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Acoustic insights into the *Rhinella marina* group: revisiting the advertisement calls of two closely related species (Anura: Bufonidae)

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Abstract

The *Rhinella marina* group comprises 11 species distributed across open and forested habitats from the southern United States to Uruguay. Previous descriptions of the advertisement calls of *R. diptycha* and *R. marina* were based on limited sample sizes and a narrow range of acoustic variables. Here, we documented the first advertisement call description of *R. marina* from Brazil, while the recordings from Rondônia constitute the westernmost acoustic record of *R. diptycha* in northern Brazil. We also compiled existing data on the advertisement calls of *R. marina* and *R. diptycha* and compared these calls with those described for other species within the *R. marina* group. To search for discrimination among *R. marina* and *R. diptycha*, we used the Random Forest (RF) model and Wilcoxon-Mann-Whitney Rank Test. Our analysis distinguished *R. marina* from *R. diptycha*, identifying acoustic differences primarily in dominant frequency, maximum frequency, number of notes per call, and maximum frequency. The calls of *R. marina* comprise 135–272 notes, with maximum frequencies of 689–775 Hz and durations of 6.8–20 s. The calls of *R. diptycha* contained 17–93 notes, with maximum frequencies of 844–1119 Hz and duration of 0.5–7.2 s. Our findings align with previous studies on *R. diptycha* and *R. marina*, except for a population identified as *R. diptycha* from Natal, Brazil, which likely corresponds to *R. marina* from Brazil. Given the extensive distribution of the *R. marina* group species across South America, particularly in Brazil, we recommend reevaluating the calls of *R. icterica*, *R. arenarum*, *R. rubescens,* and *R. cerradensis.* Finally, we emphasize the need for comprehensive descriptions of the calls of *R. achavali* and *R. veredas.*

Key words: Amazon Basin, bioacoustics, Cerrado, species distribution, taxonomy, vocalization

Introduction

The *Rhinella marina* group (Anura: Bufonidae) comprises 11 species of large neotropical toads characterized by well-developed cephalic glands (Pereyra *et al.* 2021; Menéndez-Guerrero *et al.* 2024). These species inhabit both open and forested areas across the Americas, from southern United States to Uruguay (Maciel *et al.* 2010; Frost 2024). *Rhinella marina* (Linnaeus 1758) and *R. diptycha* (Cope 1862) are closely related and morphologically similar species with a wide distribution throughout South America (Pereyra *et al.* 2021; Frost 2024). *Rhinella marina* had its type locality restricted to Suriname and occurs in the Amazon Basin (Müller & Hellmich 1936; Frost

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2024), although the taxonomic status of some populations remains unresolved (Vallinoto *et al.*, 2017). Additionally, it has been introduced worldwide, causing significant ecological disruption (Letnic *et al.* 2008; Fukuda *et al.* 2015). *Rhinella diptycha*, recognized as a distinct taxon from *R. marina* (Pereyra *et al.* 2021), inhabits most area of the the Paraná and Paraguay river drainages (Lavilla & Brusquetti 2018), but also extends from northeastern Brazil to Rio Grande do Sul, encompassing Paraguay, Bolivia, Argentina, and Uruguay (Frost 2024).

As with many Neotropical species, the advertisement calls of *Rhinella marina* and *R. diptycha* are poorly characterized. *R. diptycha* is commonly found in the Cerrado biome, particularly in Minas Gerais (Cruz *et al.* 2009; Maciel *et al.* 2010; Neves *et al.* 2019; Eterovick *et al.* 2020) and has also been reported in Rondônia (Bernarde 2007; Turci & Bernarde 2008). Descriptions of the advertisement call of *R. marina* are available from Peru (Cocroft *et al.* 2001) and Ecuador (Menéndez-Guerrero *et al.* 2024). The calls of *R. diptycha* have been described from Brazilian samples by Garda *et al.* (2010; as *R. jimi*) and Silva *et al.* (2008; as *R. schneideri*), with additional data from Bolivia provided by Köhler *et al.* (1997) and from Argentina by di Tada & Sinsch (2023). In this context, standardized descriptions and comparative analyses based on original digital recordings of these calls are essential for refining taxonomy and improving species differentiation (Gerhardt & Huber 2002; Köhler *et al.* 2017).

In this study, we provide data on the advertisement calls of two Brazilian populations of *Rhinella marina* from the eastern Brazilian Amazon and significantly expand the sample size for *R. diptycha*, including populations from the western Brazilian Amazon and southeastern Brazil. To assess geographic variation, we also compiled existing data on the advertisement calls of *R. marina* from Peru and Ecuador, as well as *R. diptycha* from Bolivia and Brazil. Finally, we compared the calls of *R. marina* and *R. diptycha* with those described for other species within the *R. marina* group, including *R. bella* (Menéndez-Guerrero *et al.* 2024), *R. cerradensis* (Maciel *et al.* 2007), *R. horribilis* (Menéndez-Guerrero *et al.* 2024), *R. icterica* (Heyer *et al.* 1990), *R. poeppigii* (De la Riva *et al.* 1996; Venâncio *et al.* 2017), *R. rubescens* (Maciel *et al.* 2007), and *R. arenarum* (Straneck *et al.* 1993).

