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ORIGINAL ARTICLE

Hybridization despite elaborate courtship behavior and female choice in Neotropical tree frogs

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Abstract

The mechanisms of hybridization can be elucidated by analyzing genotypes as well as phenotypes that could act as premating barriers, as the reproductive interactions among heterospecifics can alter the evolutionary history of species. In frogs, hybrids typically occur among species that reproduce explosively (in dense aggregations) with few opportunities for mate selection but are rare in species with elaborate courtship behaviors that may prevent erroneous mating. Using 21 microsatellite markers, we examined hybridization in the prolonged-breeding tree frogs *Bokermannohyla ibitiguara* and *B. sazimai* sampled within a contact zone in the Brazilian savanna (72 tadpoles; 74 adults). We also compared acoustic and morphological data. We confirmed both parental species genetically; STRUCTURE results confirmed 14 hybrids, 11 of which were second-generation according to NEWHYBRIDS, all with intermediate values of genetic dissimilarities compared to the parentals. Morphological and acoustic analyses revealed that hybrids showed variable but not necessarily intermediate phenotypes. Moreover, 2 hybrids exhibited call types different from parentals. The reproduction of *B. ibitiguara* involves territorial and aggressive males, elaborate courtships with acoustic and tactile stimuli, choosy females, and opportunistic strategies. Our study uncovers a rare case of viable hybridization among closely related frogs with such a combination of complex courtship behaviors and mate choice. We discuss the likely directionality and mechanisms behind this phenomenon, and highlight the importance of investigating hybridization even in species that show elaborate reproduction and female choice to advance our understanding of animal diversification.

Key words: heterospecific mating, hybrid zone, Hylidae, microsatellites, prolonged-breeding

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INTRODUCTION

Hybridization, defined as reproductive events between members of genetically distinct populations (Barton & Hewitt 1985), is an evolutionary process that can promote adaptive introgression, adaptive genetic variation, and even speciation (Allendorf *et al*. 2001; Burke &

Arnold 2001; Mallet 2007; Abbott *et al*. 2013; Comeault & Matute 2018). However, hybrids fall along a fitness continuum, and evidence for both increased and decreased hybrid fitness has been gradually accumulating (Burke & Arnold 2001; Nolte & Tautz 2010; Dagilis *et al*. 2019). The context for positive and negative consequences of hybridization is complex because hybrids can arise either naturally or artificially, i.e., as a result of anthropogenic environmental change (Allendorf *et al*. 2001; Detwiler *et al*. 2005; Seehausen *et al*. 2008). Therefore, the persistent hybridization between species offers an opportunity to evaluate its consequences and the implications for ecology and evolution of organisms (Gröning & Hochkirch 2008; Zamudio *et al*. 2010; Abbot *et al*. 2013).

Increased availability of molecular methods has enabled assessments of hybridization even in non-model species (Sequeira *et al*. 2011; Abbott *et al*. 2013; Pereyra *et al*. 2016). However, because genetic incompatibility and morphological/behavioral divergences seem to have different evolutionary rates (Grant & Grant 1997; Lougheed *et al*. 2006), incorporating phenotypes into the analysis becomes crucial to understand mechanisms of heterospecific reproductive interactions that lead to hybridization. Often, differences between species' phenotypes, when properly recognized by their individuals, constitute good pre-mating barriers (Coyne & Orr 2004; Kozak *et al*. 2009), and heterospecific matings will arise in cases of incomplete recognition among closely related species (Spencer *et al*. 1986; Schaefer & Ruxton 2015), leading to hybridization.

Different breeding contexts may facilitate or preclude heterospecific matings. For instance, anuran amphibians show temporal mating strategies along a continuum ranging from explosive to prolonged breeding with associated behaviors, in which female mate selection, individual recognition, and male territoriality tend to progressively increase from the former to the latter (Wells 1977a, 2007). Frogs are well known to erroneously mate in situations of explosive breeding; males and females form dense aggregations, males can actively search and grab females indiscriminately and may dislodge other males already in amplexus, leaving little opportunity for female choice (Brown 1977; Ficetola & De Bernardi 2005; Pearl *et al*. 2005; but see cryptic female choice in Reyer *et al*. 1999; Hettyey *et al*. 2009). This has been well documented for true toads, which typically have explosive breeding events with the aforementioned behaviors (Wells 1977a; Sullivan 1986; Haddad *et al*. 1990, 2007; Bezerra & Cascon 2011; Thomé *et al*. 2012). In contrast, hybridization is much rarer in frogs with prolonged breeding seasons (Gerhardt *et al*. 1994; Simões *et al*. 2012), especially those with complex courtships and calls, in which close contact and female choice are believed to act as successful premating isolation mechanisms (Wells 1977a, 2007). In those systems, females often rely on different traits to select potential partners (Ryan 1980; Nali & Prado 2014a; Moreno-Gómez *et al*. 2015). Male calls are among the most important signals for mate and individual recognition (Lougheed *et al*. 2006; Turin *et al*. 2018), and call characteristics are often related to morphological advantageous features, such as large body size (McClelland *et al*. 1996; Morais *et al*. 2012; Nali & Prado 2014a) as well as male genetic quality (Welch *et al*. 1998; Forsman & Hagman 2006).

Neotropical frogs comprise most of the species with known complex courtship behaviors (Montanarin *et al*. 2011; Nali & Prado 2012; Centeno *et al*. 2015; Faggioni *et al*. 2017; Nali *et al*. 2021;). However, they are underrepresented in the study of genetic hybridization, and few studies have employed an integrative framework that explicitly includes genotypes, morphological features and behavioral traits, such as calls (Sequeira *et al*. 2011; Vargas-Salinas & Amézquita 2013; Pereyra *et al*. 2016). Here, we investigated hybridization between 2 tree frog species in the genus *Bokermannohyla* that co-occur in the Brazilian savanna, southeastern Brazil. Morphological and molecular phylogenetic analyses suggest that both species belong to different species groups (Faivovich *et al*. 2005). *Bokermannohyla ibitiguara* (Cardoso 1983) belongs to the *Bokermannohyla pseudopseudis* species group (Faivovich *et al*. 2005) and is a prolonged breeder, with complex courtship involving acoustic and tactile stimuli between males and females, territorial and aggressive males, choosy females, and complex calling behaviors that vary according to the social context (Nali & Prado 2012, 2014a,b; Turin *et al*. 2018; Nali *et al*. 2021). *Bokermannohyla sazimai* (Cardoso & Andrade 1982) belongs to the *Bokermannohyla circumdata* species group (Faivovich *et al*. 2005) and is also a prolonged breeder, although reproductive behaviors for this species have not been studied in detail (Cardoso & Andrade 1982; Haddad *et al*. 1988). Males of both species emit advertisement calls that are similar in general structure, i.e., a composite call with a sequence of long pulsed notes that are followed by a sequence of short notes (Carvalho *et al*. 2012; Nali & Prado 2014a; Turin *et al*. 2018).

