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Advertisement calls of *Boulenophrys daxuemontis* Liu, Li, Cheng, Wei, Wang & Cheng, 2025 (Anura, Megophryidae) from a new distribution record in Nayong County, Guizhou Province

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Abstract

Our study reports a new distribution record for the recently described species *Boulenophrys daxuemontis* in Nayong County, Guizhou Province, expanding its known range. Furthermore, we provide the first systematic description and quantification of its advertisement call characteristics. Its advertisement call consists of a series of repeated pulsative calls, with a mean call duration of 144.91 ± 24.55 ms and a mean dominant frequency of 3.56 ± 0.12 kHz. Additionally, a detailed quantitative comparison was conducted between its advertisement calls and those of its sister species, *Boulenophrys jiangi*. Principal component analysis revealed that parameters such as pulse rate, number of pulses per call, and dominant frequency separated the two species in call space, effectively distinguishing these morphologically similar sister species. These unique acoustic features provide evidence supporting the taxonomic status of *B. daxuemontis*. While our sample size is limited, these findings provide foundational data for future study. Our study not only enriches the fundamental biological database for the genus *Boulenophrys* but also offers important reference data for biodiversity assessment, conservation, and acoustic identification of cryptic amphibian species in the region.

Key Words

acoustic comparison, bioacoustics, frogs, new distribution record, southwest China

Introduction

The Chinese horned toads of the genus *Boulenophrys* Fei, Ye & Jiang, 2016 are the largest clade of the subfamily Megophryidae Bonaparte, 1850, currently comprising 81 species (Frost 2026). Species of the genus *Boulenophrys* are mainly distributed in southern China (excluding Tibet) and extend southward to Vietnam, Laos, Thailand and Myanmar (Frost 2026). Molecular phylogenetic frameworks proposed many cryptic species in the genus (Chen et al. 2017; Lyu et al. 2023), which indicate that the species diversity of *Boulenophrys* is underestimated

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(Liu et al. 2018). Specifically, in the past five years, 40 new species have been described in *Boulenophrys*, accounting for 50% of the species in this genus (Frost 2026). However, most published species descriptions rely on morphological and molecular methods. Nevertheless, many species of the genus *Boulenophrys* exhibit subtle interspecific morphological differences, and certain plastic traits (e.g., body coloration) can vary with environmental conditions (Wang et al. 2014). This phenomenon has increased the difficulty of accurate species identification, especially for cryptic or recently diverged taxa (Shen et al. 2022). Therefore, acoustic diagnostic features can serve as a valuable complementary tool to morphometric and molecular approaches for species identification, particularly in cases where morphological differentiation is limited or ambiguous (Rivera-Correa et al. 2022; Batallas and Brito 2023).

In anuran, nearly all species are capable of producing vocal signals (Köhler et al. 2017). Vocalizations in anuran have been extensively studied in the fields of behavioral ecology and species identification (Zhu et al. 2021; Shen et al. 2023; Liu et al. 2024). To better understand the link between frog behavior and vocal communication, researchers categorize frog calls into four functional types: feeding calls, aggressive calls, reproductive calls, and defensive calls (Köhler et al. 2017). Advertisement calls are a type of reproductive call with species specificity (Toledo et al. 2015; Köhler et al. 2017), which makes it a reliable basis for the systematic and identification of cryptic species (Liu et al. 2018).

Boulenophrys daxuemontis Liu, Li, Cheng, Wei, Wang & Cheng, 2025 is a newly described species in Sichuan Province, China (Liu et al. 2025a). During field surveys in 2025, we investigated the *Boulenophrys* population in Nayong County, Guizhou Province. We collected samples and recorded bioacoustics data. Here, we confirm the presence of *B. daxuemontis* in Nayong County and provide its advertisement calls. Furthermore, we compare the advertisement call of *B. daxuemontis* with that of its sister species, *Boulenophrys jiangi*. Our findings provide acoustic evidence that supports the taxonomic differentiation of *B. daxuemontis* and contributes basic data for future behavioral and ecological studies on *Boulenophrys* species.

