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Geographic structure and acoustic variation in populations of *Scinax squalirostris* (A. Lutz, 1925) (Anura: Hylidae)

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Abstract. The advertisement call contains spectral and temporal information, which are used by frogs in different social contexts. We analysed the variation of advertisement calls among eleven populations of *Scinax squalirostris* along its broad distribution area, examining geographical patterns (latitude, longitude and altitude) related to this variation. We found that parameters responsible for advertisement call variation among populations were call duration and number of pulses. Individuals from Cristalina municipality (state of Goiás, Brazil) showed the greatest amount of differences in acoustic parameters among populations studied, suggesting that it could even be a distinct species. We found a geographic pattern between dominant frequency and latitude, with lower values found further north. The variability of call parameters of *S. squalirostris* populations was not explained by geographic distance alone, suggesting that stochastic events, such as vicariance and/or local selective pressures could have influenced their vocal repertoire historically.

Key words: advertisement call, geographic pattern, geographical distance.

Introduction

Anurans use vocalizations in different contexts, such as specific recognition, female's attraction, territoriality and aggressive interactions (Gerhardt 1994, Bastos & Haddad 1995, Guimarães & Bastos 2003). Several species of neotropical frogs hold complex vocal repertoires consisting of different vocalization types (e.g. Bastos & Haddad 1995, Twomey & Brown 2008, São-Pedro et al. 2011). Advertisement calls are the main signals given by males during the breeding season (Wells 1977) containing spectral and temporal information, which are important to specific recognition (Ryan 1985, Cocroft & Ryan 1995). Advertisement calls also possess adaptations and features that prevent or minimize interference to acoustic communication (Grafe 1996, Martins et al. 2004, 2006).

Because of the importance of advertisement calls as a mechanism of prezygotic barriers to interspecific reproduction, call properties have been largely used in systematic studies (e.g. De la Riva et al. 1996, Garcia et al. 2001). Geographic variation in acoustic parameters of advertisement calls has also been observed (Gergus et al. 2004, Bernal et al. 2005). Besides, several hypotheses attempt to explain the mechanisms through which geographic variation occurs in frog's vocalization, especially the model of isolation by distance proposed by Wright (1943), which predicts that geographically populations, more related to others should be separated by smaller genetic and phenotypic distances.

Scinax squalirostris A. Lutz, 1925 is a treefrog with a broad distribution in southern South America, including midwestern, southeastern, and southern Brazil, in addition to southern Paraguay, Uruguay, northern Argentina and eastern Bolivia (Brandão et al. 1997, Leite et al. 2008, Cruz et al. 2009, Frost 2013). Although its distribution is continuous in the Humid Chaco and Pampas, it occurs only in 'sky islands' fields (areas of high altitudes) or swamps in the Atlantic Forest and Cerrado biomes and it is found to be disjunct. This information is based on literature and evaluation of museum/collection records. The advertisement call was first described by Barrio (1962) who later also tested the influence of temperature in call parameters (Barrio 1963). Pombal et al. (2011) also presented call features of populations of *S. squalirostris* to provide comparisons to those of *S. pusillus*.

Herein, we analysed the advertisement call variation of *Scinax squalirostris* populations along its wide distribution area. Moreover, we evaluated which of the call parameters vary more among populations and also analysed geographic structure patterns.

Materials and Methods

We analysed the variation of advertisement calls among eleven populations of *Scinax squalirostris* from the following localities: Bolivia (La Paz municipality), Uruguay (Artigas department), and Brazil (Congonhas do Norte municipality, Minas Gerais state; Cristalina municipality, Goiás state; Serra da Bocaina National Park, São Paulo state; Passos Maia municipality, Santa Catarina state; Porto Alegre, São Borja, São Francisco de Paula, Rio Grande and Viamão municipalities, Rio Grande do Sul state) (Fig. 1). For information regarding coordinates, number of individuals and calls analysed per locality see Table 1.