Material and methods

Advertisement calls were recorded using MicroTrack II, Marantz 670, 671, or PMD 661 digital recorders (set at sampling rates of 44.1 kHz and 16-bit resolution), coupled with Sennheiser ME66 or ME67 directional microphones. Microphones were positioned approximately 5–10 m from the calling males. Calls of four males of *Rhinella marina* were recorded from Santa Bárbara do Pará (1°11’41.22” S, 48°16’14.99” W; 21 m elevation) in the state of Pará, and two males from Serra do Navio (0°53’51.75” N, 52° 0’20.47” W; 150 m elevation) in the state of Amapá, Brazil. Our sample of calls of *R. diptycha* included seven males from Costa Marques (12º26’42” S, 64º13’38” W; 140 m elevation) in the state of Rondônia, three males from Araguari (18°24’51.98” S, 48°11’44.93” W; 921 m elevation), and three males from Ituiutaba (19° 0’26.90” S, 49°26’51.75” W; 605 m elevation) both in the state of Minas Gerais, Brazil. We deposited the call records in the frog collection at the Museu de Biodiversidade do Cerrado, Universidade Federal de Uberlândia, Brazil. Voucher labels and localities are available in APPENDIX 1.

We analyzed the calls using Raven Pro v. 1.6 software for Windows from the Cornell Lab of Ornithology (K. Lisa Yang Center for Conservation Bioacoustics 2022). Temporal acoustic variables were analyzed on oscillograms and spectral variables on spectrograms with the following settings: window size 512 samples, Hann window, contrast 50 %, brightness 50 %, and time grid overlap 50 %. Oscillogram and spectrogram illustrations were generated using Seewave v.2.2.3 (Sueur *et al.* 2008) in the R platform (R Studio v.4.3.1 2023). The Seewave settings were as follows: Hanning window, 90% overlap, and 512 points resolution (FFT). The dominant frequency, minimum frequency, and maximum frequency was measured using ‘peak frequency’, ‘frequency 5%’, and ‘frequency 95%’ options (Charif *et al.* 2010), respectively. Measurements of the following acoustic variables, according to Köhler *et al.* (2017), were taken: call duration, number of notes per call, note duration, pulses per note, duration of the most and least energetic pulses, inter-note interval, inter-call interval, note repetition rate per second, and pulse repetition rate per second.

We used the randomForest function (RF) of the *randomForest* v4.7-1.2 package (Liaw & Wiener 2002) to search for acoustic discrimination between the two species. This function constructs multiple classification trees (e.g., 1000) using bootstrap samples of the data. The proximityPlot function (rfPermute v2.5.2 package, Archer 2021) was used to generate proximity scores through multidimensional scaling. The most important variables were ranked using the varImpPlot function and tested for significance using the Wilcoxon-Mann-Whitney test. These tests were conducted using R software (R Studio v.4.3.1 2023).

Results

The values of the temporal and spectral features of the advertisement calls of *Rhinella marina* and *R. diptycha* from Brazil are summarized in Table 1, along with comparative data from the literature. The advertisement calls of *R. marina* and *R. diptycha* consisted of a long trill of concatenated pulsed short notes (Table 1). Trills rapidly reach the maximum amplitude and maintain it until the end, with no noticeable frequency modulation along the call duration (Figs. 1A, 2A).

TABLE 1. Temporal and spectral features of the advertisement call of *Rhinella marina* and *R. diptycha.* For populations recorded in this study, values of summary statistics are presented as Mean±SD (min–max). For the remaining populations, values were transcribed as provided in the original publications. N = number of males analyzed.

