

Geographical Variation in the Advertisement Calls of *Leptobrachella ventripunctata* (Anura: Megophryidae) in Southwestern China

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Abstract In anurans, advertisement calls (ACs) are an essential form of intraspecific communication. This study evaluates geographical variation in the ACs of *Leptobrachella ventripunctata* in the Guizhou Plateau, southwestern China, and explores correlations between call characteristics, body size, and environmental factors. ACs are simple calls of *L. ventripunctata*, and apparent differences were observed in the ACs among different geographical populations of *L. ventripunctata*. The Call duration (CD) revealed a significant positive correlation with altitude and a significant negative correlation with temperature and humidity. Moreover, the Dominant frequency (DF) exhibited a significant negative correlation with altitude and the habitat closure degree and a significant positive correlation with temperature. These variations in ACs between different geographical populations of *L. ventripunctata* may critically impact the adaptive evolution of species, and the calls may also be relevant for environmental selection.

Keywords anuran, bioacoustics, environmental factors, geographical divergence

1. Introduction

Bioacoustics is a subfield of biophysics and is vital in studying animal behavior (Koehler *et al.*, 2017). Acoustic signals are helpful for taxonomic research and reflect the evolutionary history of species and environmental selection (Duarte-Marín *et al.*, 2023; Mendoza-Henao *et al.*, 2023; Booker *et al.*, 2023). Consequently, the study of regional variation in acoustic signals is an important area of research in bioacoustics (Velásquez *et al.*, 2018; Zhao *et al.*, 2023). The geographical variation of acoustic signals influenced by environmental factors highlights evolutionary pressures and adaptive strategies critical for mating, communication, and predator avoidance (Ryan *et al.*, 1990; Wells, 2007). Extensive research has been conducted on the geographical variance of acoustic signals across taxonomic groups, including birds, mammals, amphibians, and insects (Ong and Shahrudin, 2022; Wu *et al.*, 2023). Vocalization plays a key role in species recognition, reproduction, and evolutionary processes in anurans (Cunningham and Birkhead, 1998; Brenowitz and Rose, 1999; Kelley, 2004). To deepen the understanding of the relationship between anuran behavior and vocal interactions, scientists have categorized anuran calls into four distinct types: feeding, aggressive, reproductive, and defensive calls. Reproductive calls include advertisement calls (ACs), courtship calls, amplexant calls, release calls, post-oviposition male release calls, and rain calls (Toledo *et al.*, 2015; Koehler *et al.*, 2017). Furthermore, the significant variability of ACs among different species makes them a useful basis for the systematic classification and identification of cryptic species (Sullivan *et al.*, 1996).

Environmental factors can influence geographical populations of the same species in the diverse ways, imposing varying constraints on the evolution of acoustic

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signals (Forrest, 1994; Goutte *et al.*, 2018). Altitude, temperature, humidity, and vegetation (habitat closure degree) are the primary determinants of amphibian physiology and ecology (Wang *et al.*, 2012; Chen, 2018; Wang *et al.*, 2022). In addition, the acoustic signals of different geographical populations of the same species can be influenced by body size and genetic differentiation (Ey and Fischer, 2009). For example, larger individuals could produce calls with lower frequencies and higher loudness, enabling acoustic signals to propagate over greater distances (Shen *et al.*, 2015; Yasumiba *et al.*, 2016). Meanwhile, genetic differentiation can lead to variations in call frequency, rhythm, and pattern among individuals of the same species (Lee *et al.*, 2016). These variations can serve as individual recognition signals, assisting mates to choose among numerous potential partners (Brenowitz and Rose, 1999).

The Acoustic Adaptation Hypothesis (AAH) proposes that signals are selected to possess optimal characteristics for transmission within the native environment of the sender, thereby circumventing environmental constraints (Morton, 1975). The AAH posits that animals in enclosed habitats emit acoustic signals with lower frequency, longer duration, and slower amplitude (Morton, 1975; Wiley and Richards, 1978; Gillard and Rowley, 2023). Gillard and Rowley (2023) also stated that *Anura* could be a more suitable clade for studying AAH due to their relatively low mobility.

Numerous studies have explored the variation of acoustic signals within vertebrate species (Ey and Fischer, 2009; Lin, 2015; Zhao *et al.*, 2023). However, there are few systematic studies on amphibian acoustics in karst regions. Species within the genus *Leptobrachella* exhibit a high degree of morphological conservatism and cannot be accurately identified based on a single morphological characteristic. The progress of phylogenetic and acoustic studies has resulted in more precise identifications (Luo *et al.*, 2020; Liu *et al.*, 2023). The *Leptobrachella ventripunctata* (*Anura*: Megophryidae), first described in 1990, is widely distributed across the mountainous areas of southwestern China (Fei *et al.*, 1990; Frost, 2023). Prior research primarily focused on the taxonomy and phylogeny of this species (Delorme *et al.*, 2006; Dubois *et al.*, 2010; Chen *et al.*, 2018). Although there have been scattered reports regarding the acoustics of *L. ventripunctata*, no comprehensive

investigation has been carried out (Yeung *et al.*, 2021; Cheng *et al.*, 2021). The geographical variation in vocal signals of *L. ventripunctata* remains unknown. This study investigated the variations in acoustic signals among different populations of *L. ventripunctata* in the karst mountains of Guizhou, and examined the mechanisms contributing to these variances.

