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Advertisement calls of *Leptobrachella suiyangensis* and *Leptobrachella bashaensis* (Anura, Megophryidae)

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Abstract

Bioacoustic information is an essential tool for anuran identification, especially cryptic species. We present the first description of the acoustic characters of *Leptobrachella suiyangensis* and *Leptobrachella bashaensis*. The findings aim to inform future ecological studies on species and taxonomy in the genus *Leptobrachella*. Recordings were obtained in the Huoqiuba nature reserve and Basha nature reserve, China. The advertisement calls of *L. suiyangensis* mainly include monosyllabic calls and polysyllabic calls, which can be divided into four types. The call of *L. suiyangensis* has a mean dominant frequency of 4.51 ± 0.16 kHz (n = 322). *L. bashaensis* has only one type of advertisement call. The advertisement call of *L. bashaensis* comprises a single note with a mean call duration of 66.01 ± 6.86 ms and a mean dominant frequency of 6.16 ± 0.08 kHz (n = 100). We compared the advertisement calls of other congeners of *L. suiyangensis* and *L. bashaensis* as these species share similar morphological characteristics and close genetic distance. Despite a high degree of morphological similarity between these species, their advertisement calls differ significantly. Furthermore, different types of calls in the genus *Leptobrachella* and the definitions of primary advertisement calls and secondary advertisement calls are discussed. The study provides basic data for further acoustic and taxonomic studies on *Leptobrachella*.

Keywords: bioacoustics; advertisement call; *Leptobrachella*; acoustic differences

Introduction

The acoustic behavior of amphibians has been commonly studied and plays an important role in species reproduction, evolution, and interspecific identification (Cunningham and Birkhead 1998; Brenowitz and Rose 1999; Kelley 2004). Vocalization is the main communication mechanism of Anura amphibians at the interspecific and intraspecific levels (Wells 2007; Köhler et al. 2017). To better study the vocal communication-related behaviors of frogs, researchers have divided frog calls into the following five types: courtship calls, advertisement calls, aggressive calls, release calls, and distress calls (Wells 1997; Tobias et al. 2004). Advertisement calls are the main vocal type of Anura amphibians, which has specificity and changes greatly among different species and can be used as the basis for systematic classification (Gerhardt 1994; Sullivan et al. 1996). Hence, advertisement calls are widely used in taxonomic research (Goicoechea et al. 2010; Wijayathilaka et al. 2016).

The Asian leaf litter toads of the genus *Leptobrachella* (Smith, 1925) are a group of forest-dependent species, widely distributed in Southeast Asia and southern China (Frost 2023; AmphibiaChina 2023). Given the low dispersal abilities of the species, a genus is an ideal group for investigating patterns of diversity and discerning the drivers of speciation. *Leptobrachella* contains 99 species that inhabit hilly evergreen forests throughout Southeast Asia, southern China, and northeastern India (Frost 2023). Many of the species are threatened with extinction, such as the critically endangered *L. botsfordi* (IUCN 2021). A high degree of morphological similarity and rampant homoplasy appears to have misled the estimates of diversity and evolutionary relationships (Chen et al. 2018). Thirty-seven species of *Leptobrachella* have been described in the last five years, accounting for 37.8% of the total number of species in this genus (Frost 2023; AmphibiaChina 2023). Despite this considerable rate of discoveries and publications, a large percentage of the vocalizations of *Leptobrachella* individuals remains unknown (Yeung et al. 2021). Both *Leptobrachella suiyangensis* and *Leptobrachella bashaensis* were described in 2020 but so far, their advertisement calls have not been reported (Luo et al. 2020; Lyu et al. 2020). Although the conservation status of *L. suiyangensis* and *L. bashaensis* has not been evaluated by the International Union for Conservation of Nature (IUCN) Red Lists, they are expected to be endangered species, since the area of occupancy and/or their extent of occurrence is very small. In this study, we describe for the first time the advertisement calls of *L. suiyangensis* and *L. bashaensis*. Furthermore, we also collated and compared these results with the call of congeners of *L. suiyangensis* and *L. bashaensis*, since it is a species group with high morphological similarity and close genetic distance.