	Rhinella diptycha (N = 1)	*Rhinella diptycha* (N = ?)	*Rhinella diptycha* (N = 4)	*Rhinella diptycha* (N = 6)	*Rhinella marina* (N = 5)	*Rhinella marina* (N = 12)	*Rhinella marina* (N = 6)	*Rhinella diptycha* (N = 13)
Reference	Köhler *et al.* 1997	Silva *et al.* 2008	Garda *et al.* 2010	di Tada & Sinsch 2023	Cocroft *et al.* 2001	Menéndez-Guerrero *et al.* 2024	This study	This study
Locality	Bolivia	São Paulo, Brazil	Rio Grande do Norte, Brazil	Cordoba, Argentina	Tambopata, Peru	Eastern Ecuador	Eastern Brazilian Amazon	Minas Gerais and Rondônia, Brazil
Call duration (s)	2.4 (1.7–2.9)		6.3 ± 2.3 (3.8 –13.9)	2.8 ± 5.9 (1.4–3.9)	8.42	5.3 ± 4.0	12.6 ± 4.0 (6.8–20.1)	2.3 ± 1.3 (0.5–7.2)
Notes per call	24–40		95.1 ± 34.1 (52 – 201)	32 ± 7 (18–46)	163	81.7 ± 61.9	198.0 ± 68.7 (135–272.0)	34.1 ± 15.0 (17.0–93.0)
Note duration (ms)	34.7 ± 1.0 (33–40)	30 ± 0 (20–30)		30 ± 4 (23–37)	25–33		27 ± 3.9 (20.0–32.0)	34.5 ± 4.0 (25.0–49.0)
Pulses per note	3	3		2.5 ± 0.5 (2–3)	5	3.2 ± 0.7	2.7 ± 0.5 (2.0–3.0)	3.5 ± 0.5 (2.0–5.0)
Inter-note interval (ms)				60 ± 5 (48–71)	27–33		34.0 ± 6.1 (27.0–48.0)	49.8 ± 5.8 (30.0–67.0)
Inter-call interval (s)				2.8 ± 8.9 (1.2–4.6)			27.2 ± 28.7 (11.2–78.0)	3.2 ± 0.9 (0.9–13.0)
Minimum frequency (Hz)	350	440					455 ± 40.3 (430–517)	629 ± 43.9 (516–656)
Maximum frequency (Hz)	900	920					732 ± 44.0 (689–775)	898 ± 76.0 (844–1119)
Dominant frequency (Hz)	700	790 ± 0 (690–870)	600 ± 51.71 (517–689)	573 ± 26 (552–653)	626	705 ± 117	566 ± 44.0 (516–603)	770 ± 38.0 (750–861)
Note rate (notes/s)		14.1		11.4 ± 0.9 (9.9–13.8)			15.8 ± 1.0 (14.0–17.0)	12.2 ± 1.3 (10.0–14.0)
Pulse rate (pulse/s)						88.9 ± 13.6	37.9 ± 7.4 (28.0–48.0)	40.3 ± 4.8 (30.0–50.0)