Using variable microsatellite markers (Nali *et al*. 2014), we first show that hybridization occurs in this system, a rare case in frog species with such a combination of elaborate reproductive behaviors; second, we provide data on morphology and calls, which combined with

natural history and behavioral descriptions for the species allowed us to discuss possible mechanisms of their reproductive interactions. Our results highlight the importance of investigating hybridization even in species that show complex courtships and female choice, which has potential implications for our understanding of micro- and macroevolutionary processes.

MATERIALS AND METHODS

Study site

The Serra da Canastra mountain range, in southeastern Brazil, is a region with a markedly seasonal climate and originally covered by Cerrado vegetation, patches of semideciduous forest, gallery forests along streams, and grassland fields at higher elevations up to 1500 m (Dietz 1984; Queirolo & Motta-Junior 2007). Our focal taxa co-occur and may breed in the same streams in the Serra da Canastra National Park (SCNP; Haddad *et al*. 1988). The SCNP is a strictly protected area consisting mostly of a topographically homogeneous high-elevation plateau of ca. 700 km^2 (the "Chapadão da Canastra", Medeiros & Fiedler 2004; MMA/IBAMA 2005; Fig. 1). *Bokermannohyla ibitiguara* is abundant in streams throughout the SCNP, forming a single genetic cluster inside the park, but also occurs in close localities throughout the Serra da Canastra range, in altitudes ranging from 700 to 1400 m (Nali & Prado 2012; Nali *et al*. 2020). Variable degrees of riparian forests are found across its habitats, with about 1 to 30% forest cover within a 500-meter radius (Nali *et al*. 2020). *Bokermannohyla sazimai* was first described from the SCNP (Cardoso & Andrade 1982) and has a much broader geographic range compared to *B. ibitiguara*, as it has been found up to 300 km north of the SCNP; *B. sazimai* also occurs along riparian environments at similar altitudes compared to *B. ibitiguara* (ca. 650 to 1400 m; Fig. 1; Cardoso & Andrade 1982; Carvalho & Giaretta 2013; this study).

Field sampling

We focused our sampling at the SCNP, a topographically homogeneous contact zone between both species. During 2013 and 2014, we sampled 8 streams in the SCNP, with an average straight-line distance of 21.3 km among them (Table S1 in supporting information online; Fig. 1), and classified individuals as *B. sazimai* or *B. ibitiguara* according to morphology and calls. We recorded male calls at approximately 1 m from the individual 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 and hour of recording were registered.

We collected a total of 146 tissue samples from adults (liver, muscle or toe clip) and from tadpoles (tail clip) and preserved them in absolute ethanol. Adults and tadpoles were euthanized with a 10% lidocaine solution and fixed in formalin; adults were preserved in 70% ethanol and tadpoles were preserved in 5% formalin (McDiarmid 1994). Tadpoles with different body sizes were sampled in each stream, reducing the probability of collecting tadpoles from a single clutch (Nali *et al*. 2020). Specimens and tissues were deposited in the Célio F. B. Haddad Amphibian Collection, Universidade Estadual Paulista "Júlio de Mesquita Filho", Rio Claro, state of São Paulo, Brazil (Table S2).

Laboratory protocols and microsatellite data

All individuals were genotyped at 21 microsatellite loci previously developed and optimized for *B. ibitiguara* from the Serra da Canastra mountain range (Nali *et al*. 2014). All markers successfully amplified for *B. sazimai* DNA under the same conditions. We extracted whole genomic DNA with DNeasy extraction columns (Qiagen, Valencia, CA, USA) following the manufacturer's protocols. PCR profiles consisted of an initial denaturation step (94°C, 5 min) followed by 35 cycles of 1 min at 94°C, 1 min at primer-specific annealing temperatures (Nali *et al*. 2014), and 1 min at 72°C, followed by a final extension (75°C, 5 min). We performed PCRs in 10 μL reaction volumes, with 1 μL of template DNA (1–10 ng), 1X buffer, 1.5 mM $MgCl_2$, 0.1 μg/μL bovine serum albumin, 0.4 μM dNTP, 0.1 μM of the forward and reverse primers, 0.3 μM of universal dye-labeled primer, and 0.25 U Taq polymerase. Each forward primer contained a 20 bp tag on the 5′ end (Nali *et al*. 2014) to allow hybridization with the fluorescently tagged third universal primer (NED, PET, VIC or 6-FAM). After amplification, we combined 1 μL of individual PCR products from up to 4 different loci, diluted with 18.85 μL Hi-Di formamide and 0.15 μL GeneScan-500 LIZ, and ran the pooled samples on a 3730 ABI Prism Genetic Analyzer (Applied Biosystems, Foster City, CA) at the Cornell Biotechnology Resource Center.

We used GENEMARKER v. 2.4.0 (SoftGenetics LLC, State College, PA) to analyze the genotype profiles of all individuals. Because each species revealed to form a single genetic cluster in that region (Nali *et al*. 2020; our results), each locus was tested for Hardy–Weinberg equilibrium separately for *B. ibitiguara* and *B. sazimai* using ARLEQUIN v. 3.5 (Excoffier & Lischer 2010).

