

The advertisement call of *Leptobrachella dushanensis* (Anura, Megophryidae)

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Academic editor: Günter Gollmann ♦ Received 9 September 2024 ♦ Accepted 15 November 2024 ♦ Published 2 December 2024

Abstract

We describe the advertisement call of the Dushan Leaf Litter Toad, *Leptobrachella dushanensis*, based on recordings obtained from three individuals from its type locality. The advertisement call of *L. dushanensis* consists of a single note with a dominant frequency of 4.84–4.91 kHz. Furthermore, we compare the differences in advertisement calls between *L. dushanensis* and its sister species, *Leptobrachella dong*. The advertisement calls of *L. dushanensis* and *L. dong* can be clearly distinguished. Our findings provide bioacoustic evidence supporting the taxonomic status of *L. dushanensis* and provide basic data for future behavioral and ecological studies on *Leptobrachella*.

Key Words

acoustic differences, bioacoustics, frogs, southern China

Introduction

The Asian leaf litter toads of the genus *Leptobrachella* Smith, 1925, are the largest clade of the family Megophryidae Bonaparte, 1950, currently comprising 105 species (Frost 2024). Species of the genus *Leptobrachella* are widely distributed, ranging from southern China, northeastern India, and Myanmar, through Thailand and Vietnam, and extending to the Malay Peninsula, Borneo, and Natuna Island (Frost 2024). During the breeding season, *Leptobrachella* species often inhabit rocks on the stream banks (Fei et al. 2005). Molecular phylogenetic frameworks proposed many cryptic species in the genus (Chen et al. 2018). In the past five years, more than 30% of new species in *Leptobrachella* have been described (Frost 2024). High levels of morphological similarity and rampant homoplasy appear to have misled estimates

of diversity and evolutionary relationships (Shen et al. 2021). Addressing the aforementioned issues requires the application of integrative taxonomy, combining methods from morphology, genetics, and bioacoustics to reveal the species diversity within the genus *Leptobrachella*.

Vocalizations of anuran amphibians have received much attention in studies of behavioral ecology and species identification (Cui et al. 2012; Zhu et al. 2021; Shen et al. 2024). In Anura, nearly all species are capable of vocalization (Köhler et al. 2017). To further understand the relationship between frog behaviors and vocal communication, researchers have divided frog calls into four types: feeding calls, aggressive calls, reproductive calls, and defensive calls. Advertisement calls, courtship calls, amplexant calls, release calls, post-oviposition male release calls, and rain calls are examples of reproductive calls (Toledo et al. 2015; Köhler et al. 2017).

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Furthermore, advertisement calls exhibit substantial variation across species, making them a viable foundation for the systematic classification and identification of cryptic species (Sullivan et al. 1996).

Leptobrachella dushanensis (Anura, Megophryidae) is a newly described species in Southern China (Li et al. 2024). Information about their acoustic signals is not available in the literature. Here, we describe the advertisement call of *L. dushanensis* for the first time to understand its characteristics. Furthermore, we focus on comparing the advertisement call of *L. dushanensis* with that of its sister species, *Leptobrachella dong*. Our findings provide acoustic evidence that supports the taxonomic differentiation of *L. dushanensis* and contribute basic data for future behavioral and ecological studies on *Leptobrachella* species.

Materials and methods

Call recordings

A total of 197 calls were recorded from three individuals of *L. dushanensis*, collected from its type locality Dushan County, Guizhou Province, China (107.63°E, 25.94°N, ca. 1200 m elev., 17.0–19.0 °C air temperature, 98% ambient humidity) on April 1, 2024, between 19:00–23:00 h. Additionally, to compare the differences in advertisement calls between *L. dushanensis* and its sister species, *Leptobrachella dong*, we also collected advertisement calls from *L. dong*. A total of 117 calls were recorded from four individuals of *L. dong*, collected from Congjiang County, Guizhou Province, China (108.28°E, 25.57°N, ca. 1190 m elev., 12.0–13.0 °C air temperature, 90% ambient humidity) on March 4, 2022, between 19:00–23:00 h. Calls from each individual were obtained using a digital recorder, a SONY ICD-PX470 (sampling rate 44.1 kHz, 16-bit resolution). Each call was recorded within a 0.5 m distance from the calling individual. All recordings were obtained from isolated individuals, without overlapping calls or mixed choruses, and saved as WAV files. The recordings are publicly available in Figshare at <https://doi.org/10.6084/m9.figshare.26969512>.