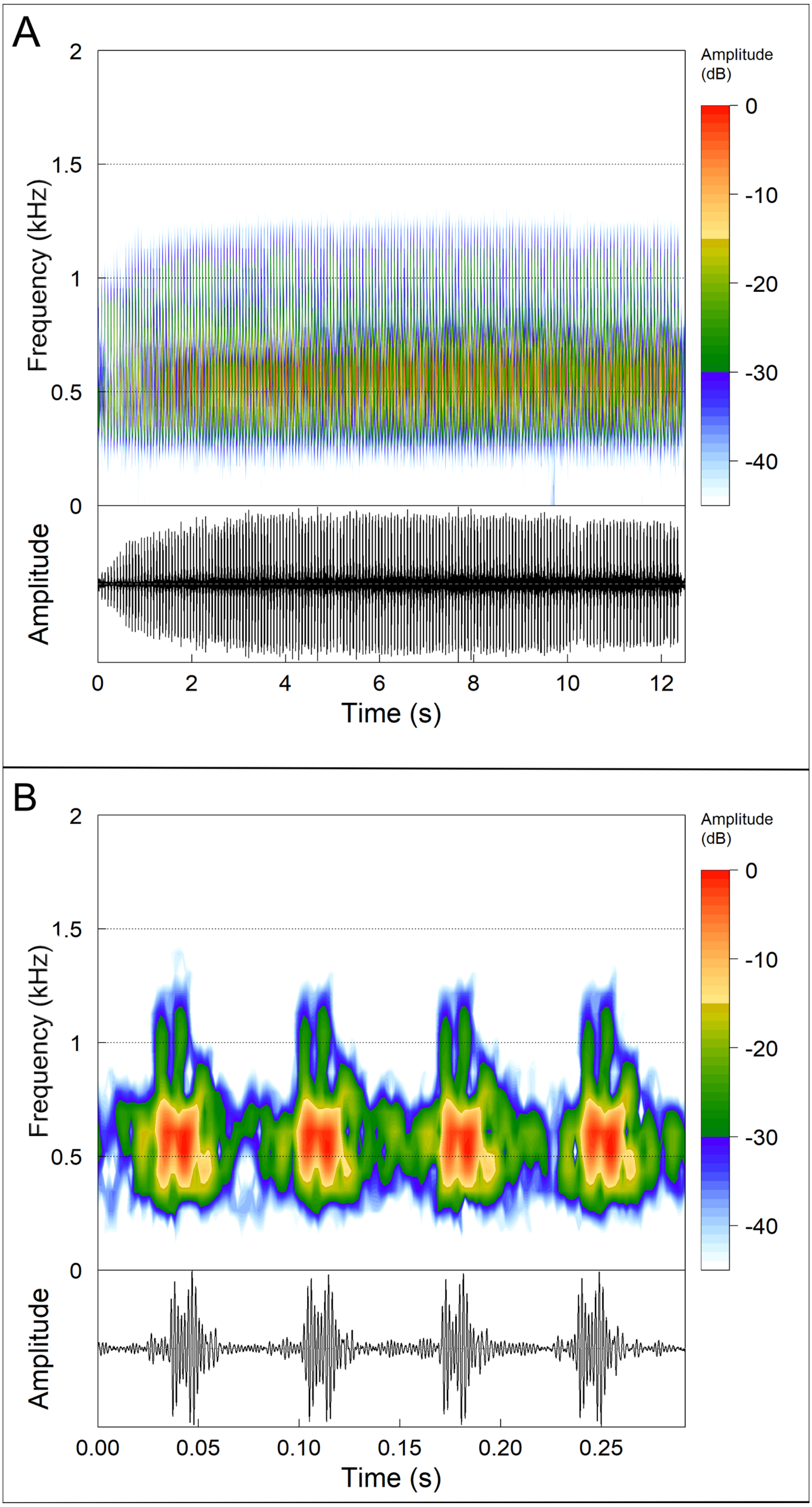

FIGURE 1. Spectrograms and corresponding oscillograms of the advertisement call of *Rhinella marina*. A) A complete call from Santa Bárbara, state of Pará, Brazil. B) Four notes from the middle portion of the call, illustrating its two-pulsed structure. Sound file: Rhinella_marinaStaBarbaraPA4aAAGm661MK2. Air temperature = 27ºC.

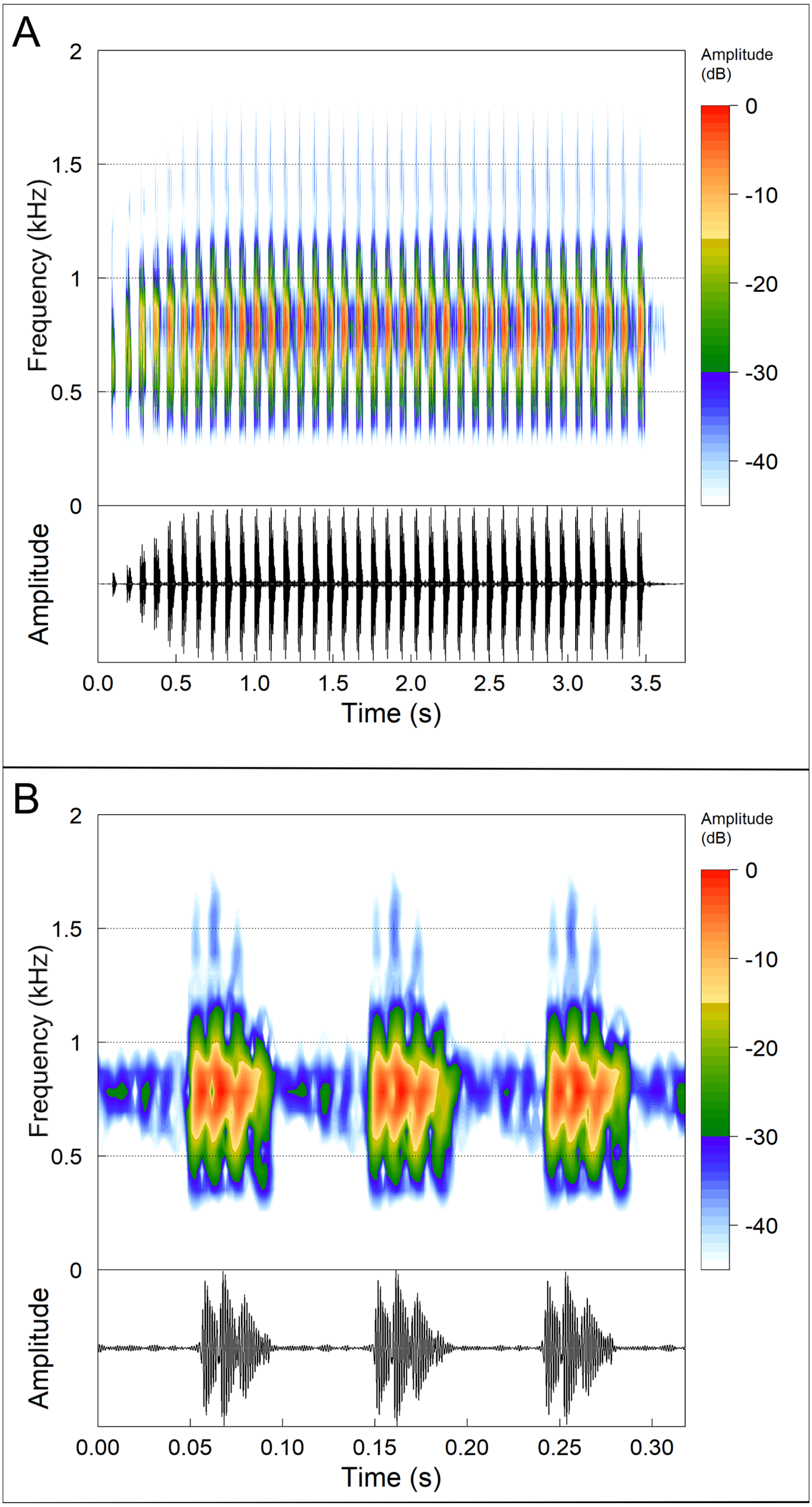

FIGURE 2. Spectrograms and corresponding oscillograms of the advertisement call of *Rhinella diptycha.* A) A complete call from Araguari, state of Minas Gerais, Brazil. B) Three notes from the middle portion of the call illustrate its three-pulsed structure. Sound file: Rhinella_diptychaAraguariMG1aAAGmt661MK2. Air temperature = 27ºC.

