults,

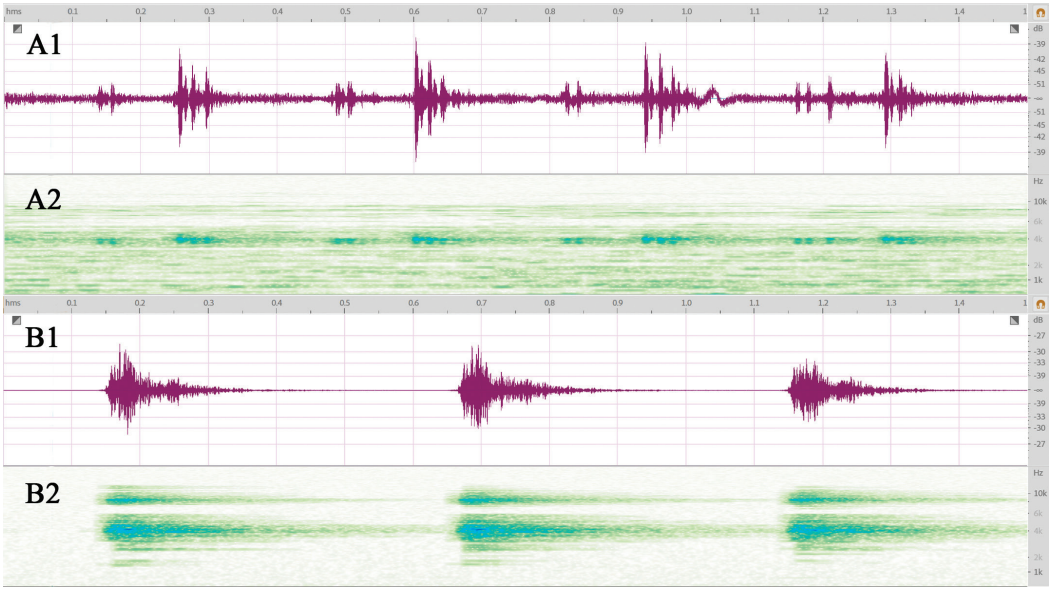

Figure 3. Two type (**A, B**) advertisement calls spectrograms of *L. yongshunensis* sp. nov., displaying the sound characteristics within 1.5 seconds (**A1, B1.** Waveforms; **A2, B2.** Spectrograms).

significant differences are exhibited between the two in ED/SVL, HDL/SVL, IOD/SVL, TD/SVL, TEY/SVL and ML/SVL. In PCA, males, females, and subadults of the two species can be clearly distinguished, leading to a distinct differentiation between the new species and *L. wulingensis* (Fig. 4). The new species still has the following morphological differences from *L. wulingensis*: head width greater than the head length (vs. head length greater than head width); tibiotarsal articulation reaching the anterior edge of the eye (vs. reaching the middle of the eye); brick-red color in dorsal surface (vs. brown); upper parts of iris bright coppery in life (vs. orange/golden).

The diagnostic characters for the new species and the *Leptobrachella* species occurring north of the Isthmus of Kra are presented in Suppl. material 1: table S6. The new species also can be distinguished from several *Leptobrachella* species (*L. arayai*, *L. dringi*, *L. fritinniens*, *L. gracilis*, *L. hamidi*, *L. heteropus*, *L. kajangensis*, *L. kecil*, *L. marmorata*, *L. maura*, *L. melanoleuca*, *L. picta*, *L. platycephala*, *L. sabahmontana and L. sola*) from south of the Isthmus of Kra, Malay Peninsula by the presence of supra-axillary and ventrolateral glands (vs absent in the latter). The new species can still be distinguished from other *Leptobrachella* species from south of the Isthmus of Kra, Malay Peninsula by its moderate body size (27.2–28.9 mm in three adult males, 26.2–31.6 mm in three adult females): *L. baluensis* (14.9–15.9 mm in males), *L. bondangensis* (17.8 mm in male), *L. brevicrus* (17.1–17.8 mm in males), *L. fusca* (16.3 mm in males), *L. itiokai* (15.2–16.7 mm in males), *L. juliandringi* (17.0–17.2 mm in males), *L. mjobergi* (15.7–19.0 mm in males), *L. natunae* (17.6 mm in one adult male), *L. palmata* (14.4–16.8 mm in males), *L. parva* (15.0–16.9 mm in males), and *L. serasanae* (16.9 mm in females).