Figure 1. Populations of *Scinax squalirostris* sampled in this study. La Paz municipality, Bolivia (A); Artigas department, Uruguay (K); Congonhas do Norte municipality, Minas Gerais state (C); Cristalina municipality, Goiás state (B); Serra da Bocaina National Park, São Paulo state (D); Passos Maia municipality, Santa Catarina state (E); Porto Alegre (I), São Borja (J), São Francisco de Paula (F), Rio Grande (H) and Viamão municipalities (G), Rio Grande do Sul state, Brazil.

Recordings were edited with frequency at 22 KHz and 16 bits resolution and analysed in Avisoft SASLab-Ligth (Avisoft Bioacustics, Kirchstr 11, 13158 Berlin, Germany), Cool Edit (Cool Edit Pro, Syntrillium Software) and SoundRuler (Marcos Gridi-Papp, Phys. Science, UCLA, USA) softwares. We measured the six following acoustic parameters: call duration (s), number of notes (notes/call), note duration (s), number of pulses (pulses/note), repetition rate (calls/minute), and dominant frequency (Hz), following Gerhardt (1998) nomenclature.

To assess which call features better explain the acoustic separation of populations, we applied a discriminant function analysis among call features and the individuals for each population. Thus, the discriminant function (DF) can be used to classify each signal as belonging to a particular individual. The percentage of signals routed to the correct individuals is considered a measure of how well the linear combination of variables distinguishes individuals (Bee & Gerhardt 2001).

We also performed a multiple regression analysis to verify if call parameters of the individuals vary according to the following geographic variables: latitude, longitude and altitude. To reduce the heterogeneity of variance and non-normal distribution, the data were expressed logarithmically (log(x)). The log was also applied to the independent variable altitude due to the large variations in data.

To evaluate possible associations between geographical distance and the acoustic parameters we performed the Mantel Test. This test is a permutation procedure to estimate the association (Z) between the elements of two distance matrices from the same sampled object (Manly 2008). It determines the significance of this association by comparison with the statistics distribution found by randomization of the order elements of the matrices (Manly 2006). Thus the calculated Z statistics, when related to distribution geographical data indicate the degree of spatial correlation of data. We used the Euclidian distance to calculate the matrices. The Mantel Test was performed with distance matrix between the latitude and longitude of sampling sites and distance matrix between the acoustic parameters.

Results

We analysed a total of 825 calls of 39 males of *Scinax squalirostris* of eleven populations. Overall mean and standard deviation of measures are: call duration 0.46 ± 0.15s; number of notes by call 11 ± 9.37; note duration 0.031± 0.022 s; number of pulses by note 7.62 ± 2.23; repetition rate 1513.12 ± 847.12 call/min and dominant frequency 4545.31 ± 391.12 Hz (Table 1). For information regarding parameters of measurements from each population see also Table 1.

Individuals from Cristalina municipality population (Fig. 2A) (16º 46′ 07″ S, 47º 36′ 48″ W; approximately 1189 m a.s.l.; datum = WGS84) showed a notable atypical pattern when compared to the other populations such as La Paz (Fig. 2B) (9º 45′ 00″ S; 65º 52′ 00″ W; approximately 3660 m a.s.l.; datum = WGS84); Porto Alegre (Fig. 2C) municipality (30º 01′ 60″ S, 51º 13′ 47″ W; approximately 3 m a.s.l.; datum = SAD69) and Congonhas do Norte (Fig. 2D) (18° 48′ 27″ S, 43° 40′ 52″ W; approximately 1050 m a.s.l.; datum = SAD69).

Table 1. Locality, coordinates, number of individuals and calls, mean and standard deviation of the six acoustic parameters of advertisement calls of eleven populations of *Scinax squalirostris*. Abbreviations: GO (Goiás state), MG (Minas Gerais state); PR (Paraná state) SP (São Paulo state); RS (Rio Grande do Sul state); BR (Brazil).