The calls of *Rhinella marina* had a duration of 6.8–20.1 s, with an average of 198 notes per call, and were spaced by intervals of 11.2–78.0 s. Each note contained up to three pulses (Fig. 1B) emitted at a rate of 30.0–50.0 pulses per second. Notes had a duration of 20.0–32.0 ms and were emitted at a rate of 14.0–17.0 notes/s. The most energetic pulse was typically the second, with a duration of 8.0–18.0 ms. The inter-note interval ranged from 27.0 to 48.0 ms. The dominant frequency was between 516 and 603 Hz. Additional details are provided in Table 1.

The calls of *Rhinella diptycha* had a duration of 0.5–7.2 s, with an average of 34 notes per call, and were spaced by intervals of 0.9–13.0 s. Each note contains up to five pulses (Fig. 2B), emitted at a rate of 28.0–48.0 pulses per second. Notes had a duration of 25.0–49.0 ms and were emitted at a rate of 10.0–14.0 notes/s. The most energetic pulse was typically the first and/or second pulse, with a duration of 5.0–12.0 ms. The inter-note interval ranged from 30.0 to 67.0 ms. The dominant frequency ranged from 750 to 861 Hz. Additional details are provided in Table 1.

The random forest model of the call features resulted in 100% accuracy between *Rhinella marina* and *R. diptycha* (500 trees, three variables tested at each split). The Minas Gerais sample of *R. diptycha* could not be discriminated from the Rondônia sample (Fig. 3). The first four most important acoustic features for discriminating species samples were the dominant frequency ($Z = 7.3$, $p < 0.01$), maximum frequency ($Z = 6.6$, $p < 0.01$), minimum frequency ($Z = 6.3$, $p < 0.01$), and number of notes per call ($Z = 5.5$, $p < 0.01$) (Fig. 4).

