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Phenotypic differentiation in populations of a gladiator tree frog: environment, genetic drift and sexual selection

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Phenotypic differentiation among animal populations is common, yet few studies have simultaneously examined the adaptive and neutral mechanisms behind it. Such evolutionary processes become more relevant in species with complex behaviours that undergo global and local selective pressures throughout their geographical range. Here we measured and compared morphological and acoustic variation across the distribution range of a Neotropical gladiator tree frog that shows elaborate reproduction (territoriality, complex courtship and female choice). We then incorporated molecular and landscape data to examine the roles of sexual selection, genetic drift and acoustic adaptation to the environment in call differentiation, i.e. the acoustic adaptation hypothesis (AAH). We found that calls varied more than morphology among populations, but differences in calls or morphological traits were not explained by genetic differentiation. We found no evidence for the AAH, but a significant relationship in the opposite direction regarding call frequencies suggests an indirect role of sexual selection. Differentiation on call traits that are associated with individual discrimination and/or female attraction also corroborated an important role of sexual selection. We show that multitrait and multimechanism approaches can elucidate intricate processes leading to phenotypic variation among individuals and populations. We emphasize that studies of species with complex reproductive behaviours across their range may provide insights into different selective pressures leading to phenotypic differentiation.

ADDITIONAL KEYWORDS: Amphibia – bioacoustics – *Bokermannohyla ibitiguara* – Brazil – complex vocalization – genetic differentiation – Hylidae – intraspecific variation – morphometry.

INTRODUCTION

Microevolutionary processes acting on populations potentially lead to speciation events or extinction of divergent lineages across time and space (Crow & Kimura, 1970; Futuyma, 2005; Wagner, 2016). When looking at the whole distribution range of a species, intraspecific variation in organismal traits is a common pattern (Elmer *et al.*, 2010; Brusa *et al.*, 2013; Talal *et al.*, 2015). As genetic differentiation normally accumulates as a result of landscape features that limit gene flow (Manel *et al.*, 2003; Pato *et al.*, 2019; Nali *et al.*, 2020), variation in populations scattered throughout a species' range may increase due to neutral evolution. Nonetheless, variation in phenotypic traits can be increased rapidly by local pressures, such as divergent sexual selection on specific traits used for mate recognition, a process that can lead to unique lineages (Gerhardt, 1999; Stuart *et al.*, 2017). Because the association among traits, the environment and population isolation are complex, accounting for multiple possible mechanisms shaping variation is crucial when investigating the evolution of lineages (e.g. González *et al.*, 2011; Warwick *et al.*, 2015), and a population-level approach to phenotypic differences will be especially informative (Pascoal *et al.*, 2017).

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Two examples of phenotypes that vary among animal populations under a composite of selective and neutral processes include external morphology and vocalizations. Variation in morphological characters, especially morphometrics, has always been reported in the field of taxonomy, but only more recently has morphology been combined with genetic variation to investigate, for example, undetected cryptic species and diversification of lineages (Elmer *et al.*, 2010; Havermans *et al.*, 2013; Warwick *et al.*, 2015; Ortega-Andrade *et al.*, 2015; Nali *et al.*, 2023). Vocalization constitutes the main communication system in many organisms (Bradbury & Vehrencamp, 2011), mediating same-sex interactions such as male–male competition and opposite-sex interactions such as female mate choice within a population (Ryan, 1990; Gerhardt, 1994; Bailey *et al.*, 2017; Pettitt *et al.*, 2020). Morphology and calls can be intimately related; for example, animals with larger body sizes emit lower-pitched calls due to physical aspects of the vocal apparatus (Ryan & Brenowitz, 1985; McClelland *et al.*, 1996; Barclay *et al.*, 1999; Nali & Prado, 2014a). This provides acoustic cues for females to select males with more advantageous sizes (Bastos & Haddad, 1996; Lu *et al.*, 2010). Although accounting for acoustic and morphological characteristics seems crucial to investigate differentiation mechanisms (Lougheed *et al.*, 2006), few studies have combined population genetics, morphological variation and calling behaviour simultaneously (but see Lougheed *et al.*, 2006; Funk *et al.*, 2011; Pato *et al.*, 2019). The combination of such traits may help disentangle the roles of neutral processes, such as genetic differentiation due to genetic drift in isolated populations, from those of different adaptive mechanisms (Wilkins *et al.*, 2013).

A well-studied adaptive mechanism shaping call differentiation is sexual selection, for which frogs have been a useful model (Gerhardt, 1994; Ryan & Rand, 2003a; Lemmon, 2009; Kaiser *et al.*, 2018). Local selection on calls can be either stabilizing, when females prefer values toward the mean to avoid heterospecific mating, or directional, when females prefer extreme values that indicate high-quality males (Ryan, 1990; Reichert, 2013; Andreani *et al.,* 2021). In addition, emission of certain parts of the call might evolve to repeal other males, also with direct consequences for male reproductive success (Endler, 1992; Nali & Prado, 2014a; Reichert, 2014). In any of these cases, call traits across a species' range will vary as a function of female selection (intersexual selection) or selection by competing males (intrasexual selection), and such mechanisms can operate depending on intermale call variability (Joshi *et al.*, 2019). In the absence of sexual selection, neutral evolution due to genetic isolation and drift may shape call differentiation (Velásquez *et al.*, 2013; Lee *et al.*, 2016). At the very least, neutral evolution may impose constraints and shape the acoustic window that could be later subject to adaptive selection (Wilkins *et al.*, 2013; Pascoal *et al.*, 2017).