Material and Methods

Call recordings

The advertisement calls of *L. suiyangensis* and *L. bashaensis* were both recorded from their type locality. A total of 322 calls were recorded from four individuals of *L. suiyangensis*, collected from the Huoqiuba Nature Reserve (E 107.08 °, N 28.47 °, 1,448 m, 15.7 °C air temperature, 93% ambient humidity) on April 27, 2022, between 19:00–23:00 and 100 calls from three *L. bashaensis* individuals collected at Basha Nature Reserve (E 108.39 °, N 25.63 °, 978 m, 16.3 °C air temperature, 83% ambient humidity) were recorded on May 1, 2022, between 19:00–20:00h. Calls of each individual were recorded 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. The recording duration was 1–4 min for all individuals. Recorded calls were always of isolated individuals and never from a mixed chorus. The recordings were saved as Wav files. Snout vent lengths (SVLs) of all recorded males were measured in situ using a precision digital caliper to the nearest 0.01 mm; one specimen from each population was taken as a reference and all other animals were released back to their original habitat following measurement. After taking photographs, they were euthanized using isoflurane and then the specimens were fixed in 10% buffered formalin. Tissue samples were taken and preserved separately in 95% ethanol prior to fixation. Specimens were deposited in the Forestry College of Guizhou University, China. Mitochondrial 12S rRNA and 16S rRNA genes were extracted and amplified from muscle samples of all samples and sequenced. The sequencing results were compared to finally determine the species collected. For the morphological identification of specimens, the procedure described by Luo et al. (2020) and Lyu et al. (2020) was followed.

Acoustic analyses

Only calls that had a high signal-to-noise ratio and were free from overlapping calls of nearby males were used for the analysis. For each recording, first, Adobe Audition 2020 audio editing software was used to reduce noise under default settings. We measured all parameters and characteristics following the procedure described by Köhler et al. (2017) and Yeung et al. (2021) including (1) call duration (ms), CD (2) dominant frequency (kHz), DF (3) note per call, NPC (4) first note pulse number, first NP (5) second note pulse number, second NP (6) inter-note intervals, NI (7) first note duration, first ND (8) second note duration, second ND (9) inter-call intervals, CI, and (10) pulse (repetition) rate, PR (Table 1). Raven Pro 1.6 was used to measure the call characteristics; temporal call characteristics were measured using Raven’s waveform display and spectral properties were measured by averaging the spectrum over the entire duration of a call (256 pt. fast Fourier transform, Hanning window). Oscillograms, spectrograms, and power spectra were graphed in the Seewave R package (Sueur et al. 2008). Descriptive statistics of call characteristics; mean, standard deviation (SD), and range were computed using SPSS 23.0.

Results

Leptobrachella suiyangensis **Luo, Xiao, Gao and Zhou, 2020**

The recorded males were calling on rocks in streams, with shrubs and bamboo forests growing nearby. The advertisement calls of *L. suiyangensis* mainly included monosyllabic calls and multisyllabic calls, which could be divided into two types, respectively. Therefore, the advertisement calls of *L. suiyangensis* could be divided into four types (Fig. 2, Table 2, 4). Type A (n = 3) included the monophonic calls from two male individuals (specimen number: SY20220427003 and SY20220427004, Table 4), and the call duration ranged from 25.30–64.70 ms; the mean call duration was 47.57 ±20.20 ms. The dominant frequency ranged from 4.13–4.82 kHz; the mean dominant frequency was 2.39 ±0.16 kHz. The mean pulse number was four, with a mean pulse rate of 47.61 ±8.77 pulses/second. Type B (n = 136) was a monosyllabic call. All four individuals emitted Type B vocals. Call duration ranged from 209.10 to 382.70 ms with a mean call duration of 291.47 ±31.59 ms. The mean interval between call duration was 422.64 ±154.88 ms. The mean dominant frequency was 4.49 ±0.15 kHz. The mean pulse number was 24.00 ±2.50, with a mean pulse rate of 75.75 ±14.83 pulses/second. Both type C (n = 138) and type D (n = 45) vocalizations were multisyllabic calls containing two syllables. All four individuals emitted type C vocals, while only three individuals (specimen number:

SY20220427003, SY20220427004, and SY20220427005, Table 4) produced type D vocals. The call duration of type C ranged from 138.30 to 284.60 ms, and the mean call duration was 179.49 ±37.56 ms. The mean dominant frequency was 4.47 ±0.13 kHz. The mean inter-call interval was 164.43 ±25.60 ms. The call duration and inter-call interval were regular and shared the same dominant frequency with type A and type B in a call series. Type D (n = 45) vocalizations had the longest call duration, with a mean call duration of 302.22 ±50.97, ranging from 220.50 to 442.00 ms. The mean dominant frequency was 4.67 ±0.16 kHz, slightly higher than the dominant frequency of the other three call types.