Snout-vent lengths (SVLs) of all recorded males were measured in situ using a precision digital caliper to the nearest 0.1 mm. One *L. dushanensis* (specimen number: DS20240401001) and one *L. dong* (specimen number: CJ20220304001) were collected for species identification, and the others were released to their original habitat after measurement. After taking photos of the two specimens, we euthanized them using isoflurane and then fixed the specimens in 10% buffered formalin. Tissue samples were taken and preserved separately in 95% ethanol before fixation. Specimens were deposited in the Moutai Institute, China. Mitochondrial COI genes were extracted and amplified from muscle samples and sequenced, and the obtained sequence was verified and uploaded to GenBank (<https://www.ncbi.nlm.nih.gov/>). The sequencing results were compared to finally determine the species collected.

Acoustic analyses

The advertisement call characteristics were analyzed with the software Raven Pro 1.6 (K. Lisa Yang Center for Conservation Bioacoustics [at the Cornell Lab of Ornithology] 2023). Temporal properties were measured using Raven's waveform display. Spectral properties were measured by averaging the spectrum over the entire duration of a call (Hann window, DFT = 512 samples, overlap = 50%, hop size = 256 samples). Only calls that had a high signal-to-noise ratio and were free from overlapping calls of nearby males were used for the analysis. We used “call-centred” terminology as summarised by Köhler et al. (2017), in which the fundamental unit was defined as a “call”; each “call” contains a single “note,” and each “note” contains several pulses. We measured all parameters and characteristics following the procedure described by Köhler et al. (2017) and Qian et al. (2023), including (1) call duration (ms), CD (2) inter-call interval, CI (3) call rate (calls/s), CR (4) note per call, NPC (5) no. of pulses per call, NP (6) pulse rate (pulses/s), PR (7) dominant frequency (kHz), DF. Oscillograms and spectrograms were generated using Seewave v.2.2.0 (Sueur et al. 2008) and TuneR 1.4.2 (Ligges et al. 2013) packages in R programme 4.2.2 (R Core Team 2021) with a “Hanning” window size of 256 samples and an overlap of 50%. Descriptive statistics of call characteristics (mean, standard deviation (SD), and range) were computed using SPSS 23.0. Furthermore, principal component analysis (PCA) was conducted to compare the differences in advertisement calls between *L. dushanensis* and its sister species, *L. dong*.

Results

Call characteristics

Males were observed calling on rocks in streams surrounded by shrubs. After molecular identification, we determined that the species captured in Dushan County is *L. dushanensis* (Fig. 1A). The GenBank accession number for the COI sequence is PQ288957 (specimen number: DS20240401001). The SVL of *L. dushanensis* ranged from 31.9 mm to 32.6 mm (Table 1). We analyzed the spontaneous vocalizations (197 calls) from three *L. dushanensis* males. The advertisement calls of *L. dushanensis* are pulse-repetition calls (Fig. 2A, B). All calls of *L. dushanensis* exhibited a consistent structure, with the amplitude reaching its peak at the beginning of the call, then gradually decreasing. About one-third into the call, the amplitude increased again but did not exceed the initial peak before gradually decreasing until the end of the call (Fig. 2B). The advertisement call of *L. dushanensis* comprised a single note with a call duration ranging from 78.19–123.78 ms (Table 1). The inter-call interval ranged from 394.88–499.59 ms. The call rate ranged from 1.78–2.31 calls/s. The pulse number ranged from 14.00–21.00, and the pulse rate ranged from 164.26–174.06 pulses/second. The dominant frequency

Table 1. Descriptive statistics for call parameters of advertisement calls of *Leptobrachella dushanensis*.

Individuals	Calls analyzed	Air temperature (°C)	SVL (mm)	Call duration (ms)	Inter-call interval (ms)	Call rate	Note per call	Note rate	No. of pulses per call	Pulse rate	Dominant frequency (kHz)
DS20220401001	75	17.0	31.9	123.78 ± 16.61 94.00–167.10	499.59 ± 49.57 410.80–594.31	1.78 ± 0.14 1.65–1.93	1	NA	21.00 ± 4.50 14.00–33.00	164.26 ± 21.72 134.30–223.40	4.91 ± 0.15 4.82–5.17
DS20240401002	77	19.0	32.3	86.76 ± 9.73 68.40–115.40	469.77 ± 77.65 303.27–669.00	2.09 ± 0.18 1.92–2.39	1	NA	15.00 ± 1.50 12.00–24.00	174.06 ± 12.40 152.01–205.72	4.87 ± 0.17 4.82–5.51
DS20240401003	45	19.0	32.6	78.19 ± 6.52 64.60–97.10	394.88 ± 68.53 288.00–588.02	2.31 ± 0.15 2.19–2.48	1	NA	14.00 ± 0.50 10.00–20.00	164.56 ± 12.13 139.32–195.67	4.84 ± 0.10 4.82–5.51
All Individuals	3/197	17.0–19.0	31.9–32.6	78.19–123.78	394.88–499.59	1.78–2.31	1	NA	14.00–21.00	164.26–174.06	4.84–4.91