A much overlooked adaptive mechanism that promotes call differentiation is adaptation to the environment (Wilkins *et al.*, 2013). The acoustic adaptation hypothesis (AAH) postulates that acoustic signals are adapted to maximize transmission success by avoiding degradation and attenuation (Morton, 1975; Hansen, 1979), as production and reception of signals are equally important to elicit behavioural responses by the receiver (Endler, 1992). Signalling across a forest habitat, for instance, may impose selective pressures on temporal and spectral parameters. Low-pitched calls should evolve in more closed environments due to their enhanced transmission across vegetation (Morton, 1975). Calls should have longer durations because longer signals across obstructed environments increase the probability of detection and create reverberations that enhance propagation distance (Slabbekoorn *et al.*, 2002; Nemeth *et al.*, 2006). Finally, the emission of fewer notes per time unit should be favoured to avoid overlapping of the reverberating signals, which could affect detection. The AAH has been investigated mostly in birds and mammals, with controversial yet more convincing support (Boncoraglio & Saino, 2007; Ey & Fischer, 2009). In frogs, results are largely inconsistent (Ziegler *et al.*, 2011; Erdtmann & Lima, 2013; Goutte *et al.*, 2018; Velásquez *et al.*, 2018; Bezerra *et al.*, 2021), such that further quantification of environmental variation is needed to understand the role of the environment in shaping frog calling behaviour (Erdtmann & Lima, 2013; Bezerra *et al.*, 2021). Moreover, studies attempting to explain call variation as a function of sexual selection and/or genetic drift did not investigate adaptation to the environment (see Wilkins *et al.*, 2013), which hampers our understanding of the multiple possible mechanisms driving population differentiation.

In this study, we investigated phenotypic differentiation in a Neotropical gladiator tree frog by simultaneously analysing, for the first time, morphology, calls, molecular data and landscape features. Our focal species, *Bokermannohyla ibitiguara* (Cardoso, 1983), inhabits streams in dense riparian forests within the Brazilian savanna. This species is territorial and has a complex courtship with tactile and different acoustic stimuli (Nali & Prado, 2012; Nali *et al.*, 2022), complex calling behaviour with different pulsed notes emitted and modulation of call parameters (Cardoso, 1983; Nali & Prado, 2014a; Nali *et al.*, 2022), and significant individual call variation and discrimination (Turin *et al.*, 2018). Additionally, populations are genetically differentiated across breeding sites

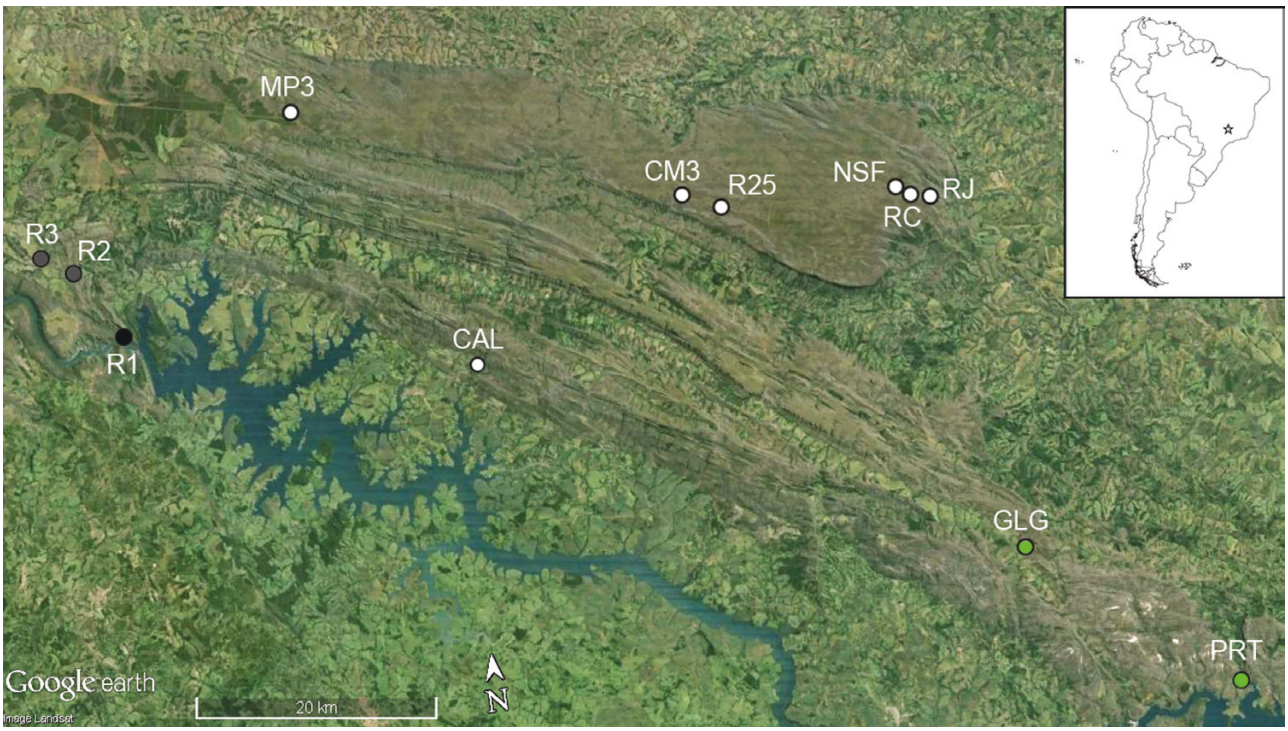

Figure 1. Sampling localities (streams) of *Bokermannohyla ibitiguara* in south-eastern Brazil (star in detail) across the Serra da Canastra mountain range. Points are coloured according to four different genetic clusters under a previous Bayesian analysis conducted by Nali *et al.* (2020) (white = PNSC cluster, green = Capitólio cluster, grey = Sacramento 2 cluster and black = Sacramento 1 cluster; see text for details). Image source: Google Earth.

with different degrees of forest cover (Nali *et al.*, 2020), and hybridization with a congener is known (Nali *et al.*, 2023). First, we analysed whether intraspecific variation in calls and morphology are partitioned among populations and genetic clusters and their degree of variation. Then, we assessed the relative importance of neutral processes (genetic drift/isolation) and adaptive mechanisms (sexual selection and the AAH) in call differentiation across its distribution range. Our study contributes to the understanding of mechanisms leading to phenotypic variation in acoustically oriented taxa, including those with complex reproductive behaviours.