This study explores three questions: 1) Are there variations in the ACs of *L. ventripunctata* among different geographical populations? 2) What are the differences, if any? 3) Is there a correlation between the main call characteristics and the habitat environment?

2. Materials and Methods

2.1. Fieldwork and acoustic recordings We recorded male *L. ventripunctata* vocalizations in their natural habitat from April 10 to May 5, 2022. We collected the ACs of four populations of *L. ventripunctata* located in Anlong County (105.6° E, 25.0° N), Ceheng County (105.5° E, 24.9° N), Huishui County (106.4° E, 25.8° N) and Ziyun County (106.2° E, 25.6° N), in Guizhou Province, China. We conducted recordings from 8:30 PM to 00:30 AM, during which the air temperature averaged 13.6 °C ± 2.6 °C, and the relative humidity was 81.4% ± 14.0%, measured using a temperature and humidity recorder (SMARTSENSOR ST8817). In addition, the latitude, longitude, and altitude of each location were recorded using the GPS Positioning System (Two-step outdoor assistant, Shenzhen 2bulu Information Technology Co. Ltd., China). ACs from 23 males of *L. ventripunctata* were recorded, including 6 males from the Anlong population, 7 males from the Ceheng population, and 5 males from both the Huishui and Ziyun populations (Table 1). Each individual's calls were recorded with a SONY ICD-PX470 digital recorder (44.1 kHz sampling rate, 16-bit depth) positioned 0.5 meters away from the calling individual and saved as WAV files. The duration of these recordings varied between 1 and 4 min for all individuals. Recorded calls were always from isolated individuals, ensuring no interference from background choruses. Frogs were carefully captured by hand and identified using the file numbers generated by the digital recorder, thus linking each frog's sound information with its morphological traits.

Table 1 Collection information of *L. ventripunctata*.

Population	Sample size (N)	Mean SVL (mm)	Longitude	Latitude	Elevation (m)	Temperature (°C)	Precipitation (%)	NDVI
Anlong	6	28.11	105.603789°	24.982511°	1 665	14.0	95	0.897767
Ceheng	7	27.26	105.545539°	24.936908°	994	17.0	97	0.888834
Huishui	5	27.34	106.480950°	25.863717°	1 010	11.0	75	0.826443
Ziyun	5	27.65	106.234956°	25.643839°	1 074	15.1	65	0.817615

All males were measured on-site with a precision digital caliper to the nearest 0.01 mm for their snout-vent length (SVL). Three individuals from each population were chosen as reference samples, while the remaining animals were returned to their natural habitat following the completion of measurements. The collected male was housed in a humid plastic container (200 mL), containing either grass blades or leaf litter. After photographing, they were euthanized using isoflurane, and the specimens were fixed in 75% ethanol. Tissue samples were separately preserved in 95% ethanol before fixation. Molecular phylogenetic analysis was performed on the collected species. Specimens were deposited at the Forestry College of Guizhou University, China. This study's experimental procedures adhere to the principles of animal ethics and have been approved by the Laboratory Animal Ethics Subcommittee of Guizhou University with the approval number of EAE-GZU-2023-E013 (Appendix 5).

2.2. Species identification To confirm the species of the specimens, both morphological comparison and molecular identification were performed. Morphological identification followed the guidelines of Fei *et al.* (2012), while molecular identification via sequencing of the mitochondrial 16S rRNA (16S) gene involves amplifying the mitochondrial 16S gene using primers P7 (5'-CGCCTGTTTACCAAAAACAT-3') and P8 (5'-CCGGTCTGAACTCAGATCACGT-3'), as outlined by Simon *et al.* (1994).

2.3. Molecular experiments and analysis The gene fragments were amplified under specific conditions: an initial denaturation at 95 °C for 4 min, followed by 36 cycles of 30 s at 95 °C for denaturation, 30 s at 51 °C for annealing, and 70 s at 72 °C for extension. Sequencing was performed on an ABI3730 automated DNA sequencer by Shanghai DNA BioTechnologies Co., Ltd. (Shanghai, China). Newly obtained sequences were deposited in GenBank, and accession numbers are provided in Appendix 1. Sequences assembly and alignment were carried out using the Clustalw module in BioEdit v. 7.0.9.0 (Hall, 1999) with default settings. Alignments were reviewed manually and revised as needed. Phylogenetic analyses were carried out using the Bootstrap method to

derive the Neighbor-Joining (NJ) tree in MEGA 11 (Saitou and Nei, 1987; Tamura *et al.*, 2021). In the NJ analysis, a bootstrap consensus tree, inferred from 10 000 replicates, was used to illustrate the evolutionary history of the taxa examined. Additionally, pairwise distances (*P*-distance) were calculated using the uncorrected *P*-distance model in MEGA 11.

2.4. Bioacoustics data processing The characteristics of the ACs were processed using the software Raven Pro 1.6. To ensure the validity and robustness of the data, only individuals with high signal-to-noise ratios and no overlap from nearby males were selected for statistical analysis. Temporal call characteristics were assessed using the waveform display in Raven, while spectral properties were examined by averaging the spectrum across the entire call duration (256 pt. fast Fourier transform, Hanning window).