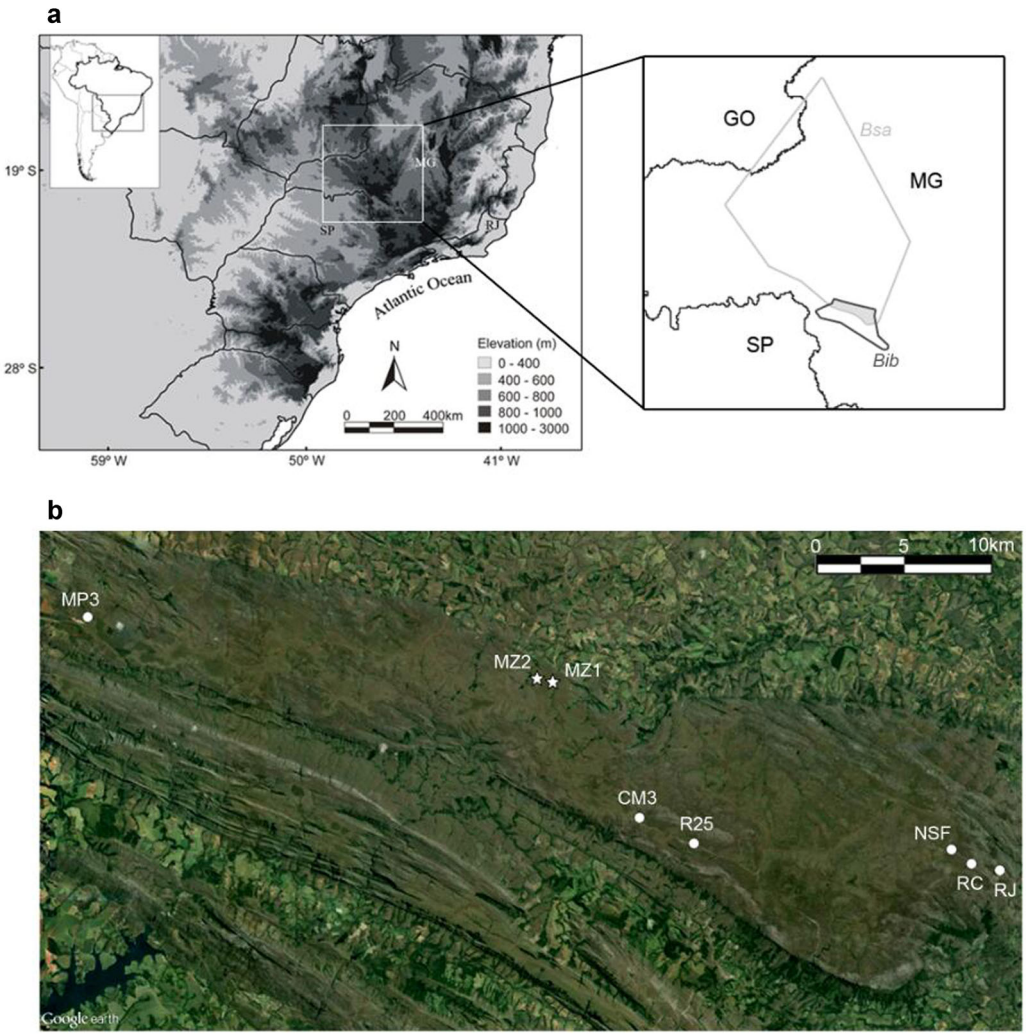

Figure 1 Geographic ranges and sampling sites of *Bokermannohyla ibitiguara* and *B. sazimai* in southeastern Brazil. (a) General locality and estimated ranges of *B. ibitiguara* (Bib) based on Haddad *et al*. (1988), Feio (2002) and Nali *et al*. (2020), and of *B. sazimai* (Bsa) based on Carvalho & Giaretta (2013). The contact hybrid zone is marked in gray. Acronyms are Brazilian states: GO = Goiás, MG = Minas Gerais and SP = São Paulo. (b) Eight sampling localities where we found mostly *B. ibitiguara* (dots) and *B. sazimai* (stars) in the hybrid zone, within the Serra da Canastra National Park (full names and coordinates in supporting information). Notice the flat plateau where the sampled populations are distributed, which is coincident with a single genetic cluster for *B. ibitiguara* (Nali *et al*. 2020).

Statistical significance was obtained from 1 million steps of Monte Carlo Markov Chains (MCMC) after 100 000 dememorization steps. We also tested for linkage disequilibrium across all pairs of loci using GENEPOP v. 4.0.9 (Rousset 2008), with 10 000 dememorization steps, and 100 batches with 5000 iterations each for each species separately. Finally, given that we optimized and developed microsatellites originally for *B. ibitiguara* sampled in that region (Nali *et al*. 2014) and not for *B. sazimai*, we checked for the presence of null alleles, scoring error due to stuttering and large allele dropout in the 2 populations containing *B. sazimai* (MZ1 and MZ2) in MICROCHECKER v. 2.2.3 (Van Oosterhout *et al*. 2004).

Genetic analyses and identification of hybrids

Bayesian analyses and resulting intermediate coefficients of membership (Q) have been used to identify hybrids between closely related species or divergent lineages (Simões *et al*. 2012; Johnson *et al*. 2015;

Malukiewicz *et al*. 2015). Therefore, we first used the software STRUCTURE v. 2.3.4 (Markov Chain Monte Carlo method; Pritchard *et al*. 2000; Falush *et al*. 2003) to determine the number of genetic clusters (K) in our sample and genetic discontinuities between collected individuals, with their respective coefficients of membership. We compared K values from 1 to 9 using 10 iterations with 200 000 burn-in steps, followed by 1 million steps, assuming an admixture model and correlated allele frequencies. The presence of genetic structure was assumed by observing the highest peak for K values in different plots of delta-K, as obtained in STRUCTURE HARVESTER (Earl & VonHoldt 2012). The cluster data were permutated for the selected K value using CLUMPAK with standard parameters (Kopelman *et al*. 2015), and bar plots were visualized in Excel. We considered individuals with $0.1 < Q < 0.9$ as potential hybrids (Vähä & Primmer 2006; Johnson *et al*. 2015). We validated the 0.9 Q-score threshold by simulating 120 genotypes (30 each F1, F2, *B. ibitiguara* backcross and *B. sazimai* backcross) using HYBRIDLAB v. 1.0 (Nielsen *et al*. 2006). We then ran a STRUCTURE analysis with these 120 simulated hybrids plus 30 randomly selected parental genotypes from *B. sazimai* and 30 from *B. ibitiguara* (5 random individuals per stream), assuming $K = 2$ and the aforementioned parameters.

Additionally, we classified individuals in the STRUCTURE run with $Q > 0.9$ as pure *B. ibitiguara* or pure *B. sazimai* and used this classification as input for the software NEWHYBRIDS v. 1.1, which can differentiate hybrid classes when using more than 20 genetic markers (Anderson & Thompson 2002). We performed 5 runs with 50 000 burn-ins and 200 000 following sweeps and averaged the probability of each individual belonging to one of the following classes: pure *B. ibitiguara*, pure *B. sazimai*, first generation hybrid (F1), second generation hybrid (F2), backcross of F1 with *B. ibitiguara* or backcross of F1 with *B. sazimai* (Anderson & Thompson 2002). Individuals were assigned to hybrid classes for which probability >0.9 (e.g., Johnson *et al*. 2015).

We calculated squared genetic distances between pairs of samples in GenAlEx (Peakall & Smouse 2012) and used this matrix in a principal coordinates analysis (PCoA) to visualize genetic dissimilarities of species and identify hybrids. This matrix was also used to create a neighbor-joining tree in R (R Core Team 2019) using the package *ape* (Paradis *et al*. 2004).