Locality (Coordinates) / Number of individuals (Number of calls analysed)	Call duration (s)	Number of notes	Note duration (s)	Number of pulses	Repetition rate (calls/min)	Dominant Frequency (Hz)
La Paz, Bolivia	0.351±0.422	8.722±10.45	0.025±0.032	6.529±7.333	1417.7±1533.13	4195.14±4507.22
(65°52′00′′W; 9°45′00′′S) / 6 (75)	(0.020–0.061)	(0.333–1.128)	(0.002–0.004)	(0.688–1.112)	(63.62–138.14)	(106.75–239.07)
Congonhas do Norte, MG, BR	0.375±0.388	13.8±15.625	0.023±0.025	4.80±5.625	2297.16±2412.60	4150.12±4340.5
(43°40′52′′W; 18°48′27′′S) / 2 (18)	(0.020–0.041)	(0.744–2.043)	(0.001–0.003)	(0.788–1.302)	(74.16–205.39)	(116.30–121.48)
Cristalina, GO, BR	0.020±0.061	45.347±48.68	0.012±0.013	1±1	4114.75±4361.50	3919.56±4195.65
(47°36′48′′W; 16°46′07′′S) / 3 (97)	(0.623–0.674)	(1.495–4.964)	(0.001–0.001)	(0–0)	(74.16–205.39)	(42.023–138.46)
Passos Maia, SC, BR	0.623±0.788	12.146±14	0.028±0.045	5.739±7.312	1062.37±1346.75	3977.93±4272.30
(52°03′32′′W; 26°46′48′′S) / 4 (122)	(0.039–0.090)	(0.485–1.215)	(0.002–0.058)	(0.383–1.227)	(29.06–310.60)	(134.69–304.51)
Porto Alegre, RS, BR	0.30±1.10	5.0±10.461	0.030±0.045	9.230±11.10	982.32±1256.56	4030.6±4916.91
(51°13′47′′W; 30°01′60′′S) / 7 (98)	(0.056–0.674)	(0.833–4.155)	(0.003–0.295)	(0.894–2.891)	(81.27–242.55)	(0–1055.59)
São Borja, RS, BR	0.187±0.240	6.90±7.857	0.017±0.018	8.0±8.45	1995.50±2226.08	4933.57±5003.70
(56°00′15′′W; 28°39′39′′S) / 2 (27)	(0.024–0.042)	(0690–0.852)	(0.002–0.003)	(0.577–0.887)	(208.27–241.048)	(65.009–152.74)
São Francisco de Paula, RS, BR	0.314±0.656	9.327±13.666	0.022±0.275	5.761±8.555	1250.31±5.034	4441.57±4700.11
(50°35′00′′W; 29°26′53′′S) / 6 (250)	(0.020–0.035)	(0.577–0.982)	(0.577–0.982)	(0.534–1.784)	(4.536–130.53)	(101.176–187.95)
Serra da Bocaina, SP, BR	0.420±0.548	11.642±14.466	0.030±0.032	7.966±9.0	1584.32±1639.68	3709.10±4256.78
(44°34′40′′W; 22°38′43′′S) / 2 (44)	(0.029–0.029)	(0.681–0.841)	(0.014–0.002)	(0.554–1.129)	(53.606–55.067)	(167.868–530.316)
Rio Grande, RS, BR	0.562±0.563	9.777±9.833	0.035±0.035	8.777±9.277	1049.73±1070.56	4555.11±4591.77
(52°05′54′′W; 32°02′06′′S) / 2 (36)	(0.619–0.064)	(0.383–0.427)	(0.003–0.003)	(0.554–1.129)	(95.113–103.713)	(189.589–232.609)
Artigas department, Uruguay	0.47±0.585	8.55±10.066	0.029±0.055	7.05±8.0	1006.39±1096.60	4593.22±5176.05
(56°28′W; 30°23′′S) / 6 (44)	(0.039–0.117)	(0.593–0.866)	(0.004–0.083)	(0.755–2.187)	(49.770–178.679)	(110.241–300.136)
Viamão, RS, BR	0.264±0.275	8.333±9.20	0.018±0.020	7.0±8.80	1822.65±2094.44	5081.11±5150.2
(51°01′24′′W; 30°04′53′′S) / 2 (14)	(0.013–0.021)	(0.447–0.50)	(0.002–0.002)	(0.707–1.30)	(129.69–155.46)	(86.250–94.756)
Overall average	0.21±0.665	7.375±46.64	0.012±0.433	1±9.367	1043.39±4267.13	3983±5115.05
39 (825)	0.026–0.123	0.291–1.951	0.0005–0.35	0–1.272	14.736–824.02	26.00–387.49