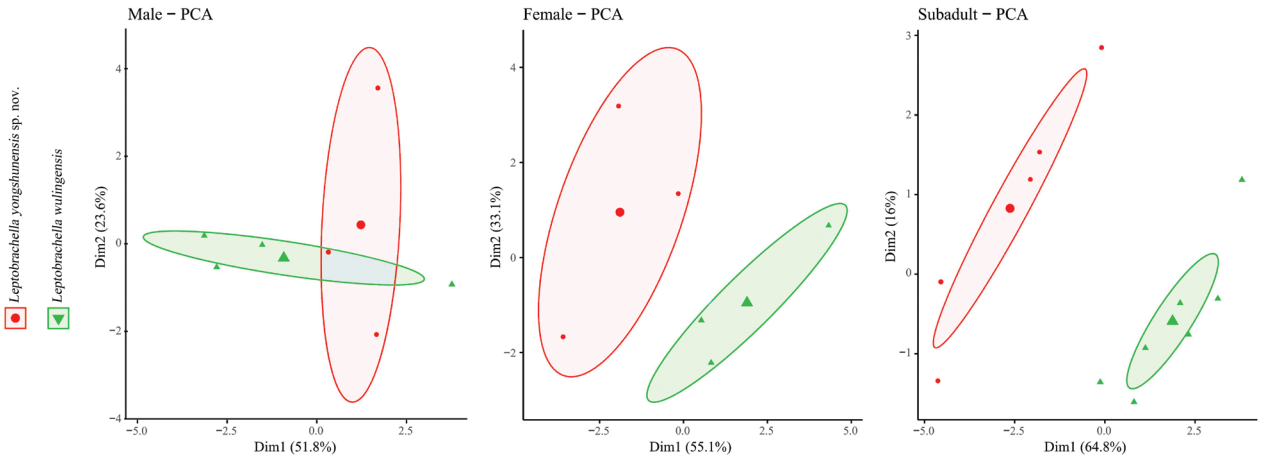

Figure 4. Plots of principal component analyses based on the morphometric measurements, distinguishing *L. yongshunensis* sp. nov. and *L. wulingensis*. The larger icons are the deduced centroid of the species.

Taxonomic account

***Leptobrachella yongshunensis* Huang, Wu, Jiang & Zhang, sp. nov.**

https://zoobank.org/6934308D-49C1-42C0-9128-78DFC3FCF70D

Fig. 5

Material examined. ***Holotype.*** China·♂; Hunan Province, Yongshun County, Hunan Xiaoxi National Nature Reserve; 28.809°N, 110.261°E, ca. 500 m a.s.l.; 5 Jul. 2024; Jie Huang leg., JSUHJ2024005. ***Paratypes.*** China·2 ♂; same locality data as for holotype·1♂; 28.756°N, 110.243°E, ca. 260 m a.s.l.; 20 Mar. 2024; Jie Huang leg.; JSUHJ2024006, JSUHJ2024007. China·1 ♀; same collected information as for holotype·1♂; JSUHJ2024004. China·1 ♀; same locality data as for holotype·1♂; 28.756°N, 110.243°E, ca. 260 m a.s.l.; 20 Mar. 2024; Jie Huang leg.; JSUHJ2024008. China·1 ♀; same locality data as for holotype·1♂; 24 Jul. 2020; Tao Wu leg.; JSUWT2020001. China·3 subadults; same locality data as for holotype·1♂; 28.756°N, 110.243°E, ca. 260 m a.s.l.; 20 Mar. 2024; Jie Huang leg.; JSUHJ2024001, JSUHJ2024002, JSUHJ2024003. China·2 subadults; same locality data as for holotype·1♂; 24 Jul. 2020, Tao Wu leg.; JSUWT2020002, JSUWT2020003.