MATERIALS AND METHODS

Study site and individual sampling

The Serra da Canastra mountain range is located in south-eastern Brazil, within South America's second largest morphoclimatic domain, the Brazilian Cerrado (Silva *et al.*, 2006). The climate is markedly seasonal, with a hot and rainy summer and a dry winter (Queirolo & Motta-Junior, 2007). This savannic formation is covered by Cerrado vegetation, patches of semideciduous forest, gallery forests along streams, and grasslands at higher elevations, up to 1500 m a.s.l. (Dietz, 1984).

Fieldwork was conducted in 12 breeding streams with different riparian forest cover throughout the distribution range of *B. ibitiguara* during the rainy season (October–March) from 2010 to 2015 (Fig. 1; Supporting Information, Table S1; Nali *et al.*, 2020). We sampled males for this study, as they are the main target of sexual selection in frogs, and found them by acoustic and visual searches at the breeding sites. We recorded calls at ~1 m distance from the males using a Marantz PMD-660 digital recorder and Sennheiser ME66 unidirectional microphone at a 16-bit resolution and 44 100 Hz (e.g. Nali & Prado, 2014a). Air temperature was measured during each sampling event with an analog thermometer. Most males were collected for morphometric measurements (Watters *et al.*, 2016), killed by spraying a solution of 10% lidocaine in the gular region, fixed in 10% formalin and preserved in 70% ethanol (McDiarmid, 1994). Individuals that were not collected were identified by unique natural marks (e.g. Nali & Prado, 2014a) or marked with the toe-clipping method (Waichman, 1992) to avoid recording the same male more than once, and we used the toes as tissue samples for genetic analyses (see below). Collected specimens

were deposited in the Célio F. B. Haddad Amphibian Collection, Universidade Estadual Paulista, Rio Claro, São Paulo, Brazil (Table S2).

Measurement of morphological and call variables

Due to some subjectivity inherent to bioacoustics (Köhler *et al.*, 2017), and also to morphometric measurements (e.g. how one handles the caliper and the specimens; Watters *et al.*, 2016), a single researcher (R.C.N.) measured voucher specimens and analysed calls, avoiding biases. A digital caliper was used to measure 12 morphometric traits (Watters *et al.*, 2016) to the nearest 0.01 mm for each specimen (Supporting Information, Fig. S1): snout–vent length (SVL), head length (HL), head width (HW), eye diameter (ED), tympanum diameter (TD), eye–nostril distance (END), internarial distance (IND), forearm thickness (FaT), forearm length (FaL), hand length (HaL), foot length (FoL) and tibial length (TiL). Specimens were measured without previous knowledge of the population, and each measurement was taken from the left side across specimens.

A total of 79 males had their calls analysed using the software Raven Pro 1.4 (Cornell Lab of Ornithology, Ithaca, NY, USA) with FFT (fast Fourier transformation) = 512 points, brightness = 70 and contrast = 70 (e.g. Nali & Prado, 2014a). Males of *B. ibitiguara* emit calls with a sequence of ca. five to six long notes, followed by a sequence of average six short notes (Cardoso, 1983; Nali & Prado, 2014a; Supporting Information, Fig. S2). Thus, we were able to measure the following call variables: (1) minimum frequency of the long note (LN min freq), (2) dominant frequency of the long note (LN dom freq), (3) duration of the long note (LN dur), (4) pulse rate of the long note (LN pulses), (5) minimum frequency of the short note (SN min freq), (6) dominant frequency of the short note (SN dom freq), (7) duration of the short note (SN dur), (8) minimum frequency of the sequence of short notes (SNseq min freq), (9) dominant frequency of the sequence of short notes (SNseq dom freq), (10) duration of the sequence of short notes (SNseq dur), (11) number of long notes per minute (LN/min), (12) number of short notes per minute (SN/min), (13) number of sequences of short notes per minute (SNseq/min) and (14) number of short notes per sequence (SN/seq). Further details on our call measurements are available in Table S3.

We measured five long notes, five short notes and up to five sequences of short notes across different calls per individual and calculated the means for each variable. In the few cases where the individuals called from inside cavities and/or at the water level (N = 5), only temporal variables were measured due to physical differences in spectral parameters in those situations (Muñoz & Penna, 2016; Muñoz *et al.*, 2020; R. C. Nali, pers. obs.). Recordings with inferior quality (e.g. with strong background water noise) were only used to measure parameters with good visualization, in Raven Pro 1.4. These quality control procedures yielded ~9.3% missing data, and consequently we had slightly different sample sizes across our analyses.