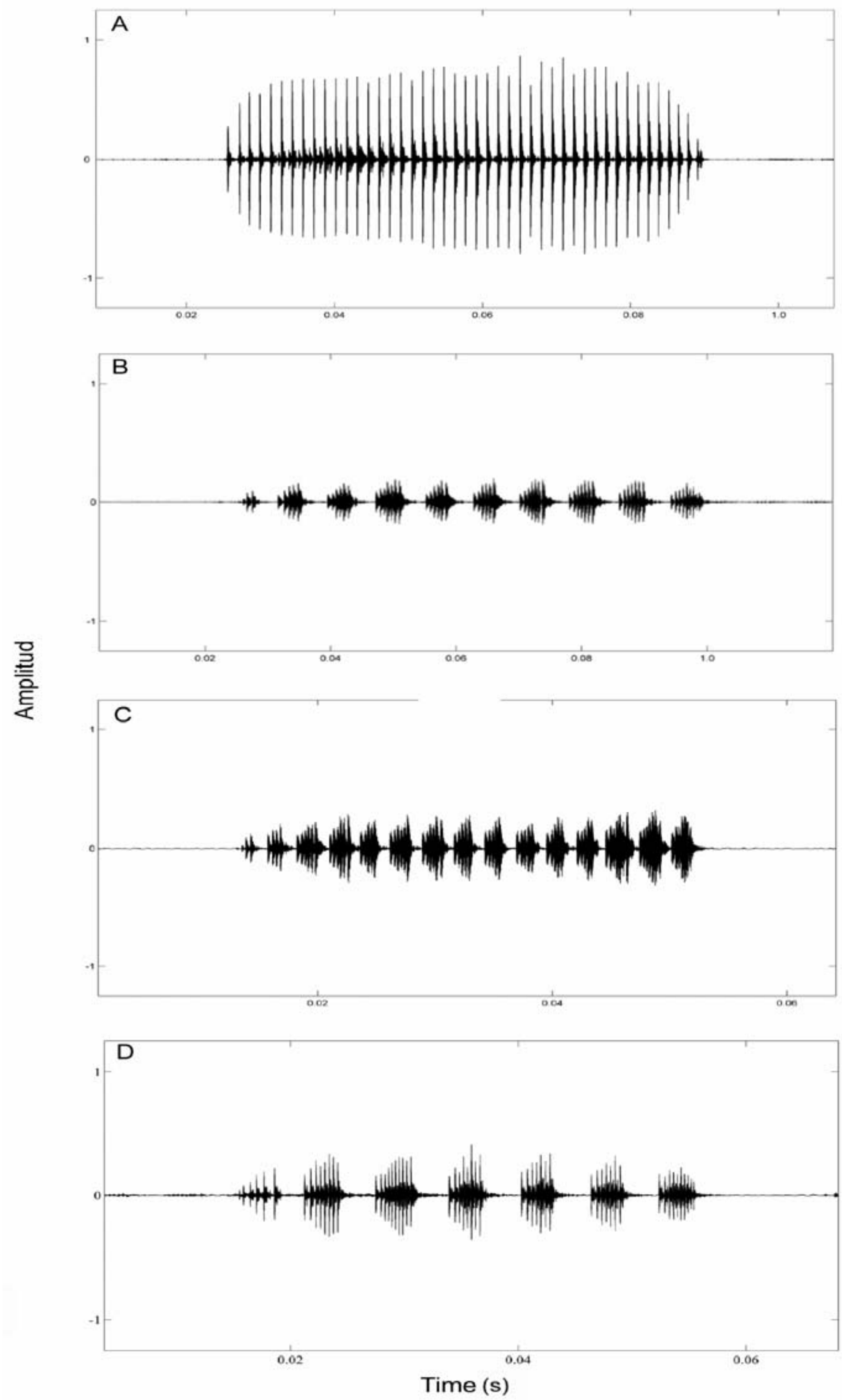

Figure 2. Oscillogram of advertisement calls of *Scinax squalirostris*. (**A**) Cristalina municipality, Goiás state, BR; (**B**) La Paz municipality, Bolivia; (**C**) Porto Alegre municipality, Rio Grande do Sul state, BR; (**D**) Congonhas do Norte municipality, Minas Gerais state, BR. *BR: Brazil.

Table 2. Results of the three canonical variables to six acoustic parameters used in the discriminant function analysis for the advertisement call of *Scinax squalirostris*.

	Discriminant Function		
Acoustic Parameters	1	2	3
Call duration	-0.08	*-0.84	-0.11
Number of notes	-0.41	-0.49	0.15
Note duration	0.13	-0.48	-0.03
Number of pulses	*0.63	0.28	*0.58
Repetition rate	-0.04	0.01	0.08
Frequency	0.07	0.39	*-0.54
Autovalue	93.56	4.79	2.10
Cumulative proportion	0.91	0.96	0.98
Canonical correlation	0.99	0.90	0.82
Lambda Wilks	0.0002	0.02	0.11
Chi square	250.98	116.77	64.97
Degrees of freedom	60	45	32
Value-p	≤0.001	≤0.001	≤0.001

Canonical variables represent correlation between acoustic parameters and the discriminant function.
* Correlations greater than 0.6

Considering the inter-population separation through acoustic parameters, the discriminant function was highly significant (Wilks lambda = 0.0002; $F_{(60,125)} = 8.52$; $P < 0.0001$). Although some degree of overlapping occurred among populations, 90% of the individuals were correctly reclassified into their original population. The discriminant function analysis of the advertisement call features included the six acoustic variables measured, which generated six discriminant functions (Table 2). The first three functions had eigenvalues above 1.00 and together explained 98.69% of the variation among males of different populations. The first discriminant function (DF) explained 91.92% of the variation and was mainly related to number of pulses (r = 0.63). The second DF explained 6.77% of the variation and was mainly related to the note duration (r = -0.84). The third DF explained 2.06% of the variation and was correlated to the dominant frequency (r = -0.54) and the number of pulses (r = 0.58). Three distinct groups were formed in the first canonical axis: one composed by Cristalina municipality (state of Goiás, Brazil), one by Congonhas do Norte municipality (state of Minas Gerais, Brazil) and another group by the remaining locations analysed (São Francisco de Paula, Rio Grande, Viamão, São Borja, Passos Maia, Porto Alegre, Serra da Bocaina, Artigas, and La Paz) (Fig. 3).

We found no geographical pattern between latitude, longitude and altitude against the acoustic parameters analysed through the multiple regressions, except the dominant frequency. Dominant frequency was influenced by latitude with lower values found further north (P = 0.04) (Table 3). We found no association between the array of acoustic parameters and geographic distance (r = -