We adhered to the “call-centered” terminology framework as outlined, which defines a single vocal event as a “call”, and a series of uninterrupted vocalizations as a “call group” (Koehler *et al.*, 2017). Due to the peculiarities of *L. ventripunctata* vocalizations, we define continuous vocalizations as “call”, and a single vocalization as a “pulse”. We measured all parameters and characteristics in accordance with the methods described by Chen *et al.* (2020) and Yeung *et al.* (2021), including Call duration (CD), Dominant frequency (DF), Inter-call interval, Pulses note per call and Pulse rate (Table 2). Oscillograms, spectrograms, and power spectrum were graphed using the Seewave package in R (Sueur *et al.*, 2008).

2.5. Data analysis Descriptive statistics of the call characteristics of different geographical populations were calculated from the individual mean, including mean, standard deviation (SD), range and percent coefficient of variation ($CV = SD \times 100/\text{mean}$; calculated as within populations CV, where SD is divided by the mean value of a population multiplied by 100) were computed in SPSS 23.0. In addition, the Kruskal-Wallis test was used to explore the differences of the four call parameters among the four populations in *L. ventripunctata*, except for the inter-call interval, and multiple comparisons were made after the fact. The Factoextra R package was utilized to perform a principal component analysis (PCA) with

Table 2 Descriptions of acoustic properties measured.

Properties of calls	Definition of call properties
Call duration (ms)	Time between onset of first pulse and offset of last pulse in a call
Dominant frequency (kHz)	The frequency with highest energy content of the entire call
Pulses note per call	Count of pulses (<i>k</i>)
Inter-call interval (ms)	The interval is between the end of the previous call and the beginning of the next call
Pulse rate (pulses/s)	$(k-1)/t$, where <i>t</i> is the time between onset of first pulse and onset of last pulse

four call characteristics, with the inter-call interval omitted from the PCA. Additionally, a discriminant function analysis (DFA) was conducted using the same call variables. The two primary call characteristics, CD and DF, were selected for correlation analysis, as follows: Spearman's correlation analysis was employed to investigate potential relationships between call characteristics and body size across different populations. Furthermore, the possible relationship between environmental factors and call characteristics by Spearman's correlation analysis, including factors such as elevation, habitat tightness, temperature and humidity (Chen, 2018; Wang *et al.*, 2022). Normalized Difference Vegetation Index (NDVI) as the best indicator factor for vegetation growth and coverage, can well reflect the characteristics and changes of vegetation

distribution (Xing and He, 2021), and it is usually related to the vegetation density and structural complexity of the understory which characterizes the habitat structure. In this study, the NDVI was used to characterize habitat closure degree, with the belief that higher NDVI values indicate greater habitat closure degree. NDVI was sourced from the Resource and Environment Science and Data Center (<http://www.resdc.cn/>), and altitude, temperature and humidity were recorded in real-time for field investigations.

3. Results

3.1. Molecular identification The aligned sequence matrix of the 16S gene contained 498 base pairs. All collected samples clustered into a clade with *L. ventripunctata* (Figure 1). The