Call parameters of *B. ibitiguara* may be influenced by male body size and air temperature (Nali & Prado, 2014a; Turin *et al.*, 2018), which are corrected in studies of population variation (Kaefer & Lima, 2012; Baraquet *et al.*, 2015). Thus, we calculated Pearson's product-moment correlations between air temperature and all 14 call parameters to remove the influence of this variable; call parameters were then adjusted to the average temperature of 20.59 °C (range = 17–25 °C; SD = 2.02; N = 79) based on the overall regression coefficients for each parameter (e.g. Pröhl *et al.*, 2007). To remove the body size effect, we performed the same for correlations with male SVL (e.g. Velásquez *et al.*, 2013); call parameters with a significant SVL effect were adjusted to the average SVL of 39.37 mm (range = 33.3–49.15 mm; SD = 3.51; N = 79). We used this fully adjusted acoustic dataset in all analyses except those of AAH (see below).

Genetic distances and genetic cluster assignment

To assess genetic differentiation among the 12 populations in this species, we used a genetic distance matrix from a previous study (Nali *et al.*, 2020), containing genotypes of 17 microsatellite markers developed for the species (Nali *et al.*, 2014) with individuals collected at the same streams and time periods. The matrix consisted of pairwise F_{ST} values $[F_{ST}/(1 - F_{ST})]$ between pairs of the same 12 populations (Rousset, 1997) and included 273 individuals (adults and larvae; see Nali *et al.*, 2020); most of the adults were also analysed for this work (Supporting Information, Table S1). Individual genotypes were also used in a Bayesian analysis in the software STRUCTURE (Pritchard *et al.*, 2000), in which delta K showed a clear peak for four genetic clusters, with a high average coefficient of membership (percentage of individual assignment = 90.3%; Nali *et al.*, 2020). Thus, each population was classified as belonging to one of the four assigned genetic clusters: Sacramento 1, Sacramento 2, SCNP and Capitólio (Table S1; Fig. 1). Detailed procedures on laboratory protocols, software parameters, quality control of markers and interpretations can be found in Nali *et al.* (2020).

Assessment and comparisons of morphological and acoustic differentiation

We used two group classifications when analysing acoustic and morphological variation: populations (12

streams) and genetic clusters (four clusters). We used Kruskal–Wallis tests to check whether morphological and call traits varied among populations and clusters in R (R Core Team, 2020). We then were able to interpret the variation of call parameters in light of previous studies with the species regarding sexual selection and call discrimination (Nali & Prado, 2012, 2014a, b; Turin *et al.*, 2018; Nali *et al.*, 2022).

To assess how the two phenotypic traits (morphology vs. calls) might differ in variation, we first calculated coefficients of variation (CVs) within each cluster and population for each morphometric and acoustic variable. Each CV was expressed as a percentage using the formula CV = SD/mean × 100. We then ran Mann–Whitney tests among call CVs vs. morphological CVs, for both populations and clusters. Thus, significantly higher CVs indicate higher variation in that specific phenotype (morphology or calls). To analyse the degree of population/cluster differentiation for each phenotype in a multivariate approach, we used canonical variate analyses (CVAs) with morphological variables pooled together and acoustic variables pooled together. Each dataset was log-transformed, scaled and centred, and then split into train (70%) and test (30%) subsets. We examined the percentages of individuals correctly assigned to each group (e.g. Lougheed *et al.*, 2006), in which lower percentages of correct classifications indicate less group differentiation according to each phenotype. All analyses were conducted in R, and the canonical roots of each analysis were plotted using *ggplot2* (Wickham, 2016).

Finally, to determine whether call differentiation and morphological differentiation were correlated or decoupled in this species, we calculated two Mahalanobis distance matrices among populations by using the canonical roots of the CVAs, one for morphology and another for calls. These distance matrices were then correlated using Mantel tests (Mantel, 1967) in PASSAGE v.2.0 (Rosenberg & Anderson, 2011), with significance assessed under 9999 permutations. To make matrices comparable in our analyses, they were all scaled, i.e. each value was divided by the largest value of that matrix (e.g. Lougheed *et al.*, 2006).

Neutral evolution and call differentiation

To test for correlations between genetic distances and call distances among populations, i.e. possible neutral evolution on calls (Wilkins *et al.*, 2013), we first calculated individual Mahalanobis distances for log-transformed acoustic variables that were significantly different among populations in the Kruskal–Wallis tests (see above; e.g. Funk *et al.*, 2009). We then used Mantel tests to investigate correlations between our genetic distance matrix (as explained previously) vs. distances of each significant acoustic parameter, scaled accordingly (dividing each value by the largest value in the matrix). Additionally, we used the aforementioned Mahalanobis matrix of acoustic distances among populations (calculated from our CVA) and ran a simple Mantel test between genetic distance and acoustic distance. We also controlled for geographical distance by using partial Mantel tests. We obtained Mahalanobis distances for each individual acoustic variable and ran Mantel tests using PASSAGE v.2.0 (Rosenberg & Anderson, 2011), with significance assessed under 9999 permutations.

Environmental influence on call differentiation

To test whether call differentiation among populations resulted from acoustic adaptation to different degrees of forest cover (the AAH), we first quantified the forested areas for each population. High-resolution satellite images for each population were extracted from the software Google Earth Pro. The images originated from CNES/Astrium satellites (pixel resolution = 0.35 m) during the years 2013 or 2014, when we conducted the majority of our fieldwork, and were visualized from ~1.5 km above the ground. We georeferenced and processed each image in ArcGIS 9.3.1 (ESRI, 2009) as follows: we drew a circle with a 500-m radius around the centroid of the population, considering our sampling points within that population, and manually classified the gallery forests within that circle (e.g. Nali *et al.*, 2020). We then calculated the percentage of forested area per locality (relative to the total circle area) and used it as a proxy; that is, higher percentages mean a more closed forested environment (e.g. Nali *et al.*, 2020). Finally, we ran linear multiple regression models with call variables that are predicted to vary by habitat type according to the AAH as dependent variables. Considering that body size and air temperature are confounding factors in the study of the AAH (Goutte *et al.*, 2018), we included percentage forest cover, male SVL and air temperature as independent variables within each model. The ten dependent call variables used in this analysis were: minimum frequencies of long notes, short notes and sequences of short notes; dominant frequencies of long notes, short notes and sequences of short notes; duration of long notes, short notes and sequences of short notes; and note repetition rate (combination of the mean number of long notes per minute and short notes per minute). Every call parameter, as well as SVL and air temperature, were averaged for the individuals collected in each particular stream, and each dataset was log-transformed for the analyses. We ran model diagnostics using the package *performance* (Lüdecke *et al.*, 2021).