***Leptobrachella bashaensis* Lyu, Dai, Wei, He, Yuan, Shi, Zhou, Ran, Kuang, Guo, Wei and Yuan, 2020**

Recorded males perched on shrubs 0.5–1 m above the ground or were calling on rocks in streams. The advertisement call of *L. bashaensis* comprised a single note with a mean call duration of 66.01 ±6.86 ms, ranging from 48.00 to 79.80 ms (Fig. 3; Table 3). The mean dominant frequency was 6.16 ±0.08 kHz, ranging from 6.03 to 6.46 kHz. The mean inter-call interval was 334.59 ±65.61 ms. The mean pulse number was 3.00 ±1.00, with a mean pulse rate of 34.40 ±4.46 pulses/second.

We describe for the first time the spectral and temporal parameters of the advertisement call of *L. suiyangensis* and *L. bashaensis*. The former was assigned to the *L. oshanensis* species group (Luo et al. 2020; Liu et al. 2023). *L. bashaensis* is nested in the *L. liui* species group, and its genetic distance from *L. suiyangensis* was far (Lyu et al. 2020). Although *L. suiyangensis* is morphologically similar to *L. bashaensis*, these species with similar morphological characteristics differ in the call structure of their advertisement calls. *L. suiyangensis* emitted several call types while

L. bashaensis emitted only one (Fig. 2, 3). The advertisement calls of *L. suiyangensis* include monosyllabic and multisyllabic calls, while *L. bashaensis* only produced monosyllabic calls.

The calls of *L. bashaensis* were simpler, with fewer pulse numbers. Finally, the dominant frequency in *L. suiyangensis* was significantly lower than that of *L. bashaensis*. The advertisement calls from the two compared species were similar to calling songs of orthopteran species (an onomatopoeic sound of a “Squeak”).

Discussion

The genus of *Leptobrachella* consists of 99 species worldwide (Frost 2023). Approximately 34 species nest in the *L. oshanensis* species group and the *L. liui* species group. The advertisement calls of 17 *Leptobrachella* species of the *L. oshanensis* species group and the *L. liui* species group have been described (Xu et al. 2005; Fei et al. 2012; Rowley et al. 2017a, 2017b; Yang et al. 2018; Ding et al. 2019; Wang et al. 2019; Li et al. 2020; Chen et al. 2021; Cheng et al. 2021; Shi et al. 2021; Yeung et al. 2021; Liu et al. 2023) and there exists a significant difference in the advertisement calls between the species in the *L. oshanensis* group and the *L. liui* group. The advertisement calls of the *L. oshanensis* species group are relatively complex (Table 5).

Most species of the *L. oshanensis* species group have multiple types of advertisement calls; *L. oshanensis* has five types of advertisement calls (Shi et al. 2021) while *L. suiyangensis* has four types. A few species (*L. bijie*, *L. purpuraventra, L. yeae* etc.) have two types of advertisement calls (Wang et al. 2019; Shi et al. 2021). In contrast, the advertisement call types of species in the *L. liui* species group are simpler. For example, both *L. liui* and *L. bashaensis* have only one type of

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advertisement call (Ding et al. 2019). The difference in *Leptobrachella* advertisement calls can help species identification rapidly in field investigations.