Material and Methods

Call recordings

A total of 297 calls were recorded from four individuals of *B. daxuemontis*, collected from Nayong County, Guizhou Province, China (26.73°N, 105.36°E, ca. 1760 m elev., 20 °C air temperature, 90% ambient humidity) on August 11, 2025, between 19:00–23:00 h. The vegetation type at the sampling site was subtropical mixed coniferous and broad-leaved forest. The dominant vegetation in this microhabitat consisted of ferns and broadleaf shrubs, with dense canopy cover resulting in low light conditions during calling periods. To compare the differences in advertisement calls between *B. daxuemontis* and its sister species, *Boulenophrys jiangi*, we also collected advertisement calls from *B. jiangi*. A total of 223 calls were recorded from four individuals of *B. jiangi*, collected from its type locality Suiyang County, Guizhou Province, China (28.20°N, 107.18°E, ca. 1430 m elev., 22 °C air temperature, 95% ambient humidity) on June 26, 2025, between 19:00–23:00 h. The habitat type at the sampling site for *B. jiangi* was mixed coniferous and broad-leaved forest, interspersed with sparse bamboo groves. Calls from each individual were obtained using a digital recorder, 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.31180990.

Species identification

Snout vent lengths (SVLs) of all recorded males were measured in situ using a precision digital calliper to the nearest 0.1 mm. One *B. daxuemontis* (specimen number: NY20250811001) and one *B. jiangi* (specimen number: KKS20250626001) 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. Thigh muscle tissue samples were taken and preserved separately in 95% ethanol before fixation. Specimens were deposited in the Forestry College of Guizhou University, China. For molecular analysis, cytochromeoxidase subunit I (COI) genes were amplifed. For COI, Chmf4 (5'-TYTCWACWAAYCAYAAAGAYATCGG-3') and Chmr4 (5'-ACYTCRGGRTGRCCRAARAATCA-3') were used following Che et al. (2012). Gene fragments were amplified under the following conditions: an initial denaturing step at 95 °C for 4 min; 36 cycles of denaturing at 95 °C for 30 s, annealing at 47 °C for 40 s and extending at 72 °C for 70 s. PCR products were purified with spin columns and then were sequenced with both forward and reverse primers, same as for the PCR. Sequencing was conducted using an ABI Prism 3730 automated DNA sequencer in Chengdu TSINGKE Biological Technology Co. Ltd. (Chengdu, China). All sequences were deposited in GenBank (https://www.ncbi.nlm.nih.gov/). The sequencing results were compared to finally determine the species collected. Our experimental procedures complied with the applicable laws on animal welfare and research in China and were approved by the Subcommittee on Experimental Animal Ethics of Guizhou University (Permit No. EAE-GZU 2023-E013).

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" and the continuous units were defined as a "call group". 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) call interval, CI (3) number of calls per call group, NCC (4) call repetition rate (calls/min), CR (5) number of pulses per call, NPC (6) pulse rate (pulses/s), PR (7) dominant frequency (kHz), DF (8) frequency 5%, fre 5%; and (9) frequency 95%, fre 95% (Table 1). 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. We tested all the data for assumptions of normality and homogeneity of variance using the Shapiro–Wilk and Levene's tests, respectively. Since for all acoustic parameters, the data from at least one species do not conform to the normal distribution, the entire dataset is not suitable for any parametric tests based on the normality assumption. We compared the call parameters of the two species using the Mann–Whitney U test (Shen et al. 2024). $P < 0.05$ was considered statistically significant. Furthermore, principal component analysis (PCA) was conducted to compare the differences in advertisement calls between *B. daxuemontis* and its sister species *B. jiangi*.

Result

Call characteristics

After molecular identification, we determined that the species captured in Nayong county is *B. daxuemontis* (Fig. 1A). All individuals of *B. daxuemontis* were found calling from low-growing vegetation, specifically on shrub leaves at a height of 0.3–0.8 m above the ground. Calling sites were located approximately 2–5 m away from the banks of small, slow flowing forest streams. The GenBank accession number for the COI sequence is PZ142573 (specimen number: NY20250811001). The SVL of *B. daxuemontis* ranged from 37.6 mm to 39.1 mm (Table 1). We analyzed the spontaneous vocalizations (297 calls) from four *B. daxuemontis* males. The advertisement call of *B. daxuemontis* was a group of repeated pulsative calls. Call amplitude was consistent across all call groups, with the exception of the initial 1–2 calls, which exhibited a lower amplitude (Fig. 2A). The initial call commenced with a moderate amplitude within each call group, followed by a distinct interval.