Etymology. The specific epithet, *yongshunensis*, is derived from the distribution of this species, Yongshun County, Hunan Province, China. The suggested common name is "永顺掌突蟾 (pinyin: yǒng shùn zhǎng tū chán)" in Chinese and "Yongshun leaf-litter toad" in English.

Diagnosis. (Table 2) *Leptobrachella yongshunensis* sp. nov. can be distinguished from its congers by the following characters: SVL 27.2–28.9 mm in males, 26.2–31.6 mm in females; black spots on flanks; toes webbing rudimentary; narrow fringes on toes; creamy white ventral body with indistinct black speckling at margins; dorsal body with sparse large warts, dense little wart grains, and longitudinal ridges; head width greater than the head length; tibiotarsal articulation reaching to the anterior edge of the eye; brick-red color in the dorsal surface; upper parts of iris bright coppery in life.

Description of holotype. (Figs 5, 6, Table 2) Adult male, body size medium (SVL 28.9 mm); head width (HDW 9.5 mm) greater than head length (HDL 7.4 mm); snout rounded in ventral and lateral views, projecting slightly beyond margin of the lower jaw; nostril closer to snout than eye; loreal region particularly oblique; eye diameter (ED 3.5 mm) slightly shorter than snout length (SL 3.7 mm); eyes notably protuberant in the dorsal and lateral views, pupil vertical, copper-colored iris; tympanum distinct, rounded; tympanum diameter (TD 1.8 mm) about half the eye diameter, upper margin in contact with supratympanic ridge; tongue notched behind; vomerine teeth absent; supratympanic ridge distinct, extending from the posterior corner of the eye to the supra-axillary gland.

Fore-limb relatively long (LAL 13.4 mm); relative length of fingers III > II = IV > I; finger tips rounded, slightly swollen; subarticular tubercles absent; inner palmar tubercle large and rounded, connected with a smaller, tear drop outer palmar tubercle. Hindlimbs slender and long (HLL 42.8 mm), heels overlapping when legs at right angle to body, tibiotarsal articulation reaching anterior corner of eye; tibia length (TL 13.8 mm) about half of snout-vent length; relative length of toes IV > III > V > II > I; toes webbing rudimentarily, narrow fringes on toes; toe tips rounded and thickened; all subarticular tubercles absent, replaced by longitudinal dermal ridges, extending on phalanges and interrupted at the articulations; inner metatarsal tubercle elongated, oval; outer metatarsal tubercle absent.

Dorsal with sparse large warts, dense little wart grains, and longitudinal ridges; upper eyelids and limbs with small tubercles; flanks with distinct larger glandular warts forming two rows; white conical spines present on lateral and ventral surface of tarsus, surface of tibiotarsal, inner-side surface of shank, and surface around cloacal region; pectoral gland and femoral gland oval; femoral glands situated on the posteroventral surface of thigh, closer to knee than to vent; supra-axillary gland raised; ventrolateral gland line distinctly visible.

Coloration of holotype in life. (Fig. 5) Dorsal surface brick-red with a dark inverted triangular marking in the interorbital region and followed by a "W" shaped coppery orange mark between axillae; three vertical bars present at the snout region; supratympanic line weak, lower edge black; granules brick-red, present on dorsum, flanks, and limbs; moderate black spots present on flanks; glandular warts on flanks white to orange; suprabrachial gland yellow; transverse bars present on lower arms and legs, as well as fingers and toes; elbow and upper arms coppery orange. Ventral surface of throat grayish pink; ventral surface of limbs dark purple; chest grayish pink with creamy white pigmentation; belly creamy white with indistinct black speckling at margins; ventrolateral glands whitish orange, femoral and pectoral glands white; iris bicolored, coppery in the upper half, fading to dark silver in the lower half.

Coloration of holotype in preservative. (Fig. 6) The background color on the dorsum faded to dark brown; dark vertical bars, transverse bars, and black spots distinct; the orange color on tubercles, glands, and elbow completely faded; ventral surfaces of limbs meat-white with dark pigmentations present at the edge of jaw and margin of belly, sparse pigmentations vaguely visible on the chin and chest; ventral surfaces of body gradually fading to reveal the color of the internal liver and other organs; iris uniformly dark gray.