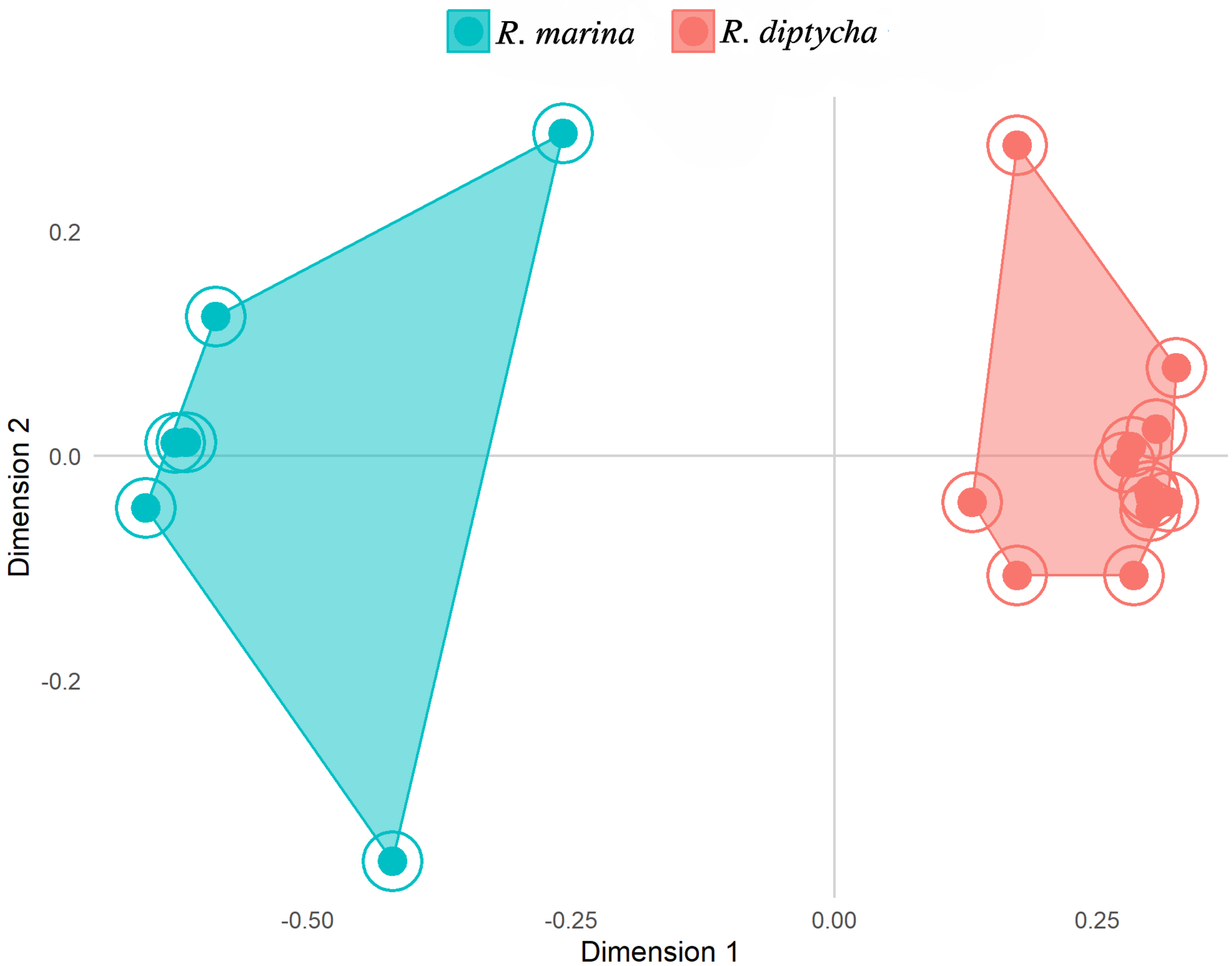

FIGURE 3. First and second dimensions of a Multidimensional Scaling analysis based on proximity scores from the randomForest analysis, using acoustic traits of *Rhinella marina* and *R. diptycha;* N = 6 and 13, respectively. Each dot represents an adult male. The circles around the dots represent how males were classified.

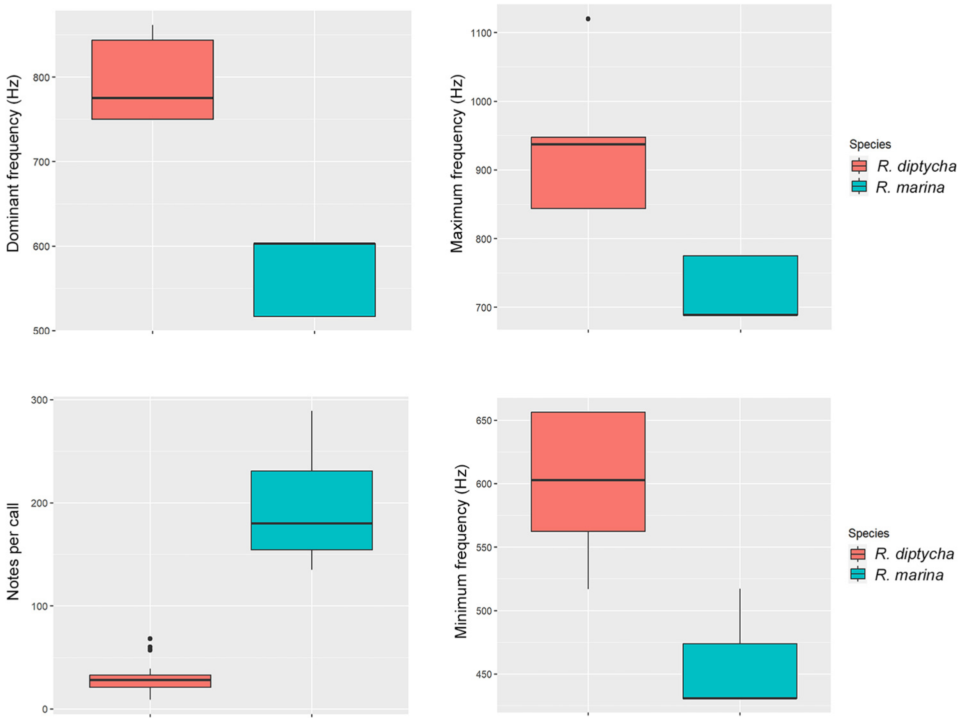

FIGURE 4. Boxplots of the call features identified as most relevant for discriminating between the two closely related species, *Rhinella marina*, and *R. diptycha*.

Discussion

Call comparisons with congeneric species of the group. All species in the *Rhinella marina* group produce advertisement calls characterized by long trills composed of concatenated, pulsed short notes, containing up to five pulses. Most of the interspecific variation occurs in dominant frequency, maximum frequency, number of notes per call, and pulse rate (Heyer *et al.* 1990; Straneck *et al.* 1993; De la Riva *et al.* 1996; Köhler *et al.* 1997; Cocroft *et al.* 2001; Maciel *et al.* 2007; Silva *et al.* 2008; Venâncio *et al.* 2017; di Tada & Sinsch 2023; Menéndez-Guerrero *et al.* 2024, this study). *Rhinella cerradensis* exhibits the longest average note duration (44.7 ms) and inter-note interval (66.5 ms; Maciel *et al.* 2007), whereas *R. bella* has the fewest notes per call (18.3 notes) and the highest pulse rate (91.3 pulses per second; Menéndez-Guerrero *et al.* 2024). The calls of *R. horribilis* (90.6 pulses per second, 83.9 notes per call; Table 2) can be distinguished from the population of *R. marina* from Brazil (this study; Table 1) by its higher pulse rate (37.9 pulses per second) and lower number of notes (198.0 notes per call), but not from the population from western Ecuador (88.9 pulses per second, 81.7 notes per call; Table 2).