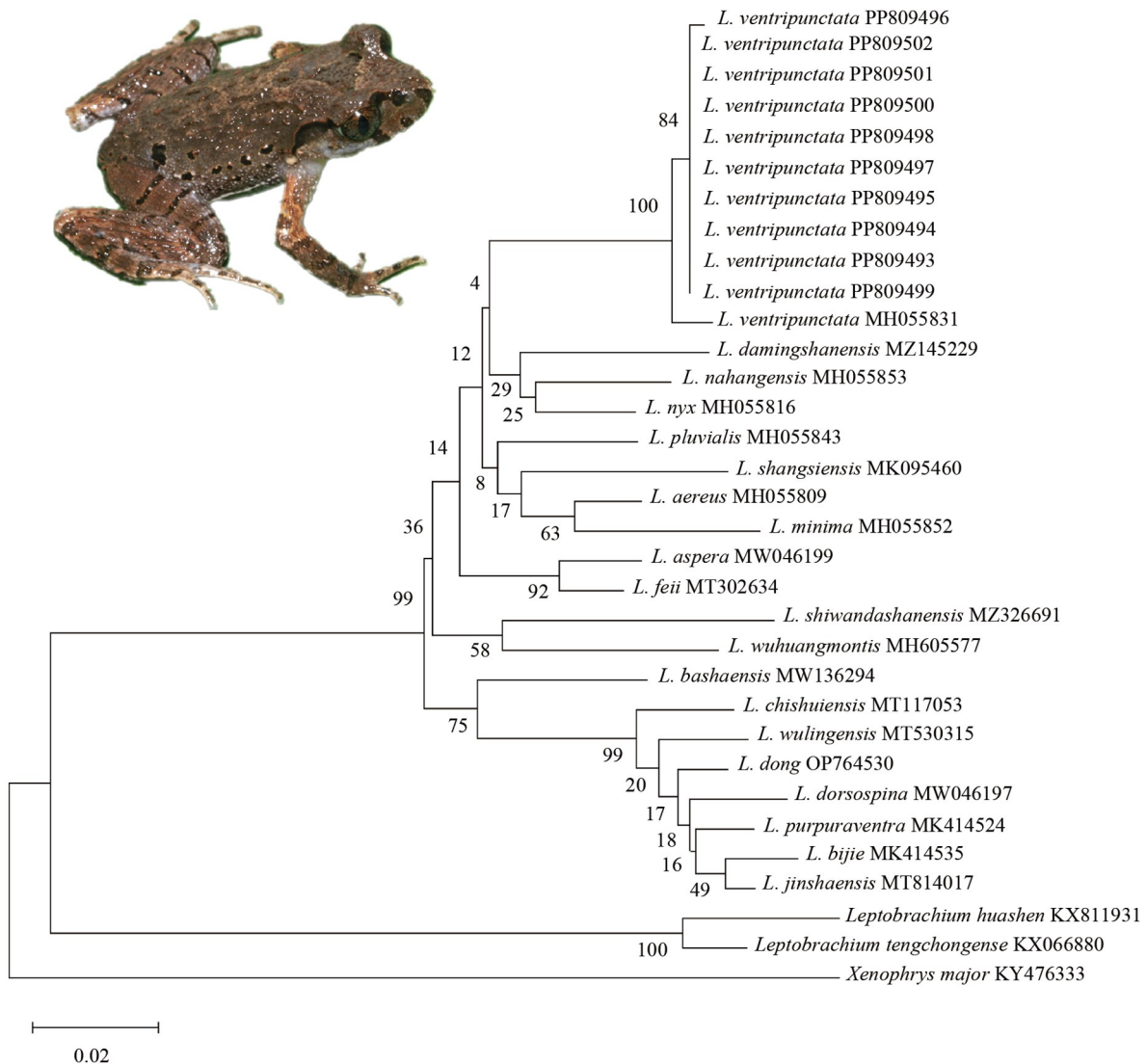


Figure 1 Neighbor-Joining (NJ) tree of *Leptobrachella* species in the karst mountains of Guizhou, and *Leptobrachium huashen*, *Leptobrachium tengchongense*, *Xenophrys major* as outgroups based on the mitochondrial 16S gene.

genetic distances between the four populations, determined by the 16S gene, ranged from 0% to 0.2% (Appendix 2). Morphological comparison and molecular identification confirm that the genetic variation among the four populations is minimal. All samples were identified as *L. ventripunctata*.

3.2. Call characteristics We analyzed a total of 1 150 calls from 23 male individuals of *L. ventripunctata*. A specific form of note was detected in male ACs, a simple call consisting of repeated pulsatile calls without noticeable harmonics (Figure 2). *L. ventripunctata* produced short trill calls with a repeated squeak sound reminiscent of the calling songs of the orthopteran species. The Ziyun and Ceheng populations exhibited the longest and shortest CD, respectively, among the

four populations (Table 3). The highest DF (6.5 kHz) was demonstrated by the Ceheng and Ziyun populations, and the lowest DF was recorded at 6.0 kHz in the Anlong population (Table 3). The descriptive statistics for individual calls of the four populations of *L. ventripunctata* are provided in Appendix 3.

3.3. The differences in call characteristics among the four geographical populations A Kruskal-Wallis test was conducted to evaluate the statistical significance of differences in four call characteristics among the four populations, with a significance threshold set at 0.05. The results indicated significant differences in the CD and the number of pulses per call among the populations, except between the Anlong and Huishui populations. Furthermore, no significant differences were observed in DF between Ceheng and Ziyun populations, however, significant differences existed among other populations (Figure 3). Multiple comparisons of the remaining parameters are presented in Appendix 4.

The PCA was conducted using a correlation matrix of four call variables (CD, DF, number of pulses per call, and pulse rate) from the four populations. The PCA revealed a partial overlap between the Anlong and the Huishui populations in PC space, with a slight overlap between the Ceheng and the Ziyun populations (Figure 4A). The first principal component (PC 1) accounted for 53.89% of the overall variability, predominantly influenced by the number of pulses in each call (factor score = 0.945) and the pulse rate (factor score = 0.796). Meanwhile, the second principal component (PC 2), primarily associated with CD (factor score = 0.806), explained 32.66% of the observed variance. Furthermore, the stepwise backward DFA (Figure 4B) correctly identified each population 73.9% as correct (100% of the Ceheng population, 80% of the Ziyun population, 60% of the Huishui population and 50% of the Anlong population). Three discriminant functions were generated, with the eigenvalues of the first two factors being 84.1 and 15.6, respectively. The centroids were -1.931 and -0.286 for the Anlong population, 2.151 and -0.476 for the Ceheng population, -1.034 and -0.310 for the Huishui population, and 0.341 and 1.320 for the Ziyun population.

3.4. Relationship between body size and call characteristics

The Anlong population exhibited the largest average snout-vent length (SVL = 28.11 ± 0.68 mm, $N = 6$), whereas the Ceheng population displayed the smallest (SVL = 27.26 ± 0.45 mm, $N = 7$). The Ziyun (SVL = 27.65 ± 0.82 mm, $N = 5$) and Huishui populations (mean SVL = 27.34 ± 1.17 mm, $N = 5$) demonstrated intermediate body size. Correlation