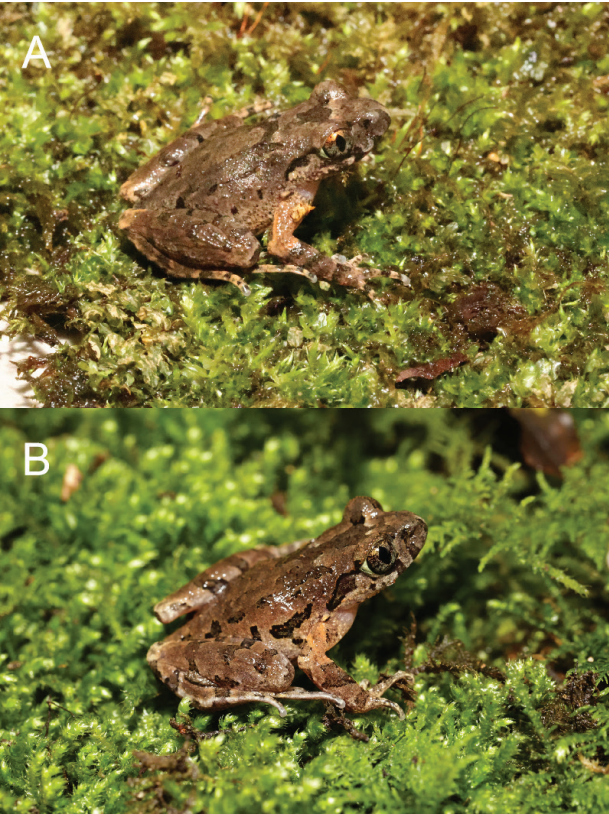


Figure 1. Photograph of the specimen. **A.** Adult male of *Leptobrachella dushanensis* (DS20240401001) from Dushan County, Guizhou Province, China; **B.** Adult male of *Leptobrachella dong* (CJ20220304001) from Congjiang County, Guizhou Province, China.

ranged from 4.84–4.91 kHz, and no obvious harmonics were found (Fig. 2B, Table 1).

After molecular identification, we confirmed that the species collected from Congjiang County is *L. dong* (Fig. 1B). The GenBank accession number for the COI sequence of *L. dong* is [PQ438741](#) (specimen number: CJ20220304001). The SVL of *L. dong* ranged from 29.4 mm to 29.8 mm (Table 2). We analyzed the spontaneous vocalizations of 117 calls from four *L. dong* males. The advertisement calls of *L. dong* are also pulse-repetition calls (Fig. 2C, D). The advertisement call of *L. dong* reaches its peak amplitude at the beginning and then gradually decreases until reaching approximately half-way through the call. Subsequently, at around two-thirds of the call duration, the amplitude suddenly increases again but does not exceed the initial peak, and then grad-

ually decreases until the end of the call (Fig. 2D). The advertisement call of *L. dong* comprised a single note with a call duration ranging from 63.54–90.82 ms (Table 2). The inter-call interval ranged from 299.20–380.01 ms. The call rate ranged from 2.23–2.72 calls/s. The pulse number ranged from 9.00–11.00, with a pulse rate of 102.13–133.77 pulses/second. The dominant frequency was 4.82 kHz, and no obvious harmonics were detected (Fig. 2D, Table 2).

Comparison of advertisement calls

The principal component analysis (PCA) of call parameters between *L. dushanensis* and *L. dong* resulted in two PCs, explaining a cumulated variance of 79.77% (Fig. 3). The PC1 axis accounted for 62.09% of the total variation and was primarily associated with temporal parameters, as it comprised the number of pulses, call duration, and pulse rate (Table 3). The PC2 axis explains 17.69% of the variation, primarily driven by the spectral parameter of dominant frequency (Table 3). The advertisement calls of the two species showed no overlap in PC space, indicating acoustic separation between the species.