Variations. Measurements of specimens are presented in Suppl. material 1: table S5. The color of the subadult dorsal body is brown or dark brown (JSUHJ2024001, Fig. 7B1; JSUHJ2024003 Fig. 8B), dorsal surface of head and trunk reddish brown; shape mark edges of specimen JSUHJ2024008 have no other color (Fig. 7A1).

Figure 5. The holotype specimen (JSUHJ2024005) of *L. yongshunensis* sp. nov. in habitat. Photo by Wan-Sheng Jiang.

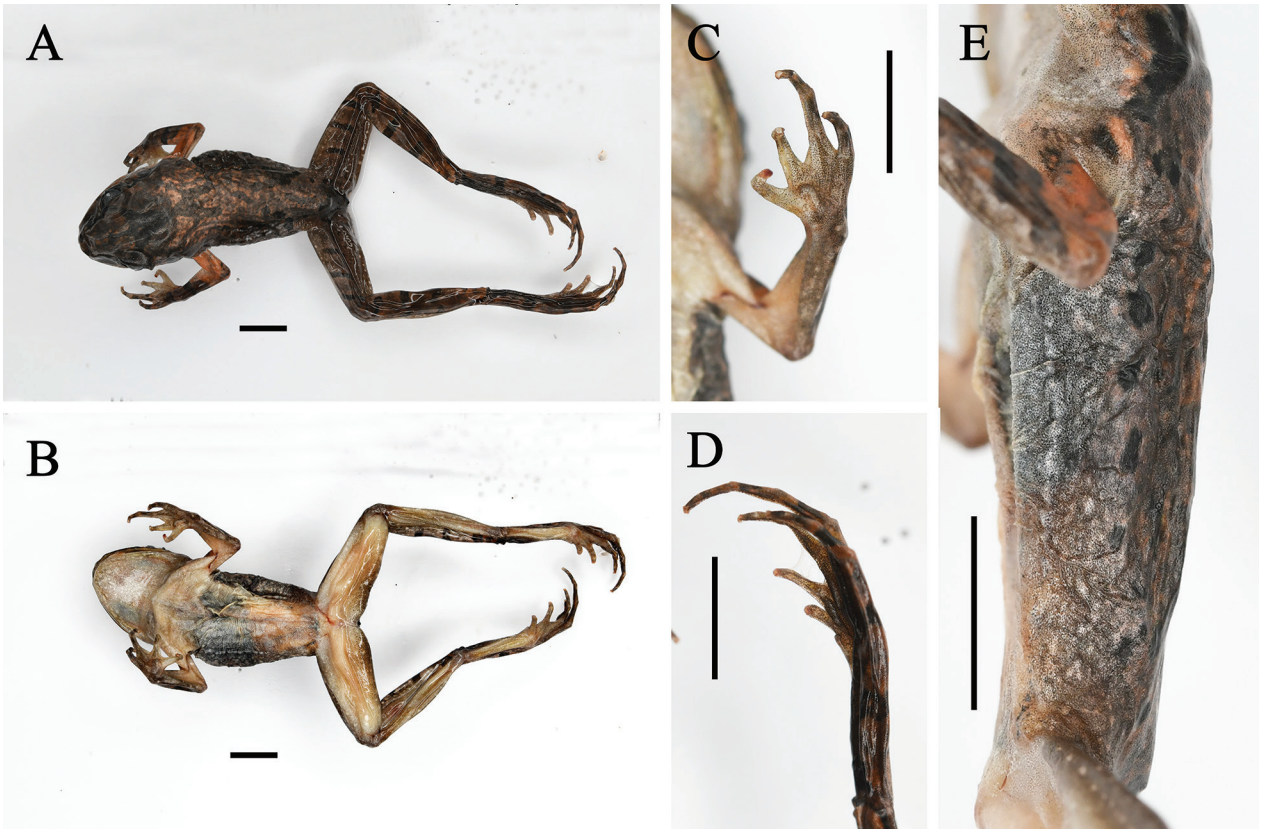

Figure 6. The holotype specimen (JSUHJ2024005, male, scale bar = 5 mm) of *L. yongshunensis* sp. nov. in preservative **A.** Dorsal view; **B.** Ventral view; **C.** Ventral view of hand; **D.** Ventral view of foot; **E.** Dorsal lateral view. Photos by You-Xiang Zhang and Jie Huang.