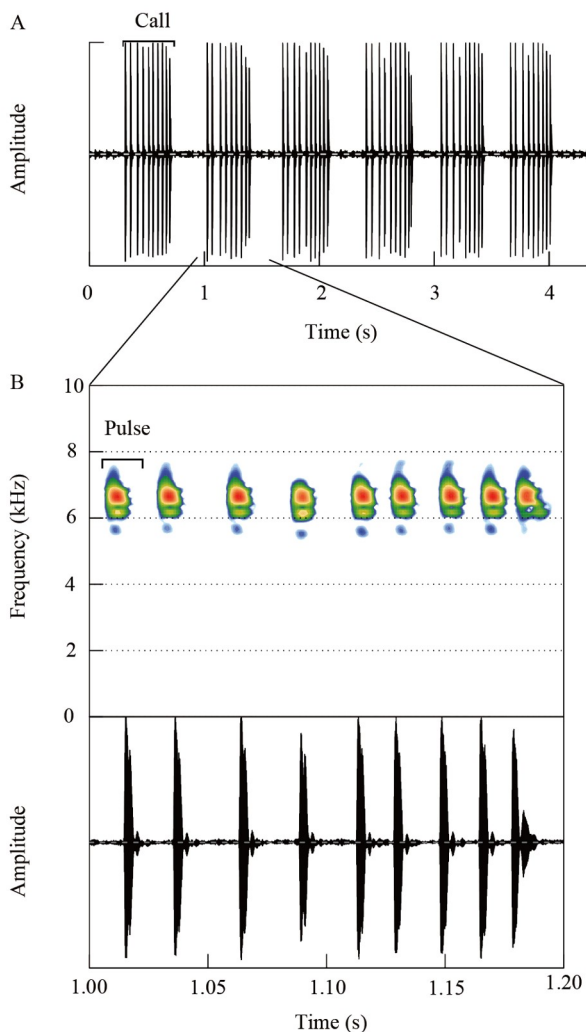


Figure 2 Schematic diagram of the measured acoustic properties. A: 4 s oscillograms showing six calls of *L. ventripunctata*. B: 0.2 s oscillograms and corresponding spectrograms showing nine pulses of one call of *L. ventripunctata*.

Table 3 Descriptive statistics for calls of the four populations of *L. ventripunctata*.

	Mean	SD	Range(min–max)	CV%
Anlong population				
CD (ms)	238	78	64–568	32.8
DF (kHz)	6.0	0.2	5.7–6.5	3.3
Pulses note per call	10 ^a	1.5 ^b	3–30	15.0
Inter-call interval (ms)	279	188	122–1 313	67.4
Pulse rate	36.5	4.2	27.7–54.2	11.5
Ceheng population				
CD (ms)	201	73	44–373	36.1
DF (kHz)	6.5	0.3	5.7–6.9	4.6
Pulses note per call	10 ^a	4 ^b	3–34	40.0
Inter-call interval (ms)	179	90	97–570	50.3
Pulse rate	51.5	11.1	27–141.6	21.6
Huishui population				
CD (ms)	232	108	65–608	46.6
DF (kHz)	6.1	0.2	5.3–6.6	3.3
Pulses note per call	10 ^a	4 ^b	3–29	40.0
Inter-call interval (ms)	751	1 026	139–5 232	136.6
Pulse rate	38.6	13.1	10.7–102.2	33.9
Ziyun population				
CD (ms)	262	84	76–482	32.1
DF (kHz)	6.5	0.2	6.1–6.9	3.1
Pulses note per call	12 ^a	2 ^b	4–24	16.7
Inter-call interval (ms)	300	199	115–1 450	66.3
Pulse rate	43.4	4.1	32.5–56.6	9.4

^a Median instead of mean. ^b Interquartile range instead of SD.

analysis was employed to evaluate the potential association between CD and DF with body size (Figure 5). The SVL for the Ceheng, Anlong, Huishui, and Ziyun populations were 28.11, 27.26, 27.34, and 27.65 mm, respectively. The analysis found no significant correlation between body size and the call characteristics across the four populations ($P > 0.05$).

3.5. Relationship between environmental factors and call characteristics Correlation analysis was performed to evaluate the relationship between environmental factors (altitude, NDVI, temperature, and humidity) and the call characteristics of *L. ventripunctata*. The analysis revealed a significant association between altitude and CD and DF in *L. ventripunctata*. Altitude exhibited a significant positive correlation with CD and a significant negative correlation with DF ($R = 0.61$, $P = 0.0019$, $N = 23$ for CD; $R = 0.55$, $P = 0.0065$, $N = 23$ for DF). DF demonstrated a significant negative correlation with NDVI ($R = 0.5$, $P = 0.016$, $N = 23$). However, no significant correlation existed between CD and NDVI ($R = 0.15$, $P = 0.49$, $N = 23$). Temperature notably impacted the DF in *L. ventripunctata* (Figure 6). A significant

positive correlation was observed between DF and sampling temperature. In contrast, the CD displayed no significant correlation with sampling temperature. Furthermore, a significant negative correlation existed between humidity and CD in *L. ventripunctata*, while no significant correlation was observed between DF and humidity.

4. Discussion

Acoustic signals are crucial for communication among anuran amphibians (Kohler *et al.*, 2017). Anuran calls are usually more stereotypical, often consisting of the continuous repetition of a single note. However, certain anuran species can produce complex vocalizations. *Boophis madagascariensis* (Anura: Mantellidae) produces 28 types of vocalizations, the highest number recorded for any anuran amphibian (Narins *et al.*, 2000). *L. ventripunctata* produces pulsatile calls, a characteristic feature of the genus and the family Megophryidae (Su *et al.*, 2020; Li *et al.*, 2022; Liu *et al.*, 2023). Nevertheless, the vocal repertoire of *L. ventripunctata* is relatively less complex than that of other species groups in this