The hybrids were checked for the presence of diagnostic alleles identified in the parental *B. sazimai* or *B. ibitiguara* individuals. Private alleles were first defined as those present in one parental species only, with a frequency higher than 5% to prevent sampling and/or genotyping errors (Oliveira *et al*. 2008). In a second approach, we considered hybrids as one group and *B. ibitiguara* and *B. sazimai* combined as another group and checked for the presence of private alleles for the hybrids only. In this case, some alleles were considered private with a frequency even lower than 5% (lowest value = 3.6%) due to the few hybrids found.

Analyzing phenotypes: morphology and calls

We incorporated data on morphology and male calls for both focal species to investigate their phenotypic similarities, which might explain heterospecific matings leading to hybridization. We measured 12 morphometric traits from each adult specimen to the nearest 0.01 mm using a digital caliper (Fig. S1 in supporting information online): 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). Each specimen was measured without a priori knowledge of collection locality, and every measurement was taken from the same side across all specimens.

We analyzed calls using Raven Pro 1.4 (Cornell Lab of Ornithology, Ithaca, NY, USA) with FFT (Fast Fourier Transformation) = 512 points, brightness = 70 and contrast = 70 (Nali & Prado 2014a). Fourteen call variables were measured: 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 pulse rate); 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); 14) number of short notes per sequence (SN/seq). Duration was measured in seconds (s), frequency in Hertz (Hz), and pulse rate was measured in 0.5 s counting from the onset of each long note (Nali & Prado 2014a). Variables 11–14 were measured within a segment of 1 minute containing short and long notes. We measured 5 long notes, 5 short notes and up to 5 sequences of short notes per individual and calculated the individual mean for each variable. To avoid potential biases, a single researcher

(RCN) measured the voucher specimens and analyzed the calls.

A number of analyses were implemented to compare phenotypic features among our groups. Mean body sizes (SVL) of the 3 groups (*B. ibitiguara*, *B. sazimai* and recognized hybrids) were compared with a Kruskal–Wallis test followed by post hoc comparisons of Student–Newman–Keuls in the software STATISTICA (StatSoft 2011). The 12 morphometric values were log-transformed and used in principal component analyses (PCA) in STATISTICA for visualization. The first component (>90% of variation, see results) was then compared among the 3 groups using the Kruskal–Wallis test followed by post hoc comparisons of Student–Newman–Keuls. Additionally, we ran a multiple analysis of variance (MANOVA) combining all 12 morphometric variables (log-transformed) to check for significant differences in morphology between the 3 groups in R (R Core Team 2019).

Call parameters in frogs may be influenced by air temperature and body size, which is known for *B. ibitiguara* (Nali & Prado 2014a; Turin *et al*. 2018). To control for temperature, all values were adjusted to the average temperature of 18.9°C across samples, based on the significant overall regression coefficients for each parameter vs. air temperature (e.g., Pröhl *et al*. 2007). We then used the same method to control for body size, adjusting the dataset to the average SVL of 39.67 mm across samples. We used this fully adjusted dataset in call analyses. The call variables were log-transformed and used in a PCA in STATISTICA for visualization. Due to a smaller sample size for hybrid calls, statistical comparisons were only possible between the 2 parental species. Given that the first component retained less than 40% of total variation (see results), we ran linear regression models in R comparing every acoustic variable between *B. ibitiguara* and *B. sazimai* instead of using the values of the first component like we did for morphometric variables.

RESULTS

Microsatellite data

All loci were highly polymorphic across our samples (average = 19.2 alleles; range = 11–42) and yielded less than 2% of missing data. Most loci were under HWE for each species, except for 7 loci in *B. ibitiguara* and 6 in *B. sazimai* (Bonferroni $P < 0.0024$). Only 1 pair of loci showed linkage disequilibrium in *B. ibitiguara* (Bi3003/Bi3836) and only 2 pairs in *B. sazimai* (Bi179/Bi1050, Bi1032/Bi1050; Bonferroni $P < 0.00024$) among 210 possible pairs. We found no evidence of large allele dropout or scoring errors due to stuttering in either population containing *B. sazimai* (MZ1 and MZ2). Only 7 loci showed evidence of null alleles in MZ1 and only 5 in MZ2, which is a common pattern in animals and have been shown not to significantly bias genetic population assignment analyses (Carlsson 2008). Due to the consistency of our markers, we included all of them in the downstream analyses. The genotypes of all individuals are deposited as supporting information online.

Genetic analyses and identification of hybrids

The Bayesian STRUCTURE analysis using the simulated genotypes in HYBRIDLAB showed that the threshold value of coefficient of membership (Q) = 0.9 used here appropriately identified hybrids; 53 of the 60 simulated parental individuals were assigned $Q > 0.9$, and 59 of the 60 F1 + F2 hybrids were assigned $0.1 < Q < 0.9$. Moreover, the average Q for parental *B. ibitiguara* and parental *B. sazimai* were above 0.9 (0.95 and 0.93, respectively). The Bayesian assignment analysis including real genotypes of *B. ibitiguara* and *B. sazimai* revealed 2 major genetic clusters compatible with the 2 species in this study (K = 2; Fig. 2; Fig. S2). In the field, 108 individuals were identified as *B. ibitiguara* and 38 as *B. sazimai*, but only 99 individuals showed coefficients of membership $(Q) > 0.9$ for *B. ibitiguara* and 33 for *B. sazimai*. Fourteen individuals showed intermediate coefficients ($0.1 < Q < 0.9$) consistent with a hybrid status (Fig. 2; Table 1; Fig. S3). Using NEWHYBRIDS, we found that 11 of the abovementioned 14 individuals were F2 hybrids, assigned with a probability >0.9 (average = 0.971; Table 1).

The first and second axes of our PCoA explained ca. 9.1% of the genetic variation. We found some genetic overlap between parental species, and the hybrids identified in the previous analyses occupied a central position in the overlap region of the PCoA (Fig. 3a). Our neighbor-joining tree revealed a separation of *B. ibitiguara* from *B. sazimai*, but with short branch lengths, and assigned hybrids were distributed throughout the tree (Fig. 3b).

Our analyses of private alleles revealed that 9 hybrids had alleles present in *B. ibitiguara* only (11 alleles diagnostic of *B. ibitiguara* across 8 loci), while a single individual had one allele at a single locus that is diagnostic of *B. sazimai* (Table S3 in supporting information online). When we considered hybrids as one group and *B. ibitiguara* and *B. sazimai* combined as another group, 9 hybrids showed private alleles, i.e., alleles absent from either *B. sazimai* or *B. ibitiguara* (10 different alleles across 10 loci; Table 1; Table S4 in supporting information online).