RESULTS

Individual sampling and dataset correction

We recorded calls of 79 males and measured 83 collected males (Supporting Information, Table S2). Air temperature was significantly correlated with all 14 call variables, and male size (SVL) was correlated with seven call variables, so the acoustic dataset was corrected accordingly for the variation analysis (Table S4).

Morphological and acoustic variation

All but two morphological variables were statistically different among the 12 populations (Supporting Information, Table S4), but only tympanum diameter was different among the four genetic clusters (Table S4; Supplemental Boxplots). Nine call parameters, including the six spectral parameters and the temporal parameters SNseq dur, LN pulses and LN/min, were significantly different among populations, with similar results among clusters (Table S4; Supplemental Boxplots).

CVs within populations for call variables ranged from 0 to 71.8% (median = 13.1%; N = 161 coefficients; Supplemental Spreadsheet), and those for morphometric variables ranged from 1.42% to 18.9% (median = 7.52%; N = 144 coefficients; Supplemental Spreadsheet). Acoustic CVs within populations were higher than those for morphology (Mann–Whitney U = 6.8; $P < 0.0001$). CVs within genetic clusters for call variables ranged from 4.3% to 61.61% (median = 14.96%; N = 56 coefficients; Supplemental Spreadsheet), and those for morphometric variables ranged from 6.26% to 16.06% (median = 8.95%; N = 48 coefficients; Supplemental Spreadsheet). Acoustic CVs within genetic clusters were also higher than those for morphology (Mann–Whitney U = 3.6; $P < 0.001$). Combined, our results show that calls varied significantly more than morphology within populations and genetic clusters, even after removing the effects of temperature and male SVL on calls.

Our CVAs showed similar results. In the analysis of call variation among genetic clusters, the first and second roots of the CVA explained, respectively, 63.13 and 22.3%, while the two first roots in the analysis of call variation among populations explained, respectively, 39.2 and 23.1%. In the analysis of morphological variation among genetic clusters, the first and second roots of the CVA explained, respectively, 74.05 and 18.08%, while the two first roots in the analysis of morphological variation among populations explained, respectively, 39.94 and 19.67%. The scatterplots of the first two roots showed morphological and acoustic overlap, but less so for calls (Fig. 2), a similar pattern observed in density plots of each first root (Supporting Information, Fig. S3). Accordingly, individuals were much more often assigned to the correct population in the CVA based on calls (35.7%) than based on morphology (15%). Regarding genetic clusters, individuals were slightly better assigned for morphology (62.5%) than for calls (55.6%). These differences agree with our Mantel tests, which showed no correlation between morphological differentiation and acoustic differentiation in this species (Table 1).

Neutral evolution and call differentiation

Our Mantel test of global acoustic distance vs. genetic distance was non-significant, even when controlling for geographical distance (Table 1). Similarly, our analyses of genetic distance vs. distances of nine individual call variables that were significantly different among populations were all non-significant (Table 2). Supporting Information Tables S5–S8 contain the scaled matrices for genetic, morphological, acoustic and geographical distances, respectively, among our 12 populations.

Environmental influence on call differentiation

The percentage of gallery forest varied from 1.12 to 29.08% among our 12 populations (Supporting Information, Table S1). In our multiple regression analyses, we found no evidence for the AAH, but in two different ways (Table 3). The six spectral variables correlated significantly but positively with percentage gallery forest, i.e. in a pattern that is opposite of what is expected from the AAH. The remaining four variables did not correlate with percentage gallery forests. Some acoustic traits, as expected (Köhler *et al.*, 2017), showed significant correlations with male SVL and air temperature.

DISCUSSION

Our results showed significant morphological and call variation across the geographical range of *B. ibitiguara*. However, calls varied significantly more than morphology among populations. Regarding genetic clusters, nine call variables were significantly different, but the only morphological difference was tympanum diameter, a phenotypic trait that is itself related to acoustic communication (Narins *et al.*, 2007). This first assessment suggests that local selective pressures on calls could be stronger compared to morphology across the distribution range (Funk *et al.*, 2011; see discussion below).