Table 1. Descriptive statistics for call parameters of advertisement calls of *Boulenophrys daxuemontis*. Data are given as mean ± SD and range (min–max).

Voucher	NY20250811001	NY20250811002	NY20250811003	NY20250811004	All individuals
Temperature (°C)	20	20	20	20	20
Humidity	90%	90%	90%	90%	90%
SVL (mm)	37.7	37.6	39.1	38.5	37.6–39.1
Call groups/Calls analyzed	26/73	22/74	23/73	22/77	93/297
Call duration (ms)	130.51 ± 10.92 86.70–149.70	161.08 ± 17.94 117.60–236.50	119.34 ± 12.00 88.20–170.10	167.25 ± 14.19 132.40–224.40	144.91 ± 24.55 86.70–236.50
Call interval (ms)	400.76 ± 96.53 224.33–645.15	375.76 ± 108.53 180.13–664.98	371.96 ± 89.96 200.17–593.86	348.96 ± 125.95 162.86–828.71	373.36 ± 107.62 162.86–828.71
No. of calls per call group	2.81 ± 0.75 2.00–4.00	3.36 ± 0.66 2.00–4.00	3.17 ± 0.65 2.00–5.00	3.50 ± 0.60 2.00–4.00	3.19 ± 0.71 2.00–5.00
Call repetition rate (calls/min)	164.61 ± 27.83 110.69–230.94	142.90 ± 13.49 110.19–170.20	163.78 ± 15.89 131.86–190.72	145.26 ± 15.00 116.82–177.19	154.69 ± 21.60 110.19–230.94
No. of pulses per call	12.00 ± 1.00 6.00–15.00	12.00 ± 1.00 8.00–16.00	13.00 ± 0.50 10.00–17.00	12.00 ± 1.50 8.00–17.00	12.00 ± 1.00 6.00–17.00
Pulse rate	82.25 ± 9.50 57.67–108.61	68.96 ± 10.98 49.23–103.09	99.78 ± 11.87 72.62–146.23	67.05 ± 9.16 47.65–92.07	79.31 ± 16.70 47.65–146.23
Dominant frequency (kHz)	3.46 ± 0.07 3.45–3.79	3.63 ± 0.08 3.45–3.79	3.54 ± 0.16 3.45–3.79	3.61 ± 0.09 3.27–3.79	3.56 ± 0.12 3.27–3.79
Frequency 5% (kHz)	2.70 ± 0.37 2.07–3.10	3.28 ± 0.08 2.93–3.45	2.90 ± 0.17 2.76–3.10	3.22 ± 0.11 2.76–3.27	3.03 ± 0.32 2.07–3.45
Frequency 95% (kHz)	4.28 ± 0.24 3.79–4.82	4.59 ± 0.24 4.31–5.34	4.35 ± 0.25 4.13–5.17	4.59 ± 0.25 4.31–5.17	4.45 ± 0.28 3.79–5.34

Figure 1. Photographs of the specimens. **A.** Adult male of *Boulenophrys daxuemontis* (specimen number: NY20250811001) from Nayong County, Guizhou Province, China; **B.** Adult male of *Boulenophrys jiangi* (specimen number: KKS20250626001) from Suiyang County, Guizhou Province, China.

All calls of *B. daxuemontis* exhibit a consistent structure (Fig. 2C), with the amplitude being relatively low at the beginning of the call and then suddenly increasing to its peak amplitude. The amplitude gradually decreases after reaching its peak until the end of the call (Fig. 2E). The advertisement call of *B. daxuemontis* comprised a single note with a mean call duration of 144.91 ± 24.55 ms. The mean call interval was 373.36 ± 107.62 ms. The number of calls from the call group ranged from 2.00–5.00. The mean call repetition rate was 154.69 ± 21.60