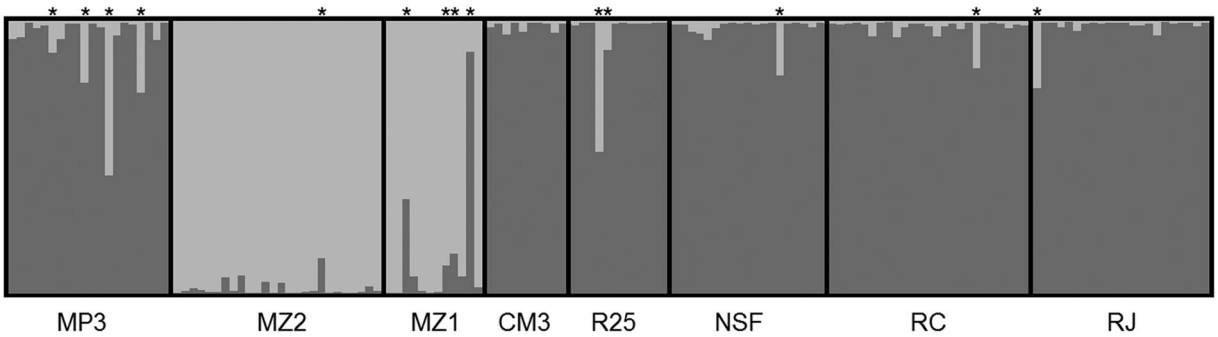

Figure 2 Bayesian STRUCTURE analysis for 8 sampled streams (ordered from west to east, see Fig. 1b) within the Serra da Canastra National Park. We found 2 genetic clusters (K = 2) representing 2 parental species (dark gray: *Bokermannohyla ibitiguara*; light gray: *B. sazimai*). Asterisks represent hybrid individuals with intermediate cluster assignments ($0.1 < Q < 0.9$).

Table 1 Hybrid individuals between *Bokermannohyla ibitiguara* (Bib) and *B. sazimai* (Bsa) identified in this study, with values for STRUCTURE (coefficient of membership; Q) and NEWHYBRIDS (probability of belonging to each category). We also show the loci with alleles absent from Bsa and Bib (specific alleles in supporting information online). Samples 17326, 18117 and 18172 were classified as hybrids by STRUCTURE only. The order of hybrids follows that of Fig. 2

	STRUCTURE		NEWHYBRIDS						
CFBH-T	Bib	Bsa	Pure Bib	Pure Bsa	First gen hybrid (F1)	Second gen hybrid (F2)	Backcross Bib with F1	Backcross Bsa with F1	Loci with private alleles
18117[T]	0.884	0.116	0.293	0.000	0.000	0.700	0.007	0.000	Bi1397, Bi3202, Bi3629[h]
18121[A]	0.775	0.225	0.003	0.000	0.000	0.996	0.001	0.000	Bi3629
18124[T]	0.435	0.565	0.016	0.000	0.000	0.982	0.001	0.000	Bi1122[h]
18128[T]	0.737	0.263	0.012	0.000	0.000	0.981	0.007	0.000	Bi383
18192[T]	0.131	0.869	0.000	0.004	0.000	0.995	0.000	0.001	Bi179
18164[A]	0.347	0.653	0.003	0.000	0.000	0.993	0.004	0.000	-
18169[A]	0.104	0.896	0.000	0.001	0.000	0.994	0.001	0.004	-
18170[A]	0.148	0.852	0.000	0.016	0.000	0.980	0.001	0.002	-
18172[A]	0.889	0.111	0.719	0.000	0.000	0.273	0.008	0.000	Bi1521[h]
17325[A]	0.521	0.479	0.010	0.000	0.011	0.953	0.024	0.001	Bi383
17326[A]	0.896	0.104	0.203	0.000	0.000	0.668	0.129	0.000	-
17261[T]	0.802	0.198	0.085	0.000	0.000	0.901	0.014	0.000	-
17290[T]	0.829	0.171	0.036	0.000	0.000	0.928	0.036	0.000	Bi4144
17242[A]	0.755	0.245	0.020	0.000	0.000	0.980	0.000	0.000	Bi639, Bi1050[h]

CFBH-T = tissue number at Célio F. B. Haddad Amphibian Collection, Rio Claro, São Paulo, Brazil; [h] homozygote at the specified locus; [T] tadpole sample; [A] adult sample.

Phenotypes: morphology and calls

We collected and measured the morphometric characteristics of 16 adult specimens of *B. sazimai* (1 female), 41 of *B. ibitiguara* (1 female), and 8 hybrids (no females; Table S5 in supporting information online). Four adult hybrids were morphologically more similar to *B. ibitiguara*, and the other 4 to *B. sazimai;* we observed that hybrids had numerically intermediate values for all 12 variables (Table S5). We recorded and measured call traits of 7 *B. sazimai* males, 38 *B. ibitiguara* males, and 4 hybrids (Table S6 in supporting information online; air temperatures = 17 to 22°C). Two of these hybrids emitted calls that were similar in general structure to the parental species, and we observed that they had numerically