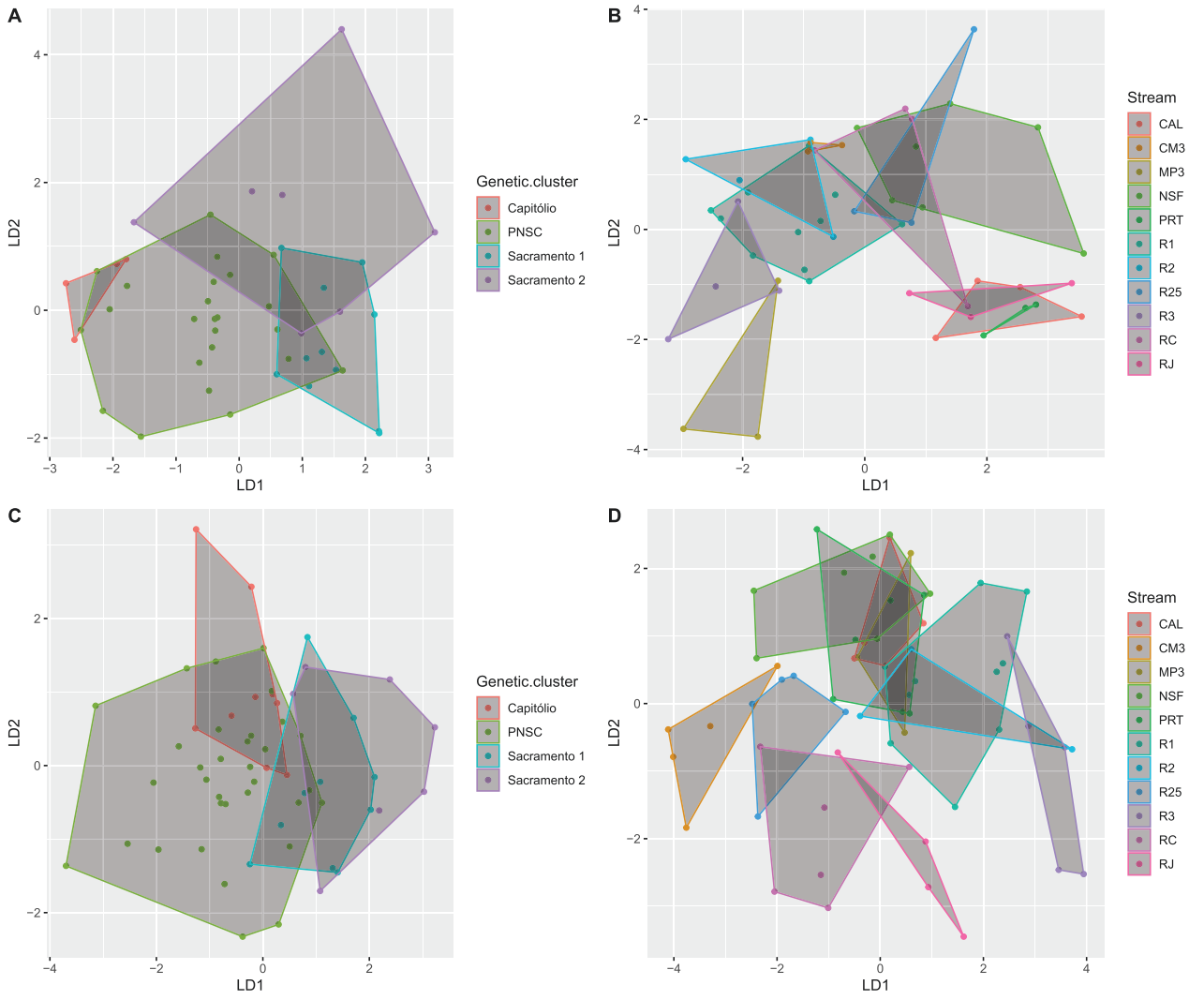

Figure 2. Acoustic (A and B) and morphological (C and D) variation in *Bokermannohyla ibitiguara*, in which each convex hull represents a genetic cluster or a population (stream) within the state of Minas Gerais, Brazil (population GLG was excluded due to insufficient acoustic data). Scatter plots were built upon the first and second loading roots of each canonical variate analysis (CVA).

Table 1. Results of Mantel tests to investigate correlations among genetic, acoustic and morphological distances in populations of *Bokermannohyla ibitiguara*, state of Minas Gerais, Brazil; geographical distances were also held constant (partial Mantel) in analyses containing genetic distance, which was dependent on geography

Distance matrix 1	Distance matrix 2	Controlled matrix	*R*	*P*	*N* populations
Geographical	Genetic	_	0.54	<0.001	12
Geographical	Acoustic	_	0.17	0.30	11
Geographical	Morphological	_	0.15	0.34	12
Acoustic	Morphological	_	0.01	0.97	11
Genetic	Acoustic	_	0.06	0.85	11
Genetic	Morphological	_	0.02	0.93	12
Genetic	Morphological	Geographical	−0.07	0.81	12
Genetic	Acoustic	Geographical	−0.03	0.93	11

Table 2. Results of the Mantel tests among genetic distances vs. Mahalanobis distances of nine call variables of *Bokermannohyla ibitiguara*, south-eastern Brazil

Call variable	Not controlling for geographical distance		Controlling for geographical distance		*N*
	R	*P*	*R*	*P*	
LN min freq	0.095	0.33	0.218	0.39	11
LN dom freq	0.096	0.69	0.213	0.31	11
LN pulses	0.155	0.45	−0.082	0.72	11
SN min freq	−0.018	0.93	−0.107	0.62	11
SN dom freq	0.249	0.23	0.119	0.60	11
SNseq min freq	0.084	0.67	−0.037	0.87	11
SNseq dom freq	0.143	0.50	0.034	0.89	11
SNseq dur	−0.036	0.94	−0.054	0.90	11
LN/min	0.179	0.38	−0.149	0.49	12

Population GLG was excluded from most analyses due to insufficient individual measurements.