Figure 7. Specimens (**A.** JSUHJ2024008, adult female; **B.** JSUHJ2024001, subadult) in life, **A1, B1.** Dorsal view **A2, B2.** Ventral view. Photos by Tao Wu.

Distribution, habitat, and life history. (Figs 1, 8) *Leptobrachella yongshunensis* sp. nov. is currently distributed in Hunan Xiaoxi National Nature Reserve, Yongshun County, Xiangxi Autonomous Prefecture, Hunan Province, China. All newly metamorphosed subadults were observed on the stone walls or wooden stakes by the stream in July (Fig. 8). On March 20, two adult males, JSUHJ2024006 and JSUHJ2024007, were observed in a crevice with flowing water, and they were chirping. Specimen JSUHJ2024008 was discovered on the rocks by the stream on March 20. Specimens JSUHJ2024004 and JSUHJ2024005 were discovered on the layer of fallen leaves on July 5, without chirping. All *L. yongshunensis* sp. nov. specimens were found distributed at 260–500 m altitudes. The new species reproduces in streams approximately around March and might conceal itself in the leaf litter layer at other times. Tadpoles completely metamorphose and crawl on rock stakes in breeding grounds in July.

Discussion

According to the advertisement call of the new species's sister group, *L. wulingensis*, as reported by Qian et al. (2023), several differences were identified in the advertisement call of Type A between *L. yongshunensis* sp. nov. and *L. wulingensis* (see Suppl. material 1: tables S7, S8). The first note has two peaks (vs. 2–4 peaks), the peak amplitudes of the first note are equal (vs. decreasing), the duration of the second note is longer than the first (vs. shorter), and the dominant frequency is higher

Figure 8. Habitat of *L. yongshunensis* sp. nov. (JSUHJ2024001, JSUHJ2024002, JSUHJ2024003), Hunan Xiaoxi National Nature Reserve, Yongshun County, Hunan Province, China. **A.** habit; **B.** ecological photo of specimen JSUHJ2024003. Photos by Jie Huang.

Table 2. Measurements of *L. yongshunensis* sp. nov. and *L. wulingensis*(mm). Abbreviations defined in text. Data of *L. wulingensis* refers from Qian et al. 2020.