The populations of *Rhinella marina* from Brazil (this study) showed the lowest reported dominant frequency at 533 Hz, followed by *R. diptycha* from Argentina (573 Hz; di Tada & Sinsch 2023), *R. diptycha* from Rio Grande do Norte, Brazil (600 Hz Garda *et al.* 2010), *R. marina* from Peru (626 Hz; Cocroft *et al.* 2001), and *R. horribilis* from Central America (633 Hz; Menéndez-Guerrero *et al.* 2024; Table 1, 2). General call features of *R. diptycha* from Rio Grande do Norte, Brazil (= *R. jimi*; Garda *et al.* 2010) are similar to those reported for *R. marina* from Brazil (this study), particularly in terms of call duration, dominant frequency, number of notes per call, and number of pulses per note (Table 1). *Rhinella arenarum* can be distinguished from all other species in the group by its highest dominant frequency (1348 Hz; Straneck *et al.* 1993; Table 2). The lowest minimum frequency was reported for *R. diptycha* from Bolivia (350 Hz; Köhler *et al.* 1997); however, we observed a distinct frequency range from populations of this study (516–656 Hz; Table 1). The calls of *R. rubescens* presented higher maximum frequency (1015 Hz; Maciel *et al.* 2007) that can be distinguished from population of this study of *R. diptycha* (898 Hz) and *R. icterica* from São Paulo, Brazil (700 Hz; Heyer *et al.* 1990).

TABLE 2. Temporal and spectral features of the advertisement call of species of *Rhinella marina* group reviewed in literature. Values were transcribed as provided in the original publications. N = number of males analyzed. Values are Mean±SD (min–max).

	Rhinella arenarum (N = 1)	*Rhinella cerradensis* (N = 1)	*Rhinella icterica* (N= ?)	*Rhinella poeppigii* (N = 1)	*Rhinella poeppigii* (N = 1)	*Rhinella rubescens* (N = 13)	*Rhinella bella* (N = 17)	*Rhinella horribilis* (N = 5)
Reference	Straneck *et al.* 1993	Maciel *et al.* 2007	Heyer *et al.* 1990	De la riva *et al.* 1996	Venâncio *et al.* 2017	Maciel *et al.* 2007	Menéndez-Guerrero *et al.* 2024	Menéndez-Guerrero *et al.* 2024
Locality	Argentina	Distrito Federal, Brazil	São Paulo, Brazil	Bulo-Bulo, Bolivia	Acre, Brazil	Distrito Federal, Brazil	Western Ecuador	Central America
Call duration (s)		1.9–12.4	4–20	1.7 ± 5.9 (0.6–2.7)	4.0	5–25	1.5 ± 0.4	6.3 ± 1.0
Notes per call	61–107	19–129	40–180	29.9 ± 10.1 (10–45)	51.8		18.3 ± 5.7	83.9 ± 11.1
Note duration (ms)		45 ± 2.4	40–60		28–57			
Pulses per note	3	3	1–3	3.3 ± 0.6 (3–5)	3.5	3	3.8 ± 0.6	3.5 ± 0.8
Inter-note interval (ms)		67 ± 2.1			14–38			
Minimum frequency (Hz)		421 ± 3.3	500					
Maximum frequency (Hz)			700			1015		
Dominant frequency (Hz)	1348 ± 18.3	839 ± 8.9	350–820	1033 ± 71.6 (907–1141)	750–1500		803 ± 75.7	633 ± 35.3
Note rate (notes/s)		9.8 ± 0.6	8.5–9.0					
Pulse rate (pulse/s)							91.3 ± 11.7	90.6 ± 13.9

Adittional renmarks. In this study, we provide a comprehensive re-description of the advertisement calls of two closely related species, *Rhinella marina* and *R. diptycha*. The calls recorded from Amapá and Pará represent the first documented description of *R. marina* from Brazil, while the recordings from Rondônia constitute the westernmost acoustic record of *R. diptycha* in northern Brazil. General call features of *R. marina* from Peru (Cocroft et al. 2001) were similar to our re-description, although those from Ecuador differed in the number of notes, call duration, and pulse rate (Menéndez-Guerrero et al. 2024) (Table 1). The acoustic features of *R. diptycha* from Icém, São Paulo, Brazil (Silva et al. 2008), matched our re-description in terms of dominant frequency and note duration. Likewise, calls from Santa Rosa de la Roca, Bolivia (Köhler et al. 1997), showed similarities in call duration, number of notes per call, and note duration. Temporal call features from Argentina (di Tada & Sinsch 2023) were similar to those from the other four localities compared, but the dominant frequency differed from these populations (Table 1).