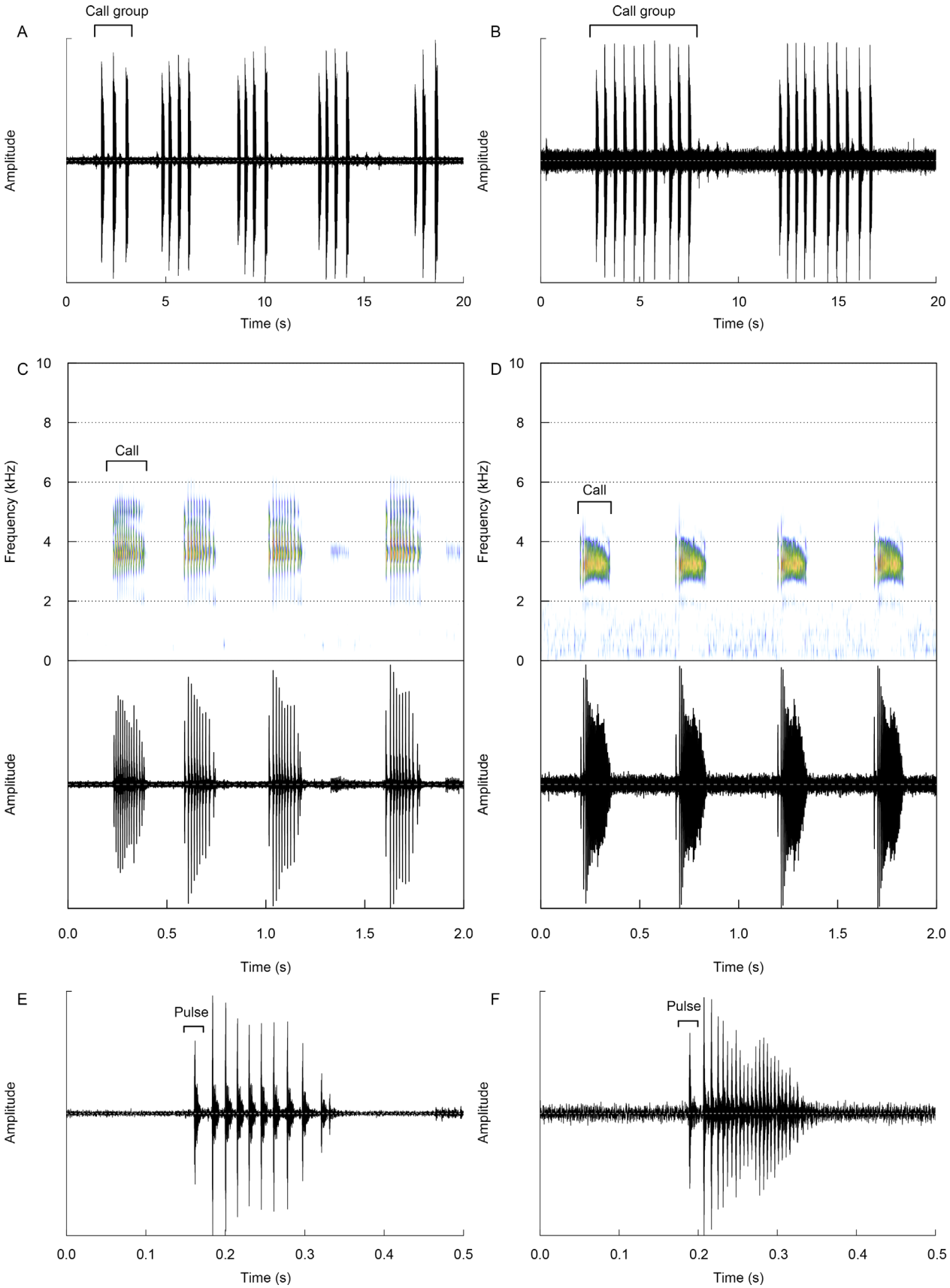

Figure 2. Advertisement calls of the two *Boulenophrys* species. **A.** 20 s oscillograms showing five call groups of *Boulenophrys daxuemontis*; **B.** 20 s oscillograms showing two call groups of *Boulenophrys jiangi*; **C.** 2 s oscillograms and corresponding spectrograms showing four calls of *B. daxuemontis*; **D.** 2 s oscillograms and corresponding spectrograms showing four calls of *B. jiangi*; **E.** 0.5 s oscillograms showing one call of *B. daxuemontis*; **F.** 0.5 s oscillograms showing one call of *B. jiangi*.

calls/min. The mean pulse number was 12.00 ± 1.00, with a mean pulse rate of 79.31 ± 16.70 pulses/s. The mean dominant frequency was 3.56 ± 0.12 kHz. The acoustic properties of the spectral and temporal structures of *B. daxuemontis* are shown in Table 1.