Character	Holotype	Range(mean ± SD)					
Species		*Leptobrachella yongshunensis* sp. nov.			*Leptobrachella wulingensis*		
Sex	male	male (n = 3)	female (n = 3)	subadult (n = 5)	male (n = 4)	female (n = 3)	subadult (n = 7)
ED	3.5	2.7 ~ 3.5 (3.1 ± 0.40)	3.1 ~ 3.7 (3.5 ± 0.33)	2 ~ 3.2 (2.5 ± 0.49)	3.3 ~ 4.2 (3.6 ± 0.41)	4.2 ~ 4.9 (4.4 ± 0.40)	2.9 ~ 3.7 (3.4 ± 0.31)
FL	18.3	12.5 ~ 18.3 (14.5 ± 3.29)	11.4 ~ 14 (13.1 ± 1.45)	7.7 ~ 10.4 (9.0 ± 1.15)	10.3 ~ 13.5 (11.5 ± 1.41)	13.1 ~ 14.6 (13.7 ± 0.81)	9.2 ~ 11.2 (10.1 ± 0.61)
HDL	7.4	7.4 ~ 9.6 (8.7 ± 1.17)	7.7 ~ 9.5 (8.4 ± 0.94)	5.3 ~ 7.8 (6.4 ± 1.12)	9.1 ~ 10.6 (9.6 ± 0.68)	10.5 ~ 12.7 (11.3 ± 1.19)	8.3 ~ 9.4 (9.0 ± 0.43)
HDW	9.5	9.5 ~ 10.8 (10.0 ± 0.72)	8.6 ~ 11.1 (9.7 ± 1.29)	6.6 ~ 8.4 (7.4 ± 0.83)	8.6 ~ 10.6 (9.3 ± 0.91)	10.3 ~ 12.5 (11.1 ± 1.22)	7.5 ~ 8.8 (8.3 ± 0.46)
HLL	42.8	42.8 ~ 43 (42.9 ± 0.12)	42.9 ~ 47.9 (46.1 ± 2.80)	26.5 ~ 35.2 (30.8 ± 4.13)	/	/	/
IND	3.4	3.1 ~ 3.4 (3.2 ± 0.15)	3 ~ 3.62 (3.3 ± 0.32)	1.7 ~ 2.8 (2.4 ± 0.41)	/	/	/
IOD	5.1	4.2 ~ 5.2 (4.8 ± 0.55)	5 ~ 5.9 (5.5 ± 0.47)	3.7 ~ 4.3 (4.0 ± 0.26)	2.7 ~ 3.2 (3.0 ± 0.24)	3.3 ~ 3.4 (3.4 ± 0.06)	2.7 ~ 3 (2.9 ± 0.13)
LAL	13.4	13.4 ~ 14.3 (13.9 ± 0.46)	13.2 ~ 15.72 (14.7 ± 1.33)	8.4 ~ 11.2 (9.7 ± 1.13)	/	/	/
SL	3.7	3.7 ~ 4.1 (3.8 ± 0.23)	3.5 ~ 4.4 (4.0 ± 0.48)	2.4 ~ 3.1 (2.6 ± 0.30)	3.1 ~ 4 (3.5 ± 0.37)	4.1 ~ 4.8 (4.4 ± 0.38)	3 ~ 3.6 (3.2 ± 0.21)
SVL	28.9	27.2 ~ 28.9 (28.3 ± 0.93)	26.2 ~ 31.6 (29.8 ± 3.13)	18.7 ~ 22.8 (20.6 ± 1.52)	24.5 ~ 32.8 (27.2 ± 3.81)	29.9 ~ 38.5 (33.2 ± 4.66)	21.8 ~ 25.4 (23.8 ± 1.41)
TD	1.8	1.8 ~ 2.6 (2.1 ± 0.46)	1.8 ~ 2.54 (2.2 ± 0.37)	1.2 ~ 1.7 (1.5 ± 0.21)	1.5 ~ 2.2 (1.8 ± 0.31)	1.7 ~ 2.2 (2.0 ± 0.25)	1.2 ~ 1.8 (1.4 ± 0.21)
TL	13.8	12.7 ~ 13.8 (13.2 ± 0.57)	13.3 ~ 15.8 (14.5 ± 1.23)	8.8 ~ 11.5 (10.1 ± 1.08)	11.5 ~ 14.3 (12.6 ± 1.26)	14.2 ~ 15.8 (14.8 ± 0.85)	9.8 ~ 12.1 (11.1 ± 0.82)
TW	3.4	1.9 ~ 3.4 (2.5 ± 0.78)	1.9 ~ 3.6 (3.0 ± 0.94)	1.6 ~ 2.2 (2.0 ± 0.28)	/	/	/
UEW	1.6	1.6 ~ 2.3 (2.0 ± 0.35)	2.6 ~ 3 (2.7 ± 0.23)	1.1 ~ 2.4 (1.7 ± 0.49)	/	/	/
TEY	1.4	1 ~ 1.4 (1.1 ± 0.23)	1.2 ~ 1.4 (1.3 ± 0.10)	0.7 ~ 1.3 (1.0 ± 0.24)	1 ~ 1.3 (1.1 ± 0.13)	1.1 ~ 1.3 (1.2 ± 0.10)	0.8 ~ 1.1 (0.9 ± 0.11)
ML	6.9	6.9 ~ 7.4 (7.2 ± 0.26)	6.3 ~ 7.5 (7.0 ± 0.59)	4.3 ~ 5.4 (4.7 ± 0.46)	6.1 ~ 8 (6.8 ± 0.84)	7.9 ~ 8.3 (8.0 ± 0.23)	5.7 ~ 6.6 (6.1 ± 0.34)

(3.811–4.042 kHz vs. 3.59–3.72 kHz). The dominant frequency (3.811–3.985 kHz) of advertisement call of the new species still varies from the following congers with recorded call (more detail see Suppl. material 1: table S8): *L. purpurus* (4.3–4.5 kHz; Yang et al. 2018), *L. bijie* (4.82–5.07 kHz; Wang et al. 2019), *L. chishuiensis* (4.21–4.55 kHz; Shi et al. 2023), *L. dong* (4.4–5.6 kHz; Liu et al. 2023), *L. oshanensis* (4.2–4.5 kHz; Shi et al. 2021), *L. purpuraventra* (4.68–4.82 kHz; Wang et al. 2019), *L. yeae* (4.71–4.95 kHz; Shi et al. 2021), *L. yingjiangensis* (5.7–5.9 kHz; Yang et al. 2018).