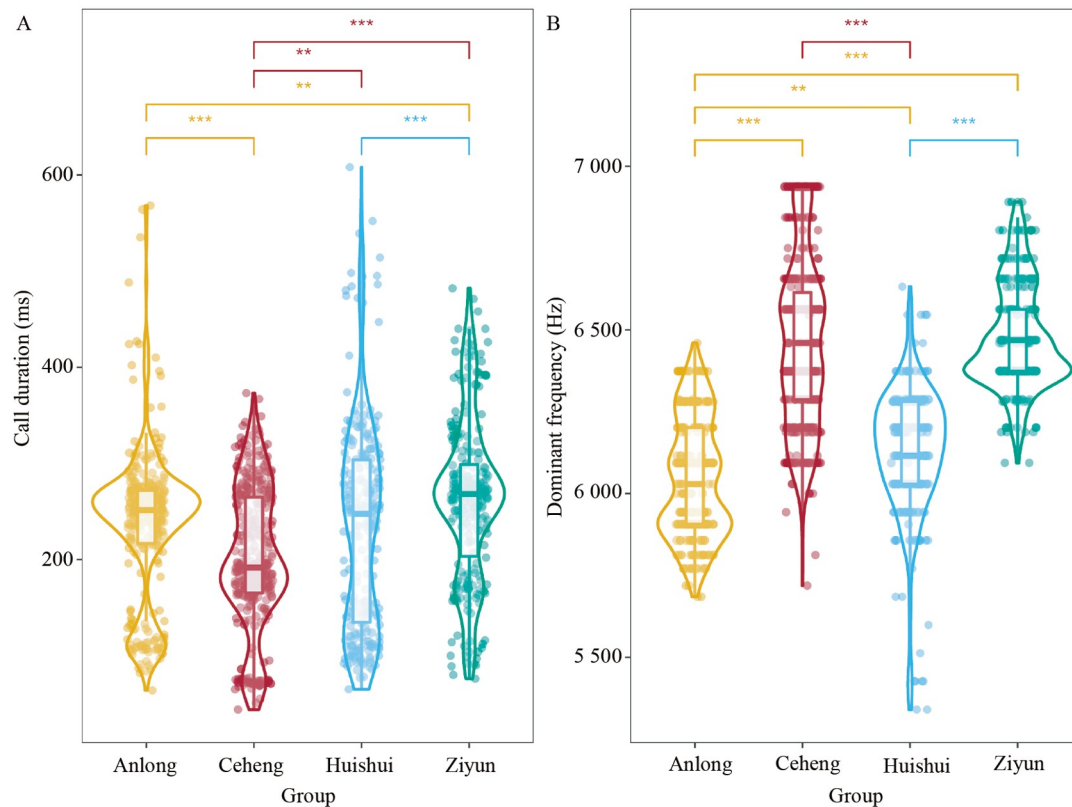


Figure 3 Acoustic comparisons among four populations of *L. ventripunctata*. *P*-value was from Kruskal test. A and B represent the Kruskal test results of the call duration and dominant frequency of the four geographical populations, respectively.

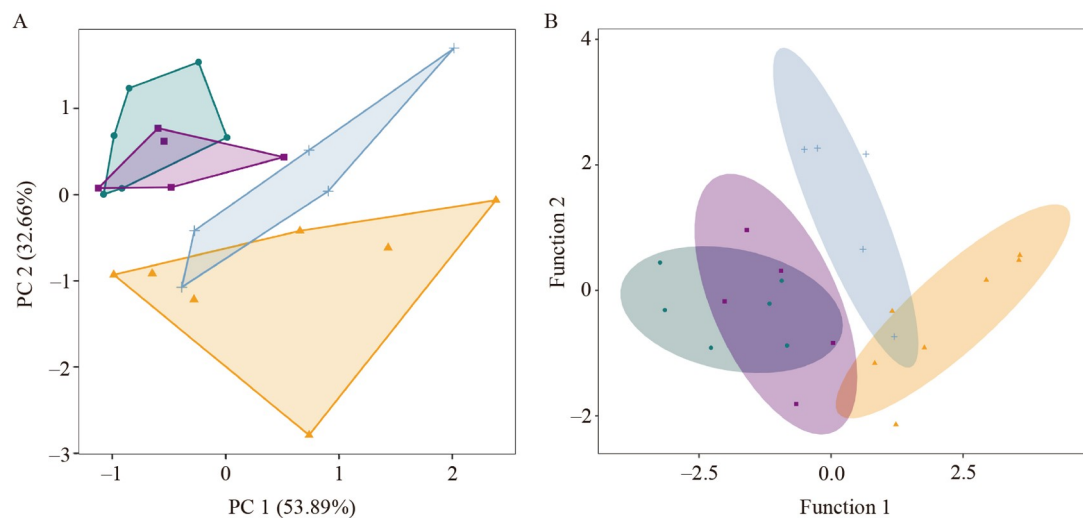


Figure 4 The principal component analysis and the discriminant function analysis of call characteristics for the four populations. Green, purple, blue, and orange areas represent Anlong population, Huishui population, Ziyun population and Chengheng population, respectively, and the points within the area are the number of individuals in each population of *L. ventripunctata*. A: Plot of principle components 1 and 2 (PC 1 (including number of pulses per call and pulse rate) vs. PC 2 (including Call duration)) of the four call characteristics (call duration, dominant frequency, number of pulses per call and pulse rate). B: Canonical variables plot of the discriminant function analysis of four call characteristics variables with 95% confidence ellipses.

