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DIVERSIFICAÇÃO E HIBRIDAÇÃO EM UM ANURO ENDÊMICO DO CERRADO: GENÉTICA, MORFOLOGIA E COMPORTAMENTO

RENATO CHRISTENSEN NALI

Tese apresentada ao Instituto de Biociências do Câmpus de Rio Claro, Universidade Estadual Paulista, como parte dos requisitos para obtenção do título de Doutor em Ciências Biológicas (Zoologia) .

Outubro - 2016

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Orientadora: Cynthia Peralta de Almeida Prado

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“It's your road, and yours alone.
Others may walk it with you,
but no one can walk it for you.”
Rumi

RESUMO

A configuração da paisagem e as características fenotípicas associadas ao reconhecimento de parceiros podem influenciar na estrutura genética das populações e nos processos de diversificação de uma espécie. Além disso, interações reprodutivas entre espécies distintas podem alterar a história evolutiva de linhagens com a formação de híbridos viáveis. Para investigar essas interações complexas, estudamos o anuro *Bokermannohyla ibitiguara*, uma espécie com reprodução prolongada, cantos elaborados, corte complexa, fêmeas seletivas e machos agressivos/territoriais. A espécie é endêmica do Cerrado e tem reprodução associada à vegetação ripária dentro e fora do Parque Nacional da Serra da Canastra (PNSC), uma zona de contato com o congênero *B. sazimai*. Amostramos vários riachos e desenvolvemos microssatélites para analisar a influência da topografia e cobertura vegetal na diferenciação genética. Comparamos, em seguida, morfologia e cantos através da área de distribuição e examinamos os papéis da seleção sexual, deriva genética e adaptação acústica a ambientes florestados (Hipótese da Adaptação Acústica; HAA) na diferenciação acústica. Testamos também a hipótese de que *B. ibitiguara* e *B. sazimai* podem hibridizar devido a semelhanças fenotípicas. Indivíduos de *B. ibitiguara* mostraram diferença genética significativa entre riachos, exceto naqueles dentro do Parque, os quais apresentaram maiores níveis de riqueza alélica e heterosigozidade. A diferenciação genética foi melhor explicada pela complexidade topográfica, bem como algumas medidas genéticas intra-populacionais. Os cantos variaram mais do que caracteres morfológicos, sugerindo uma maior pressão seletiva nesse fenótipo comportamental. Parâmetros acústicos associados com discriminação individual e/ou atração de fêmeas foram significativamente diferentes entre populações. Nem diferenciação genética nem cobertura de vegetação ripária (HAA) explicaram as diferenças acústicas entre populações, mas cantos que variaram em contraste à HAA indicaram um papel da competitividade de machos associada com aumento da densidade. Dez por cento dos indivíduos dentro do PNSC eram híbridos, apresentando valores de dissimilaridade genética sobrepostos, mas intermediários comparados às espécies parentais *B. ibitiguara* e *B. sazimai*. Características morfológicas e acústicas não foram intermediárias, mas um mosaico de fenótipos, incluindo um canto distinto em dois híbridos. Enfatizamos que a topografia pode ser um forte promotor de estruturação genética em anfíbios, e que diferenças observadas dentro e fora do Parque

podem estar relacionadas à complexidade do relevo. Estratégias de conservação devem incorporar não apenas distúrbio de hábitat, como também a complexidade topográfica, principalmente em regiões ameaçadas como o Cerrado. Nossa análise fenotípica sugere que a seleção sexual promove diferenciação acústica nessa espécie por meio de reconhecimento individual, preferência de fêmeas e competitividade entre machos. Um enfoque com vários caracteres e mecanismos foi importante para explicar esses intricados processos. Além disso, são poucos os registros de híbridos em anuros com comportamento elaborado e/ou período reprodutivo prolongado, mas esse fenômeno deve ocorrer, provavelmente, devido a similaridades nos sistemas de reconhecimento e estratégias alternativas de acasalamento, como machos satélites. A diversificação multifacetada de *B. ibitiguara* contribui para o conhecimento nas áreas de conservação, evolução e ecologia comportamental. Acreditamos que nossos resultados podem ser aplicados a diferentes animais e biomas, devido às associações generalizadas entre indivíduos com a paisagem, com coespecíficos (do mesmo sexo ou do sexo oposto), e com outras espécies simpátricas.

Palavras-chave: Amphibia. Bioacústica. Conservação. Genética da paisagem. Seleção sexual.