During the recording process, we made several behavioral observations. Males called sporadically rather than in continuous choruses, often with long silent intervals between call groups. When a male began calling, it was typically in response to the call of a nearby male. Additionally, males were highly vigilant. Calling ceased immediately in response to our approach or any noise. The four recorded individuals of *B. daxuemontis* were relatively dispersed, with an estimated distance of approximately 3 to 10 meters between calling males.

After molecular identification, we confirmed that the species collected from Suiyang County is *B. jiangi* (Fig. 1B). The GenBank accession number for the CO*I* sequence of *B. jiangi* is PZ138357 (specimen number: KKS20250626001). The SVL of *B. jiangi* ranged from 37.0 mm to 38.4 mm (Table 2). We analyzed the spontaneous vocalizations of (223 calls) from four *B. jiangi* males. The call structure of the *B. jiangi* is similar to that of the *B. daxuemontis*. (Fig. 2C, D). Call amplitude was consistent across all call groups, with the exception of the initial call, which exhibited a lower amplitude (Fig. 2B). The second call experiences a sudden increase in amplitude modulation, directly reaching the peak amplitude of the entire call group. All calls of *B. jiangi* exhibit a consistent structure (Fig. 2D), with a lower amplitude at the beginning of the call and then suddenly increasing to its peak amplitude. After reaching the peak amplitude, the amplitude gradually decreases. When it decreases to about 1/2 of the entire call, the amplitude gradually increases again, but does not exceed the peak amplitude. Then it rises to about 3/4 of the entire call, and the amplitude gradually decreases until the end of the call (Fig. 2F). The advertisement call of *B. jiangi* comprised a single note with a mean call duration of 113.27 ± 22.53 ms. The mean call interval was 309.75 ± 90.13 ms. The number of calls from the call group ranged from 5.00–15.00. The mean call repetition rate was 156.87 ± 27.46 calls/min. The mean pulse number was 24.00 ± 2.00, with a mean pulse rate of 214.96 ± 48.99 pulses/s. The mean dominant frequency was 3.42 ± 0.25 kHz. The acoustic properties of the spectral and temporal structures of *B. jiangi* are shown in Table 2.

Comparison of advertisement call

The Mann–Whitney U test revealed significant differences in call parameters between the *B. daxuemontis* and *B. jiangi*, with the exception of the call repetition rate (Table 3). The call duration, call interval, dominant frequency, frequency 5%, and frequency 95% of *B. daxuemontis* were significantly higher than those of *B. jiangi*, while the number of calls per call group, number of pulses per call, and pulse rate of *B. daxuemontis* were significantly lower than those of *B. jiangi* (Table 3). The principal component analysis (PCA) of call parameters between *B. daxuemontis* and *B. jiangi* resulted in five PCs, explaining a cumulated variance of 95.77% (Table 4). The PC1 axis accounted for 45.12% of the total variation and was primarily associated with temporal parameters, as it comprised the number of pulses per call

Table 2. Descriptive statistics for call parameters of advertisement calls of *Boulenophrys jiangi*. Data are given as mean ± SD and range (min–max).

Voucher	KKS20250626001	KKS20250626002	KKS20250626003	KKS20250626004	All individuals
Temperature (°C)	22	22	22	22	22
Humidity	95%	95%	95%	95%	95%
SVL (mm)	37.0	38.0	37.3	38.4	37.0–38.4
Call groups/Calls analyzed	9/71	6/56	4/52	4/44	23/223
Call duration (ms)	144.65 ± 7.18 127.70–168.30	102.17 ± 5.11 91.40–116.50	99.52 ± 3.25 94.40–108.70	93.01 ± 7.54 81.00–110.70	113.27 ± 22.53 81.00–168.30
Call interval (ms)	362.74 ± 98.92 230.97–732.14	227.80 ± 37.26 158.73–364.37	319.81 ± 46.07 252.19–517.74	318.00 ± 92.60 186.86–653.49	309.75 ± 90.13 158.73–732.14
No. of calls per call group	7.89 ± 1.76 5.00–10.00	9.33 ± 0.52 9.00–10.00	13.00 ± 1.83 11.00–15.00	11.00 ± 1.83 9.00–13.00	9.70 ± 2.40 5.00–15.00
Call repetition rate (calls/min)	132.31 ± 11.71 114.69–149.93	196.60 ± 7.86 182.89–204.36	152.47 ± 4.29 148.49–157.50	156.92 ± 7.25 148.20–164.60	156.87 ± 27.46 114.69–204.36
No. of pulses per call	25.00 ± 1.00 22.00–34.00	22.00 ± 1.00 19.00–26.00	22.00 ± 0.50 20.00–29.00	29.00 ± 1.50 25.00–38.00	24.00 ± 2.00 19.00–38.00
Pulse rate	167.21 ± 14.57 145.13–244.81	207.55 ± 10.41 185.19–231.82	216.23 ± 17.86 178.07–283.40	299.95 ± 17.23 256.18–349.49	214.96 ± 48.99 145.13–349.49
Dominant frequency (kHz)	3.18 ± 0.15 3.10–3.45	3.45	3.63 ± 0.31 3.45–4.13	3.51 ± 0.13 3.45–3.79	3.42 ± 0.25 3.10–4.13
Frequency 5% (kHz)	2.41 ± 0.12 1.72–2.76	2.76	2.80 ± 0.16 2.76–3.45	2.76	2.66 ± 0.20 1.72–3.45
Frequency 95% (kHz)	4.07 ± 0.13 3.79–4.13	4.37 ± 0.33 4.13–4.82	4.76 ± 0.21 4.13–4.82	4.42 ± 0.13 4.13–4.48	4.38 ± 0.33 3.79–4.82