Multivariate analyses showed call and morphology differences, with more overlap in morphological than in call traits (Fig. 2). Call traits allowed more accurate assignment of individuals to populations of origin. Sexual selection on mate recognition traits (e.g. calls) is expected to precede selection on phenotypes not exclusively involved in reproduction, such as morphology, potentially leading to population differentiation and increased speciation (Hoskin *et al.*, 2005; Lougheed *et al.*, 2006; Guerra & Ron, 2008; González *et al.*, 2011). Given that female frogs recognize and select males based on call properties (Gerhardt, 1994; Murphy & Gerhardt, 2002; Baugh & Ryan, 2010), our findings suggest that sexual selection might be leading to call variation in *B. ibitiguara*, although only playback experiments with males to evaluate intrasexual selection have been conducted in this species (Nali & Prado, 2014a). Other frogs show more intense call variation among lineages when compared to morphology, including tree frogs in the family Hylidae (e.g. Lougheed *et al.*, 2006; Funk *et al.*, 2011). *Bokermannohyla ibitiguara* has a highly male-biased operational sex ratio (Nali & Prado, 2012; Nali *et al.*, 2020; R. C. Nali, pers. obs.), where females choose among many active calling males at breeding sites (Nali & Prado, 2012), as seen in other frog species (Murphy & Gerhardt, 2002; Schwartz *et al.*, 2004). Calls can travel great distances, despite degradation, to be perceived and discriminated (Bradbury & Vehrencamp, 2011), while direct evaluation of morphological traits (e.g. male size) requires closer interactions (Ritz & Köhler, 2010). Thus, selecting calls that vary among individuals may be a first filter that highly increases female efficiency in selecting partners (Joshi *et al.*, 2019; Pettitt *et al.*, 2020).

While variability in phenotypes may result from local mechanisms such as female preferences (Kwiatkowski & Sullivan, 2002; Kaliontzopoulou *et al.*, 2007; Maan & Cummings, 2008; Akopyan *et al.*, 2017), differentiated call traits alone are not sufficient to show that they are sexually selected. Rather, this mechanism can be inferred when these traits are linked to female preferences, individual discrimination and/or male competition (Akopyan *et al.*, 2017; Joshi *et al.*, 2019). The number of long notes per minute differed across populations, and we know that long notes of the advertisement call play a role in female attraction in this species (Nali & Prado, 2014a), with courtship calls (the call directed specifically to females during courtship) consisting of long notes only (Nali *et al.*, 2022). The emission of more long notes per minute may increase female attraction in this species (Nali & Prado, 2014a), enhancing male fitness via sexual selection, and female preference for calls with higher repetition rates are known for other frogs (Sullivan, 1983; Forester & Czarnowsky, 1985; Schwartz, 1986). As a crucial component of vocal effort (Leary *et al.*, 2008), pulse rate is an important trait for female selection that can be increased in situations such as during courtships (Joshi *et al.*, 2019), which is precisely the case for our focal species (Nali *et al.*, 2022). The remaining variables that differed among populations (duration of the sequence of short notes and dominant frequency) contribute greatly to individual discrimination in this species (Turin *et al.*, 2018), and thus females may rely on those variables to select potential mates (Akopyan *et al.*, 2017). Males can also use these signals to recognize conspecific competitors (intrasexual selection; Bee *et al.*, 2001). The degree of male competition and territoriality can drive differential evolution of calling signals, especially in species with complex courtships, in which males need to rapidly assess mates and competitors to avoid predation and increase the number of mating events (Endler, 1992). Indeed, *B. ibitiguara* exhibits male competition with vocal duels and elaborate

Table 3. Multiple linear regressions between ten call variables (dependent variables, in bold) vs. percentage gallery forest, air temperature and male snout–vent length (SVL) across *Bokermannohyla ibitiguara* populations, state of Minas Gerais, Brazil

Traits	Whole-model R^2	Single-variable t	Single-variable P
LN min freq	0.80**		
Percentage gallery forest		2.82	0.0257
Air temperature		−4.36	0.0033
Male SVL		−0.89	0.4031
LN dom freq	0.75*		
Percentage gallery forest		3.09	0.0175
Air temperature		0.14	0.8928
Male SVL		−2.01	0.0849
SN min freq	0.86***		
Percentage gallery forest		2.44	0.0445
Air temperature		−1.28	0.2401
Male SVL		−6.25	0.0004
SN dom freq	0.80**		
Percentage gallery forest		3.39	0.0116
Air temperature		0.01	0.9911
Male SVL		−2.70	0.0306
SNseq min freq	0.87***		
Percentage gallery forest		2.84	0.0250
Air temperature		−1.50	0.1781
Male SVL		−6.52	0.0003
SNseq dom freq	0.72**		
Percentage gallery forest		3.01	0.0196
Air temperature		−0.53	0.6117
Male SVL		−2.44	0.0445
LN dur	0.57*		
Percentage gallery forest		−0.34	0.7460
Air temperature		−1.90	0.0944
Male SVL		1.58	0.1539
SN dur	0.43		
Percentage gallery forest		−0.60	0.5627
Air temperature		−2.05	0.0742
Male SVL		−0.71	0.4969
SNseq dur	0.54*		
Percentage gallery forest		0.85	0.4213
Air temperature		−3.08	0.0179
Male SVL		0.29	0.7811
Note repetition rate	0.46*		
Percentage gallery forest		2.21	0.0577
Air temperature		0.64	0.5378
Male SVL		0.95	0.3678

Significance of the whole model is stated next to each adjusted R^2 (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$). Population GLG was excluded from the analyses with spectral variables due to a single individual with measured call frequencies.

courtships (Nali & Prado, 2012, 2014a, b; Nali *et al.*, 2022). This should promote call differentiation even at a relatively small spatial scale. Although call plasticity itself can lead to differentiation (Ziegler *et al.*, 2011), our arguments above indicate that, if present in this frog species, call plasticity may maximize call differentiation for sexually selected traits. Even with our correlational inferences, we emphasize that playback experiments with females would be crucial in this species to corroborate a definitive link with sexual selection.