Most of the vocal characteristics can be explained by phylogenetic relationships and habitat occupation (Bosch and De la Riva 2004). Multiple studies have shown that differences in vocalizations between Anura can be elucidated through phylogeny (Mclean et al. 2013; Cocroft and Ryan 1995). For *Leptobrachella*, phylogenetic relationships seem to play a greater role than environmental influences. The *L. suiyangensis* belongs to the *L. oshanensis* species group. Previous studies have shown that the species in the *L. oshanensis* species group have more complex advertisement calls (Shi et al. 2021; Rowley et al. 2013). However, the genetic distance between *L. bashaensis* and *L. oshanensis* species group is far, and they are not in the same branch. Therefore, the call structure and type of *L. bashaensis* differ significantly from those of *L. oshanensis* species groups.

Among cryptic species, the use of acoustic diagnostic features for identification could be an alternative to morphometric and molecular diagnosis (Köhler et al. 2005; Vences and Köhler 2008). Although morphologically similar, *L. suiyangensis* and *L. bashaensis* have calls with different features, supporting the specificity of their acoustic signals at the species level. However, the magnitude of cryptic diversity and their advertisement calls remain largely unknown.

Due to the diverse types of advertising calls in the *Leptobrachella*, Rowley et al. (2013) defined for the first time the primary advertisement call (PACs) and secondary advertisement call (SACs). These terms have been used by Yeung et al. (2021). In general, the advertisement calls containing two types were defined as PACs and SACs, and the latter were similar to the dominant PACs in their note structures but with more notes per call and longer call durations, thus sounding to the human ear like prolonged PACs (Rowley et al. 2013; Rowley et al. 2017a, 2017b). SACs are rare and irregular. In this study, we were unable to apply the terminology of PACs and SACs because most species of the *L. oshanensis* species group do not have two types of advertisement calls. Therefore, we cannot describe its advertisement call with simple PACs and SACs. Hence, whether PAC and SAC need to be redefined requires large amounts of data for verification.

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Table 1. Descriptions of acoustic parameter measured.

Parameter (units)	**Discription**
Call duration (ms)	Time between onset of first pulse and offset of last pulse in a call.
Dominant frequency (kHz)	Maximum frequency using Raven's selection spectrum function over the duration of the entire call.
Notes per call	The number of monosyllabic notes contained in a multisyllabic call.
First note pulse number	The number of pulses contained in the first note of a multisyllabic call.
Second note pulse number	The number of pulses contained in the second note of a multisyllabic call.
Inter-note intervals (ms)	The time interval between two adjacent notes.
First note duration (ms)	The duration of the first note in a multisyllabic call.
Second note duration (ms)	The duration of the second note in a multisyllabic call.
Inter-call intervals (ms)	The time interval between two adjacent calls.
Pulse (repetition) rate	Instantaneous pulse rate. Number of pulses repeated in a defined period of time within a note.

Table 2. Descriptive statistics for acoustic characteristics of advertisement calls of *Leptobrachella suiyangensis*.

Call parameters	**Call type**			
	A (n = 3)	B (n = 136)	C (n = 138)	D (n = 45)
CD (ms)	47.57 ±20.20 25.30 ~ 64.70	291.47 ±31.59 209.10 ~ 382.70	179.49 ±37.56 138.30 ~ 284.60	302.22 ±50.97 220.50 ~ 442.00
DF (kHz)	4.48 ±0.34 4.13 ~ 4.82	4.49 ±0.15 4.13 ~ 4.82	4.47 ±0.13 4.13 ~ 4.82	4.67 ±0.16 4.48 ~ 4.82
NPC	1	1	2	2
1st NP	4 2.00 ~ 4.00	24.00 ±2.50 13.00 ~ 34.00	2.00 ±1.00 2.00 ~ 6.00	3.00 ±1.50 1.00 ~ 6.00
1nd NP	NA	NA	4.00 ±0.50 2.00 ~ 5.00	16.00 ±3.00 9.00 ~ 21.00
NI (ms)	NA	NA	80.73 ±25.87 36.20 ~ 159.90	45.49 ±18.10 24.70 ~ 137.10
1st ND (ms)	NA	NA	42.00 ±20.04 23.36 ~ 100.96	42.75 ±19.93 13.22 ~ 80.09
2nd ND (ms)	NA	NA	56.76 ±10.77 25.96 ~ 92.41	213.97 ±41.71 148.19 ~ 299.04
CI (ms)	NA	422.64 ±154.88 182.85 ~ 975.99	164.43 ±25.60 128.20 ~ 260.22	NA
PR	47.61 ±8.77 39.53 ~ 56.93	75.75 ±14.83 46.40 ~ 105.19	32.58 ±6.29 18.83 ~ 48.08	57.89 ±9.31 27.15 ~ 73.27

Table 3. Descriptive statistics for acoustic characteristics of advertisement calls of *Leptobrachella bashaensis*.