Discussion

From the perspective of phylogenetic relationships, *L. dushanensis* was assigned to the *L. oshanensis* species group (Li et al. 2024). Previous studies have shown that species within the *L. oshanensis* species group typically produce more complex advertisement calls (Wang et al. 2019; Shi et al. 2021; Shen et al. 2023). However, unlike other species within the species group, we only recorded simple calls of *L. dushanensis* and did not notice any complex calls. Compared to the published calls of the other species in the *L. oshanensis* species group, the advertisement call of *L. dushanensis* differs considerably, reinforcing the specific identity of this species. For example, the species of *Leptobrachella yae*, *Leptobrachella jinyunensis*, *Leptobrachella chishuiensis*, *Leptobrachella bijie*, *Leptobrachella jinshaensis*, *Leptobrachella suiyangensis*, *Leptobrachella purpuraventra*, *Leptobrachella wulingensis*, and *Leptobrachella oshanensis* exhibit multiple types of advertisement calls (Wang et al. 2019; Li et al. 2020; Cheng et al. 2021; Shi et al. 2021; Shen et al. 2023;

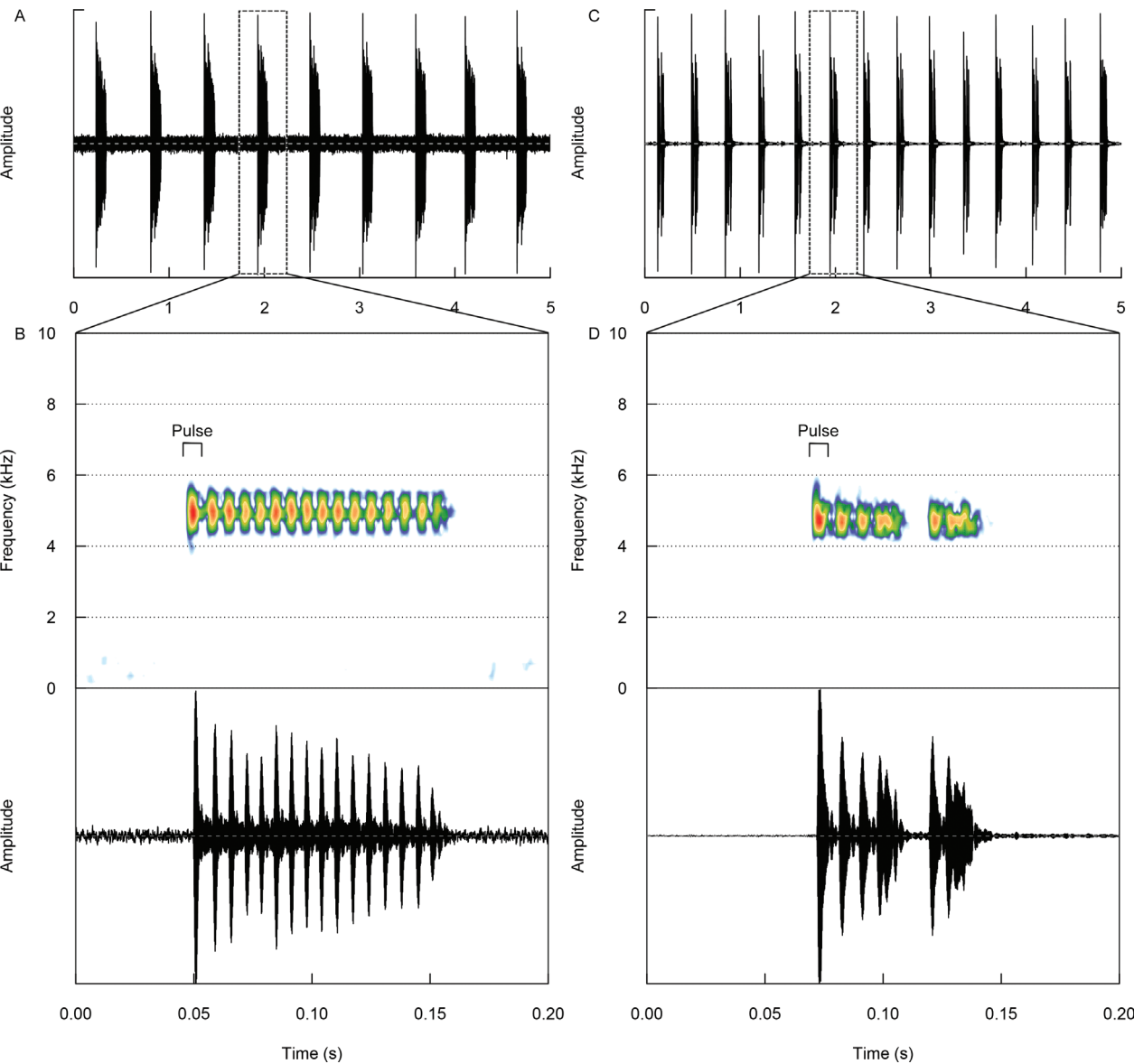


Figure 2. Advertisement calls of the two *Leptobrachella* species. **A.** 5 s oscillograms showing nine calls of *Leptobrachella dushanensis*; **B.** 0.2 s oscillograms and corresponding spectrograms showing one call of *L. dushanensis*; **C.** 5 s oscillograms showing fourteen calls of *Leptobrachella dong*; **D.** 0.2 s oscillograms and corresponding spectrograms showing one call of *L. dong*.