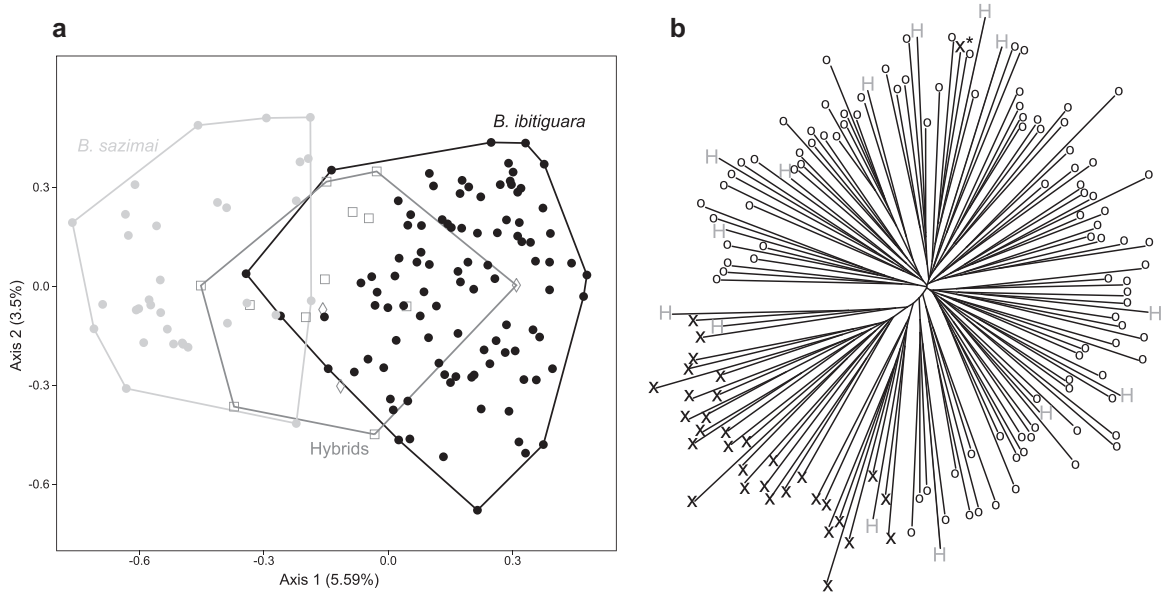

Figure 3 Genetic dissimilarities among *Bokermannohyla ibitiguara*, *B. sazimai* and identified hybrids based on squared genetic distances between pairs of samples. (a) Principal coordinates analysis with convex hulls delimiting parental species and overlapping hybrids. The more conservative estimates of hybrids (squares) were identified by STRUCTURE and NEWHYBRIDS, and the more lenient estimates of hybrids (diamonds), only by STRUCTURE. (b) Unrooted neighbor-joining tree showing a clear but not very pronounced separation between *B. ibitiguara* (o) and *B. sazimai* (x), except for a single *B. sazimai* individual (x*). Hybrids (H) were distributed throughout the tree.

intermediate values for 10 out of the 14 call variables (Table S6). However, the other 2 hybrids emitted a different type of short note, absent from both *B. ibitiguara* and *B. sazimai* calls, which sounded more like whines, with irregular pulse structure (Table S7, Fig. S4 and sound file in supporting information online). The raw phenotypic data of all individuals are deposited as supporting information online.

Body sizes of *B. ibitiguara* were larger (mean SVL = 39.6 mm) compared to those of *B. sazimai* (mean SVL = 31.8 mm), and body sizes of hybrids were intermediate (mean SVL = 34.9 mm) with a higher variance; SVLs were significantly different (Kruskal–Wallis $H = 33.6$; $P < 0.0001$), with post hoc tests significant between *B. ibitiguara* vs. hybrids and *B. ibitiguara* vs. *B. sazimai* (Fig. S5 in supporting information online). Our MANOVA showed that the 3 groups were significantly different regarding morphology ($F_{2,64} = 3.77$, $P < 0.001$). The first principal component of the PCA accounted for ca. 91% of the morphological variation (Fig. 4a), and it was mostly related with SVL, HL, HW, FoL and TiL. Our Kruskal–Wallis tests showed significant differences in morphology for PC1 ($H = 37.81$, $P < 0.0001$); post hoc differences were seen between *B. ibitiguara* vs. hybrids ($P = 0.0213$) and *B. ibitiguara* vs. *B. sazimai* ($P < 0.0001$).

The first and second principal components of the PCA accounted for ca. 51% of the acoustic variation (Fig. 4b). The first component (33.16% explanation) was predominantly related with dominant and minimum frequencies, and the second component (17.84% explanation) was mostly related with SN/min, SNseq dur and SN/seq. Only the 2 hybrids with calls that were similar to the parentals were included in the PCA, since the other 2 hybrids with different call types prevented the measurement of all needed variables, resulting in missing data that is not allowed in this analysis. Our linear regression analyses indicated that, compared to *B. ibitiguara*, *B. sazimai* emitted calls with higher minimum and dominant frequencies, shorter duration of the long note, and more long notes per minute (Table S8).

Altogether, we observed a separation of *B. ibitiguara* and *B. sazimai* according to both morphological and call characteristics, albeit less for calls, and the hybrids were distributed across the phenotypic space of both parental species, also less for calls (Fig. 4).

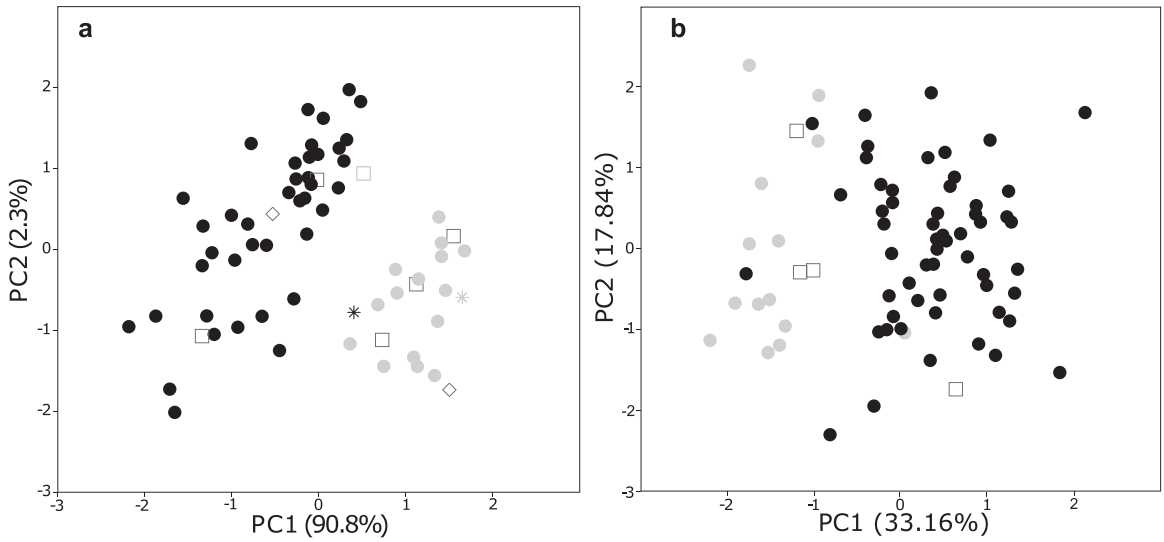

Figure 4 Principal component analyses with adults of *Bokermannohyla ibitiguara* (black dots), *B. sazimai* (gray dots), and putative hybrids (squares are conservative hybrids, identified by STRUCTURE and NEWHYBRIDS; diamonds are lenient hybrids, identified only by STRUCTURE) using (a) morphological variables, including 2 females (asterisks), and (b) acoustic variables (only males).