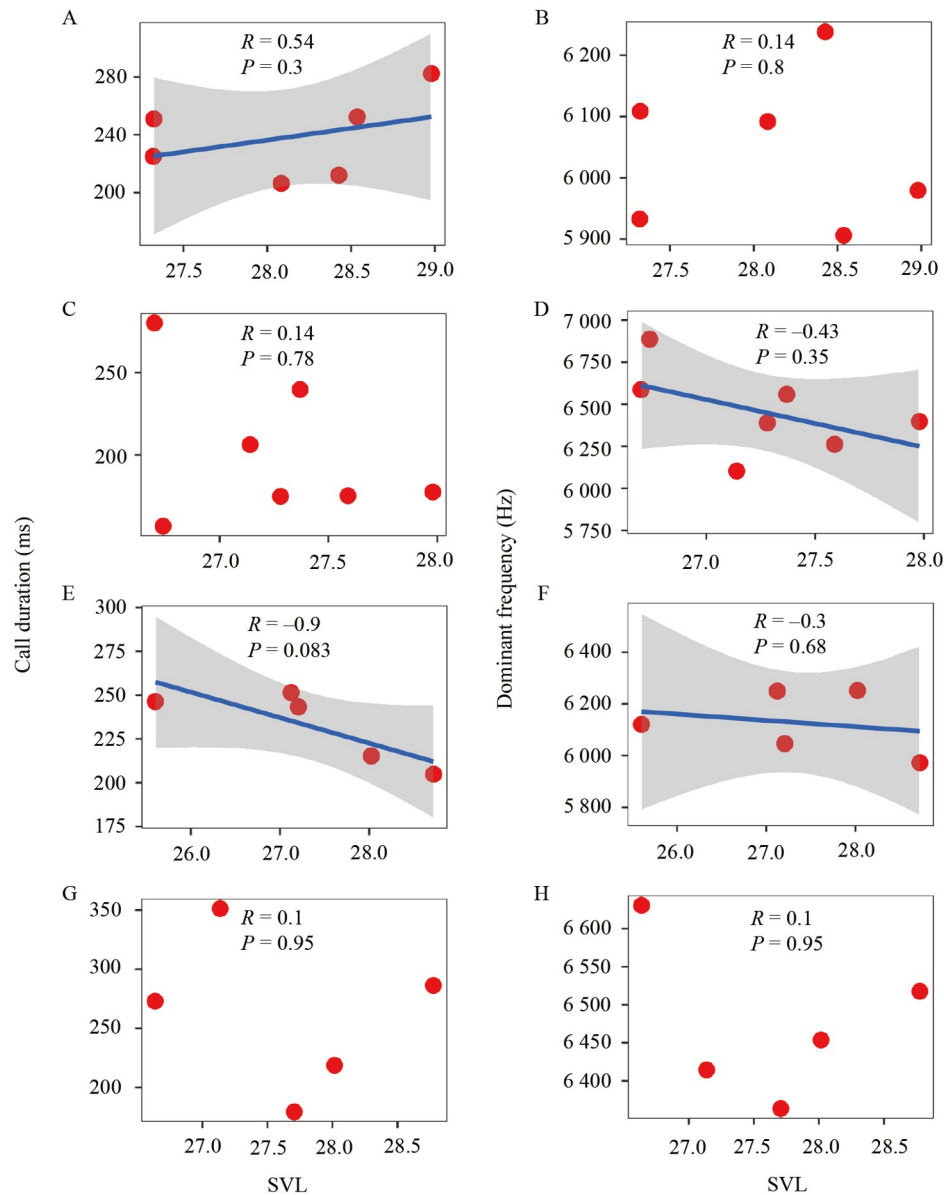


Figure 5 Relationship between two main call characteristics (Call duration and Dominant frequency) and SVL for four populations of *L. ventripunctata*. A–B: Anlong population; C–D: Ceheng population; E–F: Huishui population; G–H: Ziyun population. Blue solid lines indicate significant result of linear regression, with grey shaded areas indicating the 95% confidence interval. Red filled circles are the value of the individual from four populations.

genus (Wang *et al.*, 2019; Shi *et al.*, 2021a, 2023b).

In this study, call characteristics analysis found that the acoustic signals of *L. ventripunctata* generated differentiation among different geographical populations. The two principal call characteristics in the time-domain and frequency-domain exhibited significant variations among certain populations. The results of this research aligned with those of earlier studies (Rodríguez-Tejeda *et al.*, 2014; Shen *et al.*, 2015). Other anuran species demonstrate notable variations in their acoustic signals, attributable to genetic drift (Irwin *et al.*, 2008)

and natural (Ryan *et al.*, 1990) or intersexual selection (Boul *et al.*, 2007).

Call characteristics in some anuran species are associated with body size and may serve as crucial signals in both male-male competition and mate selection (Liu *et al.*, 2018; Robertson, 1986; Giacoma *et al.*, 1997). The larger the individual (such as SVL), the lower the DF, the longer the CD, and the higher the pulse rate (Táranó, 2001). Providing individuals an advantage in mating selection, resource possession, or male competition. However, this view was