Table 2. Descriptive statistics for call parameters of advertisement calls of *Leptobrachella dong*.

Individuals	Calls analyzed	Air temperature (°C)	SVL (mm)	Call duration (ms)	Inter-call interval (ms)	Call rate	Note per call	Note rate	No. of pulses per call	Pulse rate	Dominant frequency (kHz)
CJ20220304001	27	13.0	29.6	82.55 ± 5.08 65.60–94.00	380.01 ± 42.06 312.95–478.13	2.23	1	NA	11.00 ± 0.00 8.00–13.00	121.87 ± 5.43 106.71–133.63	4.82
CJ20220304002	30	13.0	29.8	63.54 ± 4.84 48.40–72.80	336.30 ± 47.67 274.14–441.65	2.57	1	NA	9.00 ± 1.13 5.00–13.00	133.77 ± 21.62 70.80–165.41	4.82
CJ20220304003	30	12.0	29.5	90.82 ± 8.50 63.10–105.60	311.65 ± 37.97 270.60–435.54	2.55	1	NA	10.50 ± 0.50 6.00–12.00	102.13 ± 7.24 79.24–119.18	4.82
CJ20220304004	30	12.5	29.4	66.56 ± 6.15 54.40–81.60	299.20 ± 27.75 242.72–360.42	2.72	1	NA	9.00 ± 0.50 5.00–10.00	112.59 ± 16.92 66.89–147.06	4.82
All Individuals	4/117	12.0–13.0	29.4–29.8	63.54–90.82	299.20–380.01	2.23–2.72	1	NA	9.00–11.00	102.13–133.77	4.82

Shi et al. 2023; Qian et al. 2023). The dominant frequency of *L. dushanensis* (4.88 kHz) is lower than that of *Leptobrachella alpien* (6.70 kHz; Xu et al. 2005). As the sister species of *L. dushanensis*, *L. dong* exhibits a similar advertisement call structure to that of *L. dushanensis*. Their

advertisement calls can be distinguished by call parameters such as the number of pulses, call duration, pulse rate, and dominant frequency (Table 3). In taxonomic studies, an increasing number of researchers are utilizing acoustic diagnostic traits to identify cryptic species (Cui et al. 2023;