Table 3. Mann–Whitney U test to examine the differences in advertisement call parameters between *Boulenophrys daxuemontis* and *Boulenophrys jiangi*. Significance was set at p = 0.05.

Call parameter	p value
Call duration (ms)	< 0.001
Call interval (ms)	< 0.001
No. of calls per call group	< 0.001
Call repetition rate (calls/min)	0.948
No. of pulses per call	< 0.001
Pulse rate	< 0.001
Dominant frequency (Hz)	< 0.001
Frequency 5% (Hz)	< 0.001
Frequency 95% (Hz)	< 0.001

Table 4. Factor loadings of the principal component analysis (PCA) on the advertisement call parameters of the *Boulenophrys daxuemontis* and *Boulenophrys jiangi*.

Call parameters	Principal components				
	1	2	3	4	5
Call duration	0.72	-0.41	-0.10	0.18	0.47
Call interval	0.37	-0.45	0.19	-0.38	-0.05
No. of pulses per call	**-0.88**	0.09	-0.04	-0.23	0.34
Pulse rate	**-0.89**	0.33	0.17	0.60	-0.03
Dominant frequency	0.52	**0.68**	0.18	0.01	0.42
Frequency 5%	0.76	0.36	**0.81**	-0.01	-0.05
Frequency 95%	0.29	**0.72**	0.21	-0.08	0.20
Eigenvalue	3.16	1.60	0.81	0.59	0.55
Variance (%)	45.12	22.80	11.50	8.48	7.87
Cumulative variance (%)	45.12	67.92	79.42	87.90	95.77

and pulse rate (Table 4). The PC2 axis explains 22.80% of the variation, primarily driven by the spectral parameter of dominant frequency and frequency 95%. PC3 axis accounted for 11.50% of the total variation, mainly driven by frequency 5% (Table 4). The scatter plots generated from the first two principal components showed complete separation between the two species along the PC1 axis, with considerable overlap observed along PC2 (Fig. 3). PC1 was primarily driven by temporal parameters, specifically the number of pulses per call and pulse rate (Table 4). This indicates that although spectral parameters also showed statistically significant differences between species (Table 3), pulse-related characteristics are the primary acoustic features distinguishing the advertisement calls of *B. daxuemontis* and *B. jiangi*.

Discussion

Over the past five years, morphological and phylogenetic analyses have facilitated the identification of species in the genus *Boulenophrys*, which has increased from forty to eighty-one (Frost 2026; Li et al. 2025; Wang HT et al. 2025; Wang RQ et al. 2025). *Boulenophrys daxuemontis* is a recently described species, with its type locality in Junlian County, Sichuan Province (Liu et al. 2025a). The discovery in Nayong County, Guizhou Province has further expanded the geographical distribution range of this species. The discovery of *B. daxuemontis* in Guizhou Province has also increased the number of amphibian