In the last five years (2020 to 2024-8-20), approximately 30 new species of the genus *Leptobrachella* have been reported (Li et al. 2024; Chen et al. 2024). Among these, merely one species, *L. wulingensis* (Qian et al. 2020), has been described in the Wuling Mountains. In 2024, Xu et al. (2024) revealed six new hidden biological hotspots in China, including the Wuling Mountains. In this study, a relatively wide river, Lishui River (澧水), separates the distribution of the new species and its sister species (Fig. 1), which may be the main reason for their isolation. Furthermore, the Wuling Mountain area is rich in water systems, and numerous rivers divide it into small areas. This discovery still implies that there might be more hidden *Leptobrachella* species.

In the current taxonomic research of the genus *Leptobrachella*, a growing number of integrated taxonomic approaches combining morphological comparison, acoustic studies, and molecular research are employed to determine species. Meanwhile, the genetic markers for the genus *Leptobrachella* are not confined to the 16s rRNA gene, and other genes, such as COI and Rag1, are gradually incorporated (Shi et al. 2021, 2023; Li et al. 2024; Chen et al. 2024). However, many species only have the information of 16s rRNA gene in the Genbank, posing a challenge for future classification. In our phylogenetic analysis, the evolutionary relationships of many species in the genus *Leptobrachella* have not been included. In future research, we hope that more mitochondrial gene fragments and even the entire mitochondrial genome can be extensively studied, and the radiation evolution of the entire genus *Leptobrachella* can be studied in detail. The advertisement call provides important information for the classification of species in the genus *Leptobrachella* (Köhler et al. 2017; Qian et al. 2023; Wang et al. 2019; Chen et al. 2024). Many *Leptobrachella* species lack sound descriptions, thus requiring further related research in the future.

On a macro scale, the diversification of the genus *Leptobrachella* is attributed to the climatic warming and wetting of the Miocene, and its colonization direction was from Sundaland to mainland Asian, from tropical archipelagos to a non-tropical continental landmass, with Borneo serving as the source of the mainland Asian lineages (Chen et al. 2018). Nevertheless, there is a dearth of research on microscale evolution, such as the evolution and interaction of species within the same domain (e. g., *L. guinanensis*, *L. shiwandashanensis*, *L. shangsiensis*, and *L. sungi* distributed in Shiwandashan National Nature Reserve), and the significance of micro geographic isolation in species differentiation. In our team's future research, a systematic study on the evolutionary history and taxonomy of *Leptobrachella* spp. will be conducted in Wuling Mountain area.

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Supplementary material 1

Additional information

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Data type: xls

Explanation note: **table S1.** Information for samples used in molecular phylogenetic analyses in this study; **table S2.** The raw data of *Leptobrachella yongshunensis* sp. nov. and *Leptobrachella wulingensis* for comparison; **table S3.** Uncorrected p-distance between *Leptobrachella* species based on the 16S gene; **table S4.** Uncorrected p-distance between *Leptobrachella* species based on the COI gene; **table S5.** Morphometric measurements of *L. yongshunensis* sp. nov.; **table S6.** Selected diagnostic characters for the species in the genus *Leptobrachella* occurring north of the Isthmus of Kra. Toes webbing was determined following Fei et al. (2012); **table S7.** The advertisement call of *Leptobrachella wulingensis* (TPW04) from Tianpingshan, Sangzhi County, Hunan Province, China. (A) 30s waveform containing 16 PACs; (B) the recorded; **table S8.** Comparisons of characters of advertisement calls of the new species, and its congers.

Link: https://doi.org/10.3897/zse.101.135586.suppl1