The random forest (RF) model successfully distinguished our samples of *Rhinella marina* from *R. diptycha*, highlighting the discriminative importance of dominant frequency, maximum frequency, minimum frequency, and number of notes per call. No significant differences were detected between the calls of *R. diptycha* from the western

Brazilian Amazon and southeastern Brazil, suggesting that call features are conserved across Brazilian populations (São-Pedro *et al.* 2011; Forti *et al.* 2018).

The noted low-frequency calls of *Rhinella diptycha*, *R. marina*, and *R. horribilis*, which have similar average body sizes, provide further evidence of the well-established negative correlation between body size and dominant frequency in anurans (Gingras *et al.* 2013; Bezerra *et al.* 2021). The divergence observed in acoustic traits among species of this group, along with the conserved external morphology, suggests that vocalizations may evolve more rapidly than morphological traits, potentially driven by sexual selection or intraspecific recognition, particularly in zones of sympatry (Sequeira *et al.* 2011; Funk *et al.* 2012). However, this hypothesis warrants further investigation.

The broad geographic distribution of neotropical toads of *Rhinella marina* group across South America, along with the presence of cryptic lineages, underscores the critical role of acoustic traits in the recognition and geographic delimitation of these species. Future studies should prioritize large, representative sample sizes and adopt a standardized terminology for acoustic descriptions. We recommend a re-evaluation of the calls of *R. arenarum*, given the limited sample size (N = 1) and small number of acoustic traits analyzed (N = 3), especially for Brazilian populations. Likewise, the calls of *R. icterica*, *R. rubescens*, and *R. cerradensis* require reassessment due to the small sample sizes and limited number of acoustic traits reported (Table 2). Additionally, we emphasize the need for comprehensive descriptions of the calls of *R. achavali* and *R. veredas*. Such efforts will facilitate the identification of diagnostic call traits and improve the accuracy of species delimitation within the *R. marina* group.

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APPENDIX I

TABLE 1. Voucher labels and localities of the analyzed sound files. Vouchers are housed in the frog collection of the Museu de Biodiversidade do Cerrado, Universidade Federal de Uberlândia (UFU).

Voucher label	**Locality**
Rhinella_marinaStaBarbaraPA1aAAGm661MK2	Santa Bárbara do Pará, Pará, Brazil
Rhinella_marinaStaBarbaraPA2aAAGm661MK2	Santa Bárbara do Pará, Pará, Brazil
Rhinella_marinaStaBarbaraPA3aAAGm661MK2	Santa Bárbara do Pará, Pará, Brazil
Rhinella_marinaStaBarbaraPA3bAAGm661MK2	Santa Bárbara do Pará, Pará, Brazil
Rhinella_marinaStaBarbaraPA4aAAGm661MK2	Santa Bárbara do Pará, Pará, Brazil
Rhinella_marina_12_10_2017_1211EduCampos	Serra do Navio, Amapá, Brazil
Rhinella_marina_30_04_2021_Pedra_Preta_Serra_Navio_2_ind	Serra do Navio, Amapá, Brazil
Rhinella_marina_30_04_2021_Pedra_Preta_Serra_Navio_1_ind	Serra do Navio, Amapá, Brazil
Rhinella_diptychaCostaMarquesRO1aAAGm671	Costa Marques, Rondônia, Brazil
Rhinella_diptychaCostaMarquesRO2aAAGm671	Costa Marques, Rondônia, Brazil
Rhinella_diptychaCostaMarquesRO3aAAGm671	Costa Marques, Rondônia, Brazil
Rhinella_diptychaCostaMarquesRO4aAAGm671	Costa Marques, Rondônia, Brazil
Rhinella_diptychaCostaMarquesRO5aAAGm671	Costa Marques, Rondônia, Brazil
Rhinella_diptychaCostaMarquesRO5bAAGm671	Costa Marques, Rondônia, Brazil
Rhinella_diptychaCostaMarquesRO6aDLB_AAGm670	Costa Marques, Rondônia, Brazil
Rhinella_diptychaAraguariMG1aAAGmt661MK2	Araguari, Minas Gerais, Brazil
Rhinella_diptychaAraguariMG2aAAGm661MK2	Araguari, Minas Gerais, Brazil
Rhinella_diptychaAraguariMG3aAAGm661MK2	Araguari, Minas Gerais, Brazil
Rhinella_diptychaItuiutabaMG2aAAGm671	Ituiutaba, Minas Gerais, Brazil
Rhinella_diptychaItuiutabaMG3aAAGm671	Ituiutaba, Minas Gerais, Brazil
Rhinella_diptychaItuiutabaMG4aAAGm671	Ituiutaba, Minas Gerais, Brazil