DISCUSSION

Genetic evidence of hybrids

Our combined genetic analyses confirmed the existence of hybrids between *Bokermannohyla ibitiguara* and *B. sazimai* within the Serra da Canastra National Park, where both species exhibit habitat overlap (Cardoso & Andrade 1982; Haddad *et al*. 1988; Nali *et al*. 2020). The occurrence of hybrids was relatively uncommon, with only 7.5% or 9.5% of the total individuals sampled, depending on the hybrid assignment method. Six tadpoles and 8 adult males were hybrids and were collected in 7 out of the 8 sampled streams. Cytogenetic studies indicate that hybridization events are possible between these species for 2 main reasons. First, both species have the same karyotypes, which seems to be highly conserved across genera of this subfamily ($2N = 24$); second, they do not have heteromorphic sex chromosomes (Catroli *et al*. 2011; Ferro *et al*. 2018), which are potential promoters of hybrid incompatibilities (Meiklejohn & Tao 2010; Austin *et al*. 2011; Johnson & Lachance 2012). Although a combined genetic sampling within and outside the hybrid zones for both species is desirable, to the best of our knowledge this is the first genetic assessment of hybridization in the speciose Neotropical subfamily Cophomantinae (Frost 2021).

Eleven of the 14 assigned hybrids were classified as being from the second generation (Table 1), which suggests sustained hybridization in this system. Due to the higher recombination of parental and hybrid genomes, F2 hybrids are expected to be more variable than F1 hybrids; our hybrids had genetic and morphological characteristics that visually overlapped with those of parental species, indicating high variability (Fig. 3; Fig. 4; Fig. S3 in supporting information online). The 2 hybrids with calls that are different from the parental species also corroborate such variability (see supporting information online). Testes morphology has been used as an indicative of male fertility in frogs (e.g., Byrne *et al*. 2002; Rudin-Bitterli *et al*. 2020), and testes of adult hybrids and parental species were similar in morphology and mass (RC Nali, unpubl. data: testes mass *B. sazimai* = 0.004 to 0.016 g; *B. ibitiguara* = 0.006 to 0.018 g; hybrids = 0.004 to 0.024 g). Thus, specifically at this level, we do not have any evidence for hybrid infertility for males (Wünsch & Pfennig 2013), although fertilization experiments are crucial to confirm it. Outbreeding depression (i.e., when hybrid individuals have reduced fitness compared to the parentals) is less likely to occur when genetic divergence is not pronounced across species (Allendorf *et al*. 2001; Fig. 3). The most comprehensible amphibian molecular phylogeny estimated a divergence time between these 2 species of less than 30 mya (Jetz & Pyron 2018), but specific phylogenies and accurate estimates of genetic divergence are unknown for the genus. Further studies would help to rule out any selection against backcrosses of hybrids and parentals in this

system (e.g., Kahilainen *et al*. 2011), or whether this is an old hybrid zone that causes backcrosses and later generation hybrids to be pooled together, although NEWHYBRIDS seems to correctly assign classes when using more than 20 markers (Anderson & Thompson 2002).

Phenotypic comparisons and mating aspects

In general, heterospecific matings in frogs are known to occur among explosive breeders, in which the reproductive season is short, individuals tend to form dense aggregations of males and females after heavy rains, and there is little or no territorial defense, female selection, or complex courtships (Wells 1977a, 2007; Sullivan 1986; Azevedo *et al*. 2003; Sequeira *et al*. 2011; Hettyey *et al*. 2014). High mate densities also seem to play a role in heterospecific matings in other taxa, such as insects (Hochkirch *et al*. 2007; Matute 2014). In contrast, in prolonged breeders, females arrive asynchronously at breeding sites and choose from available calling males, which typically defend a territory and may engage in courtships that could act as premating barriers and hamper hybridization (Wells 1977a, 2007). Nonetheless, we found hybrids not only between prolonged breeders (e.g., Gerhardt *et al*. 1994), but also in a scenario where mating is preceded by an elaborate courtship for at least one of the species involved (Nali & Prado 2012; Nali *et al*. 2021). Simões *et al*. (2012) found hybrids in the Brazilian Amazon between *Allobates hodli* Simões *et al*. 2010, and *Allobates femoralis* (Boulenger, 1884), a species with a complex courtship, although observed in a different population (Montanarin *et al*. 2011). Recent molecular evidence suggests that the nominal *A. femoralis* includes many separated lineages and at least 2 cryptic species in the Amazon (Grant *et al*. 2017; Motta *et al*. 2018). Thus, ours is one of the first cases of hybridization between frog species with such a combination of complex courtship behaviors and female choice (Nali & Prado 2012; Nali *et al*. 2021).

Details of courtships are still unknown for most *Bokermannohyla* species, including *B. sazimai*, but recent studies have shown that complex courtships within this genus are more common than previously thought (Nali & Prado 2012; Lima *et al*. 2014; Centeno *et al*. 2015; Zornosa-Torres & Toledo 2019). The courtship of *B. ibitiguara* involves acoustic and tactile stimuli between males and females, courtship calls that are modifications of advertisement calls, and inspection of nests by the females followed either by mate acceptance or rejection (Nali & Prado 2012; Nali *et al*. 2021). Males aggressively defend territories and oviposition sites employing calls and physical combats (Nali & Prado 2012, 2014a,b). The strong selection of males by females likely leads to differential pressures on call characteristics (Nali & Prado 2012, 2014a; Turin *et al*. 2018; Nali *et al*. 2021) along the whole species range (Nali 2016). Considering this scenario, our results suggest that hybridization occurs not due to simple misidentification with no opportunity for female choice, such as in "mating balls" of explosively breeding species (Sequeira *et al*. 2011; Pereyra *et al*. 2016); rather, it may occur throughout the rainy season, when males call and defend territories to attract females. We hypothesize that hybridization in this system passes a presumably high selective behavioral filter, possibly with the occurrence of misdirected courtships (Taylor *et al*. 2017). Since males of *B. ibitiguara* are much more abundant at the reproductive sites compared to females, i.e., there is a highly male-biased operational sex-ratio (see Nali & Prado 2012; Nali 2016; this study), males of this species may try to engage in courtships with females of *B. sazimai* when present. Hybridization was associated with differential mate densities in 2 explosively breeding spadefoot toads (Pfennig & Simovich 2002), thus scarcity of conspecific mates might also be a selective pressure promoting hybridization in prolonged-breeding species with elaborate behaviors (Wilson & Hendrick 1982). Hence, this mechanism deserves further investigation in this system.