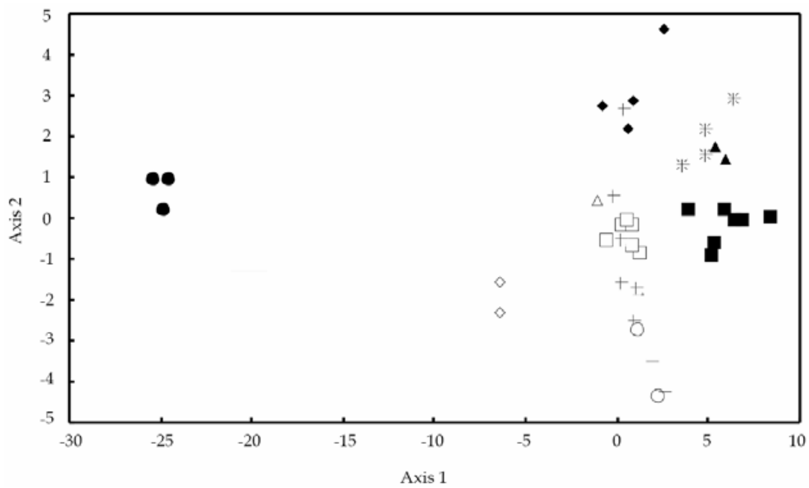

Figure 3. Population groups showed by the first two discriminant axis. Localities are shown by the following symbols: ⁎ Uruguay; □ Bolivia; ◊ Congonhas do Norte municipality, Minas Gerais state; Δ Bocaina National Park, São Paulo state; ● Cristalina municipality, Goiás state; ■ Porto Alegre municipality, Rio Grande do Sul state; ♦ Passos Maia municipality, Santa Catarina state; ▲ Rio Grande municipality, Rio Grande do Sul state; + São Francisco de Paula municipality, Rio Grande do Sul state; ○ Viamão municipality, Rio Grande do Sul state; - São Borja municipality, Rio Grande do Sul state. The last nine localities are found in Brazil.

Table 3. Multiple regression (partial correlation and the whole model) with call parameters and dependent variables and latitude and longitude as independent variables. *Significant results, with p<0.05.

Acoustic parameters	$r_{partial}$	p	R^2_{model}	$F_{(3.7)}$	P
Call duration	–	–	0.07	0.18	0.9
Altitude	0.2	0.61	–	–	–
Latitude	-0.09	0.82	–	–	–
Longitude	0.2	0.56	–	–	–
Number of notes	–	–	0.41	1.64	0.26
Altitude	0.56	0.1	–	–	–
Latitude	0.17	0.59	–	–	–
Longitude	0.41	0.22	–	–	–
Note duration	–	–	0.3	0.99	0.45
Altitude	-0.51	0.16	–	–	–
Latitude	0.19	0.58	–	–	–
Longitude	-0.5	0.89	–	–	–
Number of pulses	–	–	0.29	0.94	0.47
Altitude	-0.49	0.18	–	–	–
Latitude	-0.22	0.52	–	–	–
Longitude	-0.22	0.54	–	–	–
Repetition rate	–	–	0.27	0.86	0.5
Altitude	0.43	0.24	–	–	–
Latitude	0.25	0.47	–	–	–
Longitude	0.25	0.49	–	–	–
Dominant frequency	–	–	0.68	5.04	*0.04
Altitude	-0.3	0.21	–	–	–
Latitude	-0.71	*0.01	–	–	–
Longitude	-0.28	0.25	–	–	–

0.023; *P* = 0.46; 10.000 permutations, Fig. 4), being considered not significant.

Discussion

The comparison of acoustic properties among different species and populations is important to interpret and comprehend ecological interactions, taxonomic position and phylogenetic relationships among species (Hartmann et al. 2002). Individuals from Cristalina municipality (state of Goiás, Brazil) showed higher and clearly the degree of differences in acoustic parameters among all populations analysed. This great difference could indicate that *Scinax squalirostris* from Cristalina municipality could represent a distinct species, as suggested by Pombal et al. (2011).