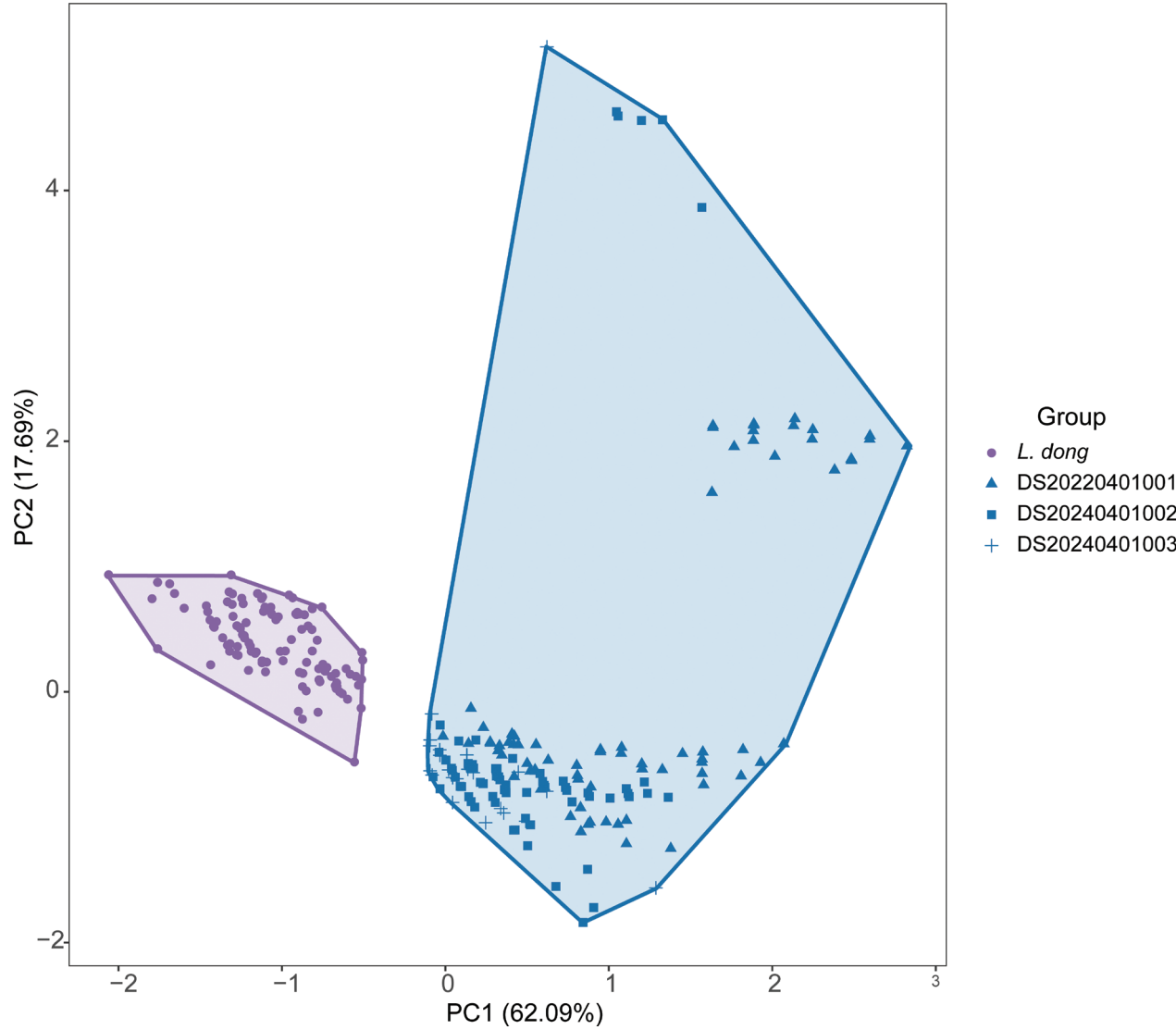


Figure 3. Plots of the first principal component (PC1) versus the second (PC2) for advertisement calls of *Leptobrachella dushanensis* and *Leptobrachella dong* from a principal component analysis.

Table 3. Factor loadings of the principal component analysis (PCA) on the advertisement call parameters of the *Leptobrachella dushanensis* and *Leptobrachella dong*.

Call parameters	Principal components				
	1	2	3	4	5
Call duration	0.830	0.074	-0.551	0.030	0.043
Inter-call interval	0.765	-0.395	0.210	0.463	-0.003
Number of pulses	0.967	-0.004	-0.178	-0.175	-0.060
Pulse rate	0.814	-0.192	0.428	-0.341	0.031
Dominant frequency	0.483	0.829	0.247	0.138	0.001
Eigenvalue	3.104	0.884	0.624	0.381	0.006
Variance (%)	62.085	17.686	12.473	7.628	0.128
Cumulative variance (%)	62.085	79.771	92.244	99.872	100.000

Chen et al. 2024). Species within the genus *Leptobrachella* are highly morphologically conserved, but *L. dushanensis* exhibits distinct advertisement call characteristics in its calls compared to other congeneric species, supporting the species-specific nature of its acoustic signals. The differentiation of advertisement calls in *Leptobrachella* can serve as evidence for their differentiation in taxonomic

status. Our study will support further studies on the behavior, physiology, and ecology of the species.

Acknowledgements

This work was supported by the projects from the National Natural Science Foundation of China (Nos. 32270498, 31960099, 32260136, and 32070426), Guizhou Provincial Science and Technology Projects (Nos. ZK[2022]540 and [2023]099), and high-level personnel research start-up funding projects of Moutai Institute (Nos. mygccrc[2022]055, mygccrc[2022]067, and mygccrc[2022]083).

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