As we observed short notes in 2 hybrids that are different and unique compared to the notes emitted by the parental species and similar calls to the parentals in 2 other hybrids, our data agrees with the idea that hybrid phenotypes are not always intermediate but rather different and sometimes unique phenotypes (Campton 1987; Allendorf *et al*. 2001). Advertisement calls of hybrid frogs may be either different (Bogart 1980) or very similar compared to parental species (Haddad *et al*. 1994; Vargas-Salinas & Amézquita 2013). We found evidence for both patterns in the same system even with a small sample size, corroborating that incomplete prezygotic mechanisms are in place. Regarding note emission by the parentals, we found that long notes were emitted more frequently by males of *B. sazimai* than *B. ibitiguara*. Detailed acoustic studies showed that long notes are specifically directed to females in *B. ibitiguara*, with males increasing emission of long notes in the presence of females (Nali & Prado 2014a; Nali *et al*. 2021). Preference for heterospecific characteristics during mate choice is rather uncommon but known for other animal taxa (Jones & Hunter 1998; McLennan & Ryan 2008; Sato *et al*. 2014). We suggest that females of *B. ibitiguara* could be attracted to the higher repetition rate of long notes emitted by males of *B.*

sazimai, which could be elucidated with controlled playback experiments (e.g., Burke & Murphy 2007).

Even when females are misguided towards heterospecific male calls, close contact between the sexes can cause the interaction to terminate prematurely (Wong & Candolin 2005; Gröning & Hochkirch 2008; Brunetti *et al*. 2015), especially when females are choosy and engage in complex courtships, as observed for *B. ibitiguara* (Nali & Prado 2012; Nali *et al*. 2021). However, heterospecific matings can be advantageous for either sex in certain contexts (Schlupp *et al*. 1994; Reyer 2008). We hypothesize that females of *B. sazimai* could be attracted to males of the larger species (*B. ibitiguara*) and be favored, because larger males might defend better territories due to fighting capabilities (Wells 1977b; Berec 2017); indeed fights have already been observed among *B. ibitiguara* males (Nali & Prado 2012, 2014a). Alternatively, circumvention of female choice could occur due to the presence of satellite behavior (Wirtz 1999; Stewart *et al*. 2017), an opportunistic strategy in which non-calling males that do not participate in the courtship steal females from a courting male (Wells 2007). As a consequence, males and females of different species could engage in heterospecific matings (Stewart *et al*. 2017). Satellite behavior is known for *B. ibitiguara* and is a common strategy in many prolonged-breeding frogs (Wells 2007; Nali & Prado 2012).

Although we found a few private alleles in hybrids, we did not observe any evidence of genetic erosion or disruption of parentals (Allendorf *et al*. 2001; Malukiewicz *et al*. 2015). Other studies with frogs have found hybridization mostly with promiscuous and explosive breeding, or else with no complex courtships with acoustic and tactile stimuli between males and females in close contact (e.g., Gerhardt *et al*. 1994; Pereyra *et al*. 2016). Our results open interesting avenues of investigation for this and other systems with complex reproductive behaviors and mate choice, especially in the Neotropics. We emphasize that employing an integrative framework with genetics, morphology and behavior will help uncover the roles of hybridization in the diversification of lineages (Montanarin *et al*. 2011). Furthermore, as we found sustained hybridization, our results also impact the construction of molecular frog phylogenies (Pyron & Wiens 2011; Duellman *et al*. 2016; Grant *et al*. 2017; Jetz & Pyron 2018). Because they usually do not take into account nondeleterious hybridization events, the resulting topologies can be questioned depending on the frequency of hybrids across taxa, including those with elaborate reproductive behaviors. We recommend that further research on hybridization should be conducted across animal taxa, with the extended possibility of using mitochondrial DNA to confirm the directionality of hybridization (Sequeira *et al*. 2011; Vanhaecke *et al*. 2012), and modern computational tools that account for that phenomenon (Elworth *et al*. 2019).

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SUPPLEMENTARY MATERIALS

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Table S1 Sampling localities within the hybrid zone of *Bokermannohyla ibitiguara* and *B. sazimai*, Serra da Canastra National Park, state of Minas Gerais, Brazil.

Table S2 Tissue accession numbers (CFBH-T) of *Bokermannohyla ibitiguara*, *B. sazimai* and hybrid individuals (in bold) from 8 localities within the Serra da Canastra National Park, state of Minas Gerais, Brazil.

Table S3 Hybrids between *Bokermannohyla ibitiguara* and *B. sazimai* found in this study, with respective private alleles from B. ibitiguara.

Table S4 Hybrids between *Bokermannohyla ibitiguara* and *B. sazimai* found in this study, with respective private alleles absent from parental individuals of both *B. ibitiguara* and *B. sazimai*.

Table S5 Morphological traits from the 2 *Bokermannohyla* parental species and their assigned hybrids from the state of Minas Gerais, southeastern Brazil (see text for complete names).

Table S6 Acoustic traits from the 2 *Bokermannohyla* parental species and hybrids with calls that were similar to them, all from the state of Minas Gerais, southeastern Brazil (see text for complete names).

Table S7 Body and acoustic measures of the 2 hybrids between *Bokermannohyla ibitiguara* and *B. sazimai* that emitted short notes different from either parental species.

Table S8 Results of the ANOVAs of the linear regression models comparing call variables of *B. ibitiguara* ($N = 38$) and *B. sazimai* ($N = 7$) in Minas Gerais State, southeastern Brazil.

Figure S1 Twelve morphometric variables measured in this study (see text for full names).

Figure S2 Plots obtained from STRUCTURE HARVESTER regarding our 146 sampled individuals of *Bokermannohyla ibitiguara*, *B. sazimai* and hybrids in southeastern Brazil.

Figure S3 Examples of adult *Bokermannohyla ibitiguara* (top left, voucher CFBH 40 563), *B. sazimai* (top right, voucher CFBH 40 614) and hybrids (bottom) found in this study.

Figure S4 Comparison among sequences of short notes of (A) *B. ibitiguara*, also with an introductory long note (voucher CFBH 35 867; SVL = 38.2 mm; air temperature = 20°C), (B) an identified hybrid (CFBH 40 569; 38.2 mm; 20°C) and (C) *B. sazimai* (CFBH 40 616; 33.3 mm; 18°C).

Figure S5 Means and standard errors of body size values (SVL) among parental *B. ibitiguara* (Bib), parental *B. sazimai* (Bsa) and hybrids found in this study.

Spreadsheet with genotypes and phenotypes of all individuals.

Sound file with calls of *B. ibitiguara*, hybrid and *B. sazimai*.

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