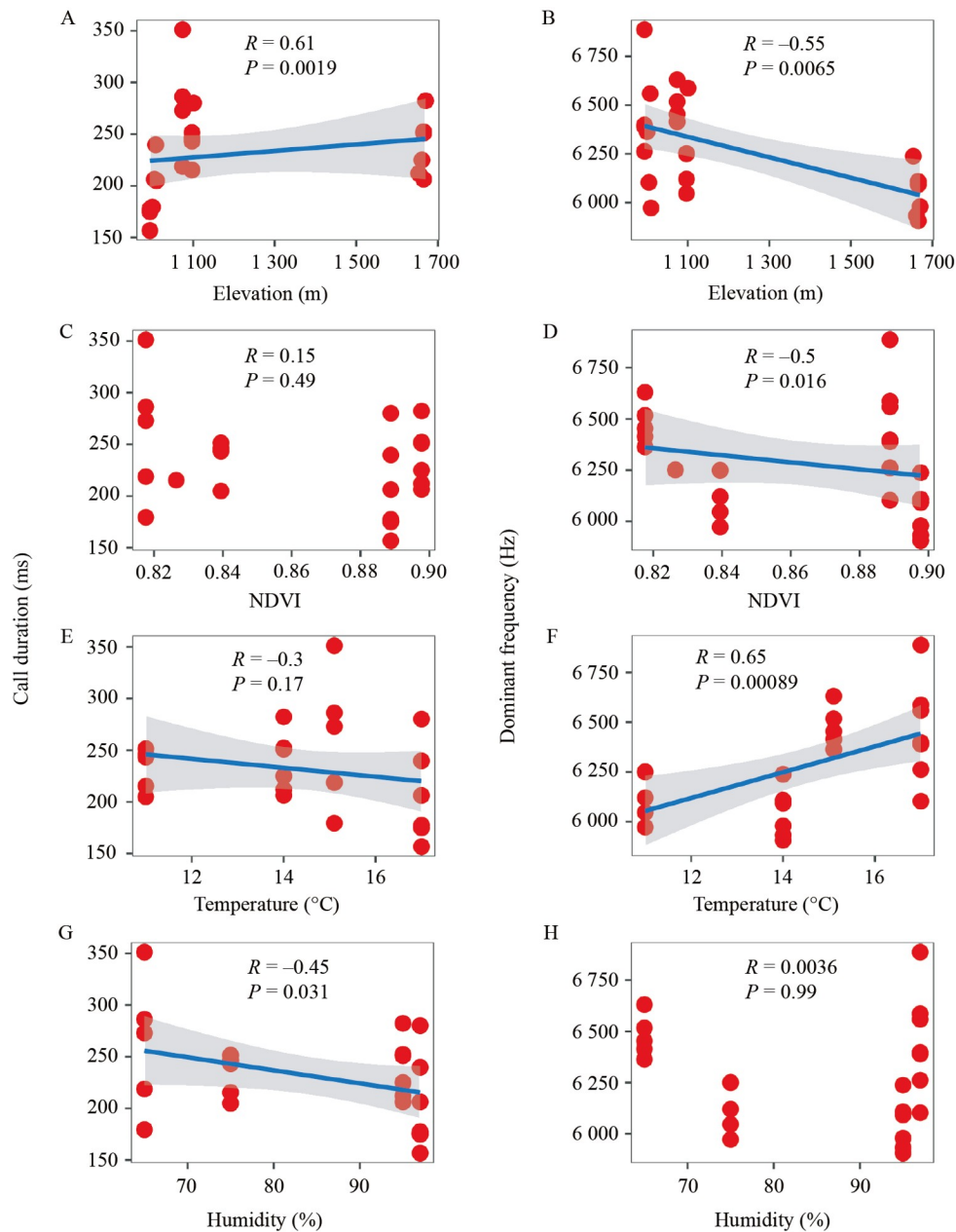


Figure 6 Relationship between two main call characteristics (Call duration and Dominant frequency) and environment factors for *L. ventripunctata*. Blue solid lines indicate significant result of linear regression, with grey shaded areas indicating the 95% confidence interval. Red filled circles are the value of the individual from four populations.

not tested in this study, and it may be due to the small difference in body size among the four populations. Some studies have indicated that there is no correlation between call characteristics and morphological traits in specific frog species (Márquez *et al.*, 2005; Wang *et al.*, 2021). Several variables influence call characteristics, including climate, microhabitat, gene flow and population history (Wei *et al.*, 2012). The different environmental pressures faced by distinct populations may explain the observed variations, leading to

unique survival strategies.

Elevation, temperature, precipitation, and vegetation are the primary determinants of amphibian physiology and ecology (Wang *et al.*, 2022; Chen, 2018). Reducing CD and the number of pulses are known adaptations to increasing ambient temperatures (Gayou, 1984; Narins and Meenderink, 2014). Our study observed a significant uptick in DF as temperature increased. Prior research has illustrated that with higher elevations, the CD of anurans lengthens while the DF reduces

(Narins and Smith, 1986; Narins and Meenderink, 2014), consistent with our results (Figure 6A–B). Ambient humidity impacted the CD and exerted no significant influence on DF (Figure 6G–H). Furthermore, the population in the area with the largest NDVI value (the actually observed more enclosed habitat) exhibited a lower DF, which decreased as the NDVI value increased. AAH maintains that the frequencies of acoustic signals in confined habitats are lower than in open habitats (Gillard and Rowley, 2023). This was a relatively intriguing discovery in this study, however, additional research is required to verify this hypothesis.

Concludingly, this study highlighted acoustic differences between different geographical populations of *L. ventripunctata* despite the small sample size. We discovered the geographical variation of the ACs of *L. ventripunctata* on a small scale, which could serve as potential research subjects that may support the AAH. These findings underscore the relationship between environmental factors and call characteristics, enhancing our understanding of acoustic signal transmission and the evolutionary process.

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Supplementary information Supplementary information for this paper is available at <https://doi.org/10.3724/ahr.2095-0357.2024.0017>

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