Call duration and number of pulses were the dynamic features that could vary more among populations. Temporal properties are found to be evolutionarily more variable than spectral ones (Ryan 1998). This reflects a greater instability of characters that are mostly under physiologic-behavioural control (such as temporal characters) compared with those under morphological control (such as spectral characters). Temporal parameters can also be influenced by abiotic factors such as temperature (e.g. Lingnau & Bastos 2007). The strong influence of the abiotic factors on the acoustic parameters has been well documented in many species of anurans (Sullivan & Malmos 1994, Giacoma et al. 1997, Navas & Bevier 2001). Besides,

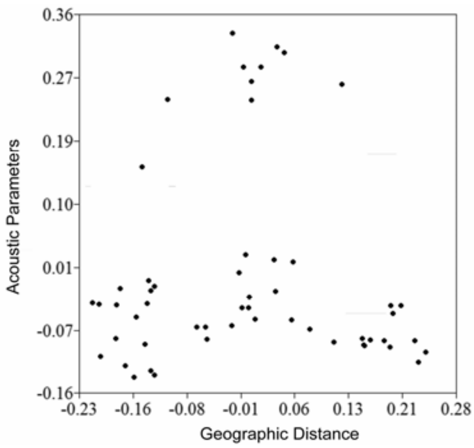

Figure 4. Relationship between geographical and acoustic parameters of the eleven populations of *Scinax squalirostris* used in this study.

temporal parameters are also regulated by individual variation, due to high-energy cost of vocalization being limited by the amount of energy that the individual holds (Zimmiti 1999).

In this work, the dominant frequency was strongly influenced by the latitude. Lower values of dominant frequency were found further north. The influence of these attributes in the spectral factors of anuran vocalization is well documented (e.g. Castellano et al. 2002, Felton et al. 2006). This is due to the relation of vocal cords and body, when larger individuals produce more calls with lower frequencies (Ryan 1986). Therefore we could not evaluate the influence of body size and mass of individuals and correlate them to call features and geographical variation. However, amphibians tend to be larger at higher latitudes, indicating that body size is a response to selective pressures. Usually, body size and mass vary with latitude and possibly the temperature (Ashton 2004). However, according to Bergmann's rule, individuals of a species tend to be smaller in warmer areas and larger in colder areas (Michael et al. 2004). Typically, low temperatures retard the development rather than growth. Thus, tadpoles that grow in colder areas have a longer period of development, metamorphosing with larger size than conspecifics growing in warmer areas (Alvarez & Nicieza 2002, Ashton 2004, Laugen et al. 2005). However, our data didn't confirm Bergmann's rule, as we found higher medium frequencies further south, suggesting that individuals further south have smaller body size, although being in a colder area. So, the southern increment of frequency may be related to changes in environmental factors such as temperature.

Geographic variation in acoustic parameters has been documented in frog species (e.g. Gerges et al. 2004, Bernal et al. 2005). One of the mechanisms involved in geographic variation occurring in animals is the isolation by distance (Wright 1943). We could not observe a geographical differentiation on the call patterns in *Scinax squalirostris*. Although a pattern of isolation by distance is commonly referred to genetic differentiation, geographic distance could also be associated with variation in phenotypic traits, such as call. Therefore geographical distance only doesn't explain the variability in acoustic parameters among populations of *Scinax squalirostris* analysed. In this context, local selective pressures may be affecting population call structure (Alexandrino et al. 2005) or more than one species fall under the specific name *squalirostris*. Differences in acoustic parameters varying geographically could be used to detect anuran cryptic species (Pombal & Bastos 1998). Therefore, the disjunct distribution pattern found in populations of this species or closely related species could be explained to occur by stochastic events other than dispersal, such as vicariance.

The taxonomy of many species has been reevaluated based mostly on morphological and/or bioacoustics analysis (Haddad & Pombal 1998, Kwet & Solé 2005, Kwet 2007, Izecksohn & Carvalho-e-Silva 2008). However, recent studies also have been conducted under a taxonomic integrative approach (e.g. Vaz-Silva & Maciel 2011) when multiple sources of evidence, such as morphology, bioacoustics, statistical, molecular and behavioural characters could be used to reveal the diversity of a lineage or organisms, including cryptic species (Padial et al. 2010). Further studies, including additional sampling in new areas are needed for better understanding the taxonomy of *Scinax squalirostris* as well as other Neotropical species of frogs. Thus, the data from this work aims to contribute to a better understanding of the real biodiversity that these populations constitute under variation of advertisement call parameters and evaluation of geographical patterns.

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