Call parameters (n = 100)	**Mean ± SD**
CD (ms)	66.01 ± 6.86
	48.00 ~ 79.80
DF (kHz)	6.16 ± 0.08
	6.03 ~ 6.46
NPC	1
NP	3.00 ± 1.00
	3.00 ~ 4.00
CI (ms)	334.59 ± 65.61
	257.69 ~ 538.39
PR	34.40 ± 4.46
	28.78 ~ 47.69

Figure 1

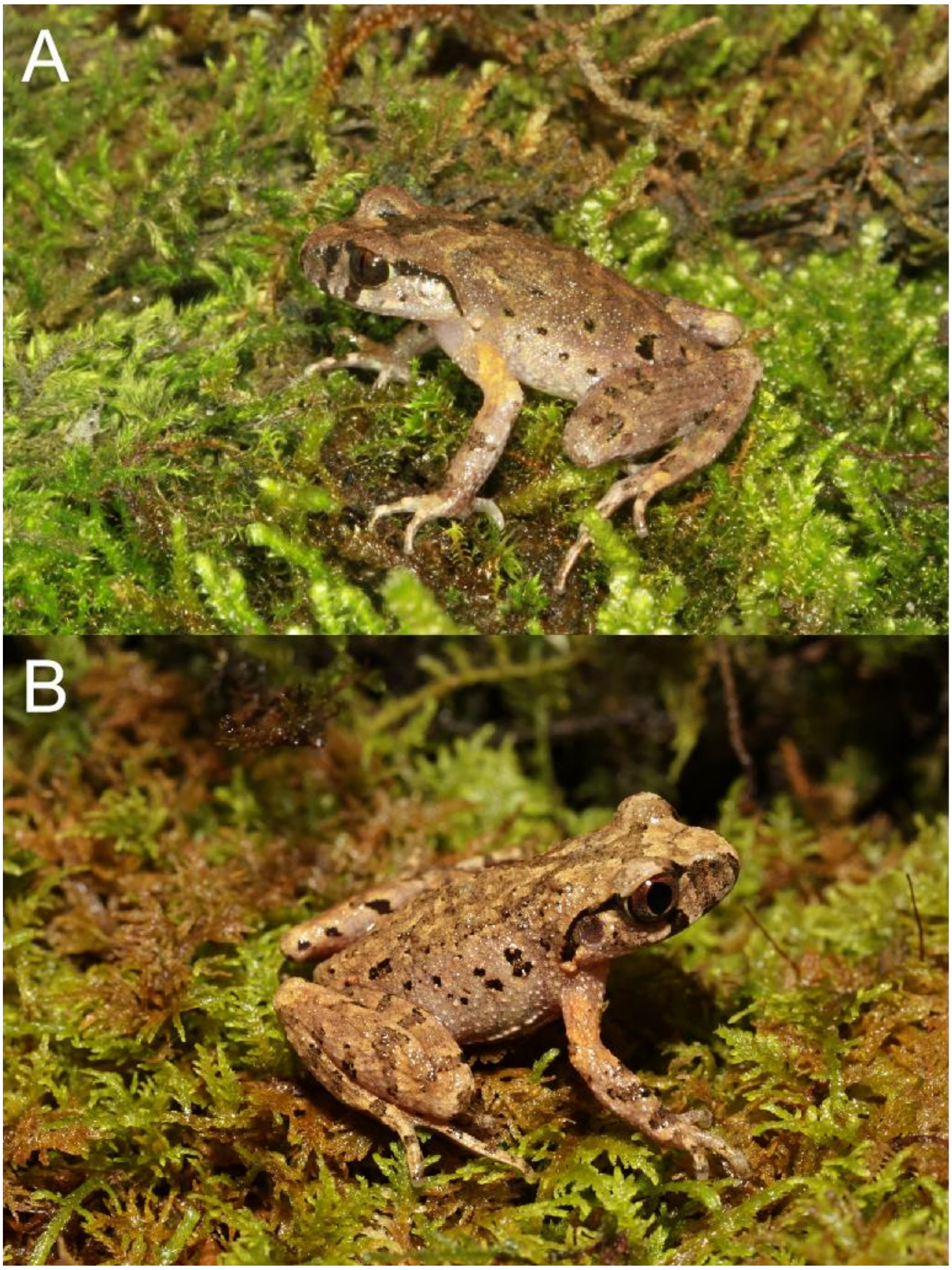

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Figure 2

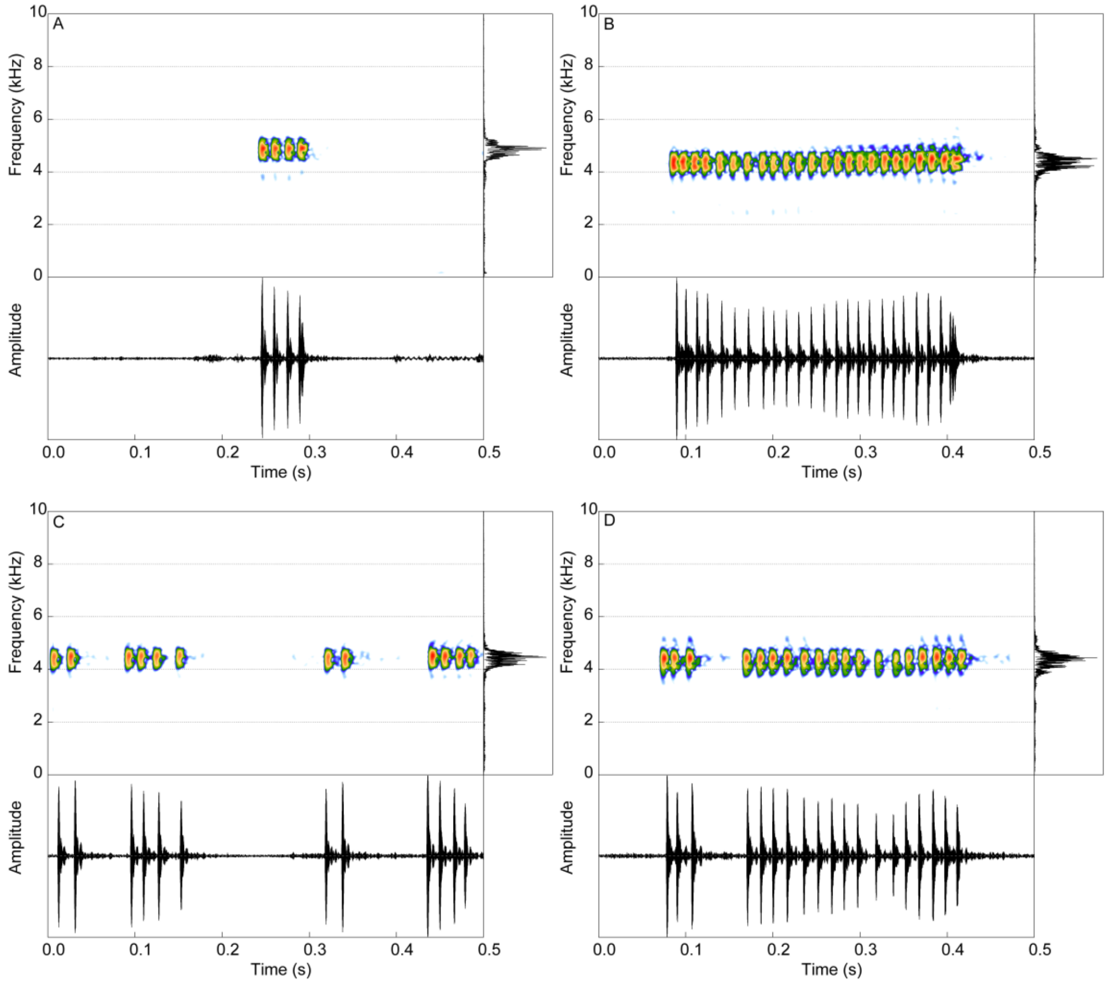

Figure 3