Other possible mechanisms that could explain population-level variance in calls were genetic drift due to isolation (neutral evolution) and ecological adaptation of call signals (the AAH). Call differences among populations were not linked to genetic

differentiation, indicating selective mechanisms on call differentiation that are related to non-random mating rather than landscape isolation effects (e.g. Boul *et al.*, 2007), the latter of which is known for this species (Nali *et al.*, 2020). Our results differ from those for another Neotropical frog (*Pleurodema thaul*), in which call and genetic distances were correlated, and call variation was attributed to genetic drift and isolation (Velásquez *et al.*, 2013). In this system, despite the influence of inter-male interactions on signal evolution, the lack of female preferences suggested a minor role for intersexual selection (Velásquez *et al.*, 2014, 2015). In *B. ibitiguara*, sexual selection pressures are strong on both sexes. Females and males engage in complex courtship with tactile and acoustic stimuli (Nali & Prado, 2012, Nali *et al.*, 2022). Females are choosy and inspect oviposition sites, deciding whether to mate with that male or move to another one (Nali & Prado, 2012; Nali *et al.*, 2022). Males are strong competitors that emit complex aggressive and modulated calls and engage in physical combats (Nali & Prado, 2012, 2014a, b). Hence, *B. ibitiguara* probably suffers much stronger selective pressures on calls across its range due to sexual selection in comparison with *P. thaul*.

Individuals of *B. ibitiguara* inhabit gallery forests along streams where breeding and oviposition occur (Nali & Prado, 2012; Nali *et al.*, 2023). Thus, call traits may have undergone ecological pressures imposed by the forest habitat. However, we found no evidence for the AAH in this species (Table 3). One possible explanation for this result is behavioural plasticity, in the form of call adjustment to the surrounding microhabitat. Previous research has shown that male tree frogs can adapt their calls to the microhabitat they use for vocalization, leading to variation in call traits (Ziegler *et al.*, 2011). Our quantitative assessment of forested areas, herein measured as percentage forest cover, was a step further compared to broad qualitative classifications commonly used for frogs (Ey & Fischer, 2009; Erdtmann & Lima, 2013; but see Goutte *et al.*, 2018), a procedure that is desirable because environmental selection on call traits might be stronger in forests, but relaxed or minimal in open habitats (Ryan *et al.*, 1990). Although the forested environment may have a minor role in shaping call diversification in this species (e.g. Castellano *et al.*, 2003; Penna & Moreno-Gómez, 2015; Goutte *et al.*, 2018), the contribution of call plasticity in the adaptation to microhabitats deserves further investigation (e.g. Ziegler *et al.*, 2018).

In more forested areas, male frogs emitted calls with higher frequencies, which was opposite to the AAH (Table 3). As these results are based on correlations, other factors may be causing this deviation from the AAH (Kalko, 1995; Sugiura *et al.*, 2006; Ey & Fischer, 2009). The short notes of the advertisement call convey a territorial/aggressive message in this species, and males lower their call frequencies to repel intruders and avoid physical combats (Nali & Prado, 2014a). Lower call frequencies are usually correlated with larger body size, which tend to be important in intra- and intersexual selection across frogs (Davies & Halliday, 1978; Asquith & Altig, 1990; Gingras *et al.*, 2013; Nali & Prado, 2014b; Reichert, 2014; Turin *et al.*, 2018). Due to their territorial behaviour, reproductive males distance themselves as much as possible within the gallery forests (Nali & Prado, 2012), resulting in more proximity in areas with less forest cover. Thus, we hypothesize that less gallery forest could lead to more intense competition among reproductive males for calling sites, oviposition sites and females in this species, which could result in the emission of calls with lower frequencies. Although further studies directly correlating male densities and call frequencies are needed, it again indicates the role of sexual selection in call variation of *B. ibitiguara.*

Evolutionary studies that integrate genotypes and phenotypes, and that employ methods to evaluate multiple mechanisms underlying population differentiation are crucial but still scarce, particularly in the megadiverse group of frogs (this study; Wilkins *et al.*, 2013; Warwick *et al.*, 2015). In organisms with resource defence or lek mating systems, which may show elaborate reproductive behaviours (Zina & Haddad, 2007; Nali & Prado, 2012; Miles & Fuxjager, 2018; Mitoyen *et al.*, 2019), genetic drift may play little role in morphological and behavioural evolution. To understand the evolution of acoustically oriented taxa, studies should rely on natural history observations allied with experiments to assess preferences for specific and variable signals and aggressive responses to them, as well as quantitative measurements of the environment, which formed the basis of our study (Endler, 1992; Ryan & Rand, 2003a,b; Ziegler *et al.*, 2011, 2018; Reichert & Gerhardt, 2013; Nali & Prado, 2014a; Goutte *et al.*, 2018; Nali *et al.*, 2022). While conducting so many procedures altogether is certainly a laborious task (Gerhardt, 2013), we emphasize that combining data helps elucidate the complex and yet poorly understood associations among the surrounding environment, behavioural phenotypes and sexual selection (Ey & Fischer, 2009; Erdtmann & Lima, 2013; Wilkins *et al.*, 2013).

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DATA AVAILABILITY

Most of the data underlying this article are available in the article and in its online Supporting Information. Any additional data will be shared on reasonable request to the corresponding author.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article on the publisher's website.

Tables S1–S8. Information on localities, sample sizes, call variables, voucher specimens, Kruskal–Wallis results and distance matrices.
Figures S1–S13. Representation of our morphometric and acoustic measurements, density plots of our canonical variate analyses, and multiple regression model diagnostics.
Boxplots. Boxplots of our acoustic and morphological variables separated by genetic clusters and populations.
Spreadsheet. Descriptive statistics and coefficients of variation for our variables separated by genetic clusters and populations.