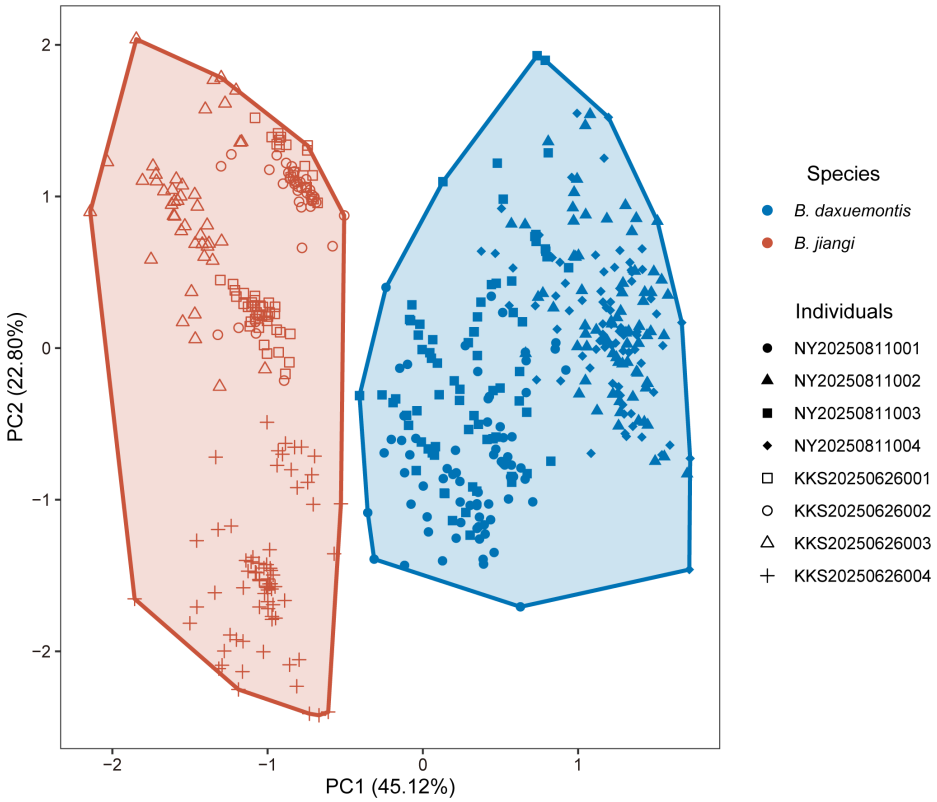

Figure 3. Plots of the first principal component (PC1) versus the second (PC2) for advertisement calls of *Boulenophrys daxuemontis* and *Boulenophrys jiangi* from a principal component analysis.

species in the province, while indicating that the diversity of amphibians in Guizhou Province is still underestimated (Li et al. 2020a; Zhou et al. 2023). The population of *B. daxuemontis* in Nayong county is approximately 140 km away from the type locality (Liu et al. 2025a). This area is located on the border of Sichuan Province, Guizhou Province, and Yunnan Province, indicating that *B. daxuemontis* may also be distributed in other nearby areas. Further investigation efforts should be intensified to explore the distribution boundaries of this species.

At present, there are 13 species of the genus *Boulenophrys* in Guizhou Province (Li et al. 2024; Liu et al. 2025b). Compared to the published calls of the other species in Guizhou Province, the advertisement call of *B. daxuemontis* differs considerably, reinforcing the specific identity of this species. For example, the call duration of *B. daxuemontis* is longer than that of *Boulenophrys chishuiensis* and *Boulenophrys congjiangensis* (Xu et al. 2020; Luo et al. 2021). Compared to *B. daxuemontis*, the species of *Boulenophrys anlongensis*, *Boulenophrys jiangi*, *Boulenophrys leishanensis*, *Boulenophrys qianbeiensis*, and *Boulenophrys spinata*, have more calls in each call group (Li et al. 2020b; Liu et al. 2020; Su et al. 2020; Shen et al. 2024). It should be noted that our advertisement call samples were not collected from the type locality of *B. daxuemontis*. Additionally, we acknowledge that our acoustic analyses are based on recordings from a limited number of individuals. Therefore, future studies should collect advertisement calls from the type locality and incorporate larger sample sizes to investigate potential geographic variation and clarify intraspecific variation patterns of advertisement calls.

In summary, our research provides a new record location for the *B. daxuemontis*. In addition, our study described the advertisement call characteristics of *B. daxuemontis* and confirmed that it exhibits species-specific advertisement call features, which supports the species specificity of its acoustic signals. Our results offer basic data for future study in behavior, ecology, and biodiversity.

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