ABSTRACT

Landscape configuration and phenotypic characteristics linked with mating recognition may influence population genetic structure and diversification processes. Moreover, reproductive interactions among different species may alter the evolutionary history of lineages with the formation of viable hybrids. To investigate these complex interactions we studied the treefrog *Bokermannohyla ibitiguara*, a species with prolonged reproduction, complex calls, elaborate courtship, choosy females and territorial/aggressive males. It is endemic to the threatened Brazilian Cerrado and breeds in streams associated with riparian forests within and outside the Serra da Canastra National Park (SCNP), a contact zone with the congener *B. sazimai*. We sampled many streams and developed microsatellite markers to analyze the roles of topography and land cover on genetic differentiation. We then compared morphology and calls throughout the range and examined the roles of sexual selection, genetic drift, and acoustic adaptation to forested habitats (Acoustic Adaptation Hypothesis; AAH) on call differentiation. We also tested the hypothesis that *B. ibitiguara* and *B. sazimai* may hybridize due to phenotypic similarities. Individuals of *B. ibitiguara* showed significant genetic differentiation among streams, except those within the Park, which had higher levels of allelic richness and heterozygosity. Genetic differentiation was best explained by topographic complexity, as were some within-population genetic measures. Calls varied more than morphology, suggesting stronger selective pressures on this behavioral phenotype. Acoustic traits associated with individual discrimination and/or female attraction showed significant population differences. Neither genetic differentiation nor riparian forest cover (the AAH) explained population acoustic differences, but call traits that varied in contrast with AAH indicated a role of male competition associated with increased density. Ten percent of individuals within the SCNP were hybrids, showing overlapping but intermediate values of genetic dissimilarities compared to parental *B. ibitiguara* and *B. sazimai*. Morphology and calls were not intermediate, but a mosaic of phenotypes, including a distinct call type in two hybrids. We underscore that topography is a strong driver of genetic structure in amphibians, and differences observed within and outside the Park may rely on the degree of topographic relief. Conservation strategies should incorporate not only habitat

disturbance, but also topographic complexity, especially in threatened regions such as the Cerrado. Our phenotypic analyses suggest that sexual selection shapes call differentiation in this species in the forms of individual recognition, female preferences and intermale competition. A multi-trait and multi-mechanism approach was nevertheless crucial to explain these intricate processes. Also, records of hybridization in frogs with elaborate behavior and/or long reproductive period are underrepresented, but may also occur likely due to similarities in mating recognition systems and alternative mating tactics, such as satellite males. The multi-faceted diversification of *B. ibitiguara* enhances our knowledge on conservation, evolution and behavioral ecology. We believe that our results can be applied to different animals and biomes, given the widespread associations observed among individuals with the landscape, with conspecifics (within and between the sexes) and with other sympatric species.

Keywords: Amphibia. Bioacoustics. Conservation. Landscape genetics. Sexual selection.

SUMÁRIO

INTRODUÇÃO GERAL

O Cerrado brasileiro é o segundo maior domínio morfoclimático da América do Sul, com uma área original de aproximadamente 2,5 milhões de quilômetros quadrados (Silva *et al.*, 2006), em sua maioria composto de savanas sazonais, campos e corredores de mata de galeria. Esse domínio é a maior savana do mundo em área geográfica e em riqueza de espécies, estando entre os 35 hotspots mundiais de biodiversidade (Mittermeier *et al.* 2004; Klink & Machado, 2005). Apesar disso, a modificação intensa nos últimos 40 anos devido ao desenvolvimento urbano e agrícola resultou em perda ou modificação de metade de sua área (Machado *et al.,* 2004). Apenas 2,2% do Cerrado é legalmente protegido (Klink & Machado, 2005), sendo considerada a savana mais ameaçada do mundo (Silva & Bates, 2002). Esta formação neotropical abriga cerca de 2600 espécies de vertebrados, incluindo mais de 200 espécies de anfíbios anuros, com altas taxas de endemismo (Klink & Machado, 2005; Vasconcelos & Rossa-Feres, 2005; Nogueira, 2006; Valdujo, 2011). Estima-se que 35% das espécies de anfíbios neotropicais encontram-se ameaçadas principalmente pela perda e fragmentação de habitats (e.g. Silvano & Segalla, 2005; Becker *et al.*, 2007; Loyola *et al.,* 2008). Somado a outros fatores como doenças infecciosas emergentes, poluição e aquecimento global, este grupo está entre os mais ameaçados atualmente, levando ao chamado Declínio Global de Anfíbios (Beebee & Griffiths, 2005; Vredenburg & Wake, 2007). Assim, estudos de diversificação populacional em anuros que objetivem integrar evolução e conservação são imprescindíveis, já que medidas de conservação para este grupo devem ser constantes (Beebee & Griffiths, 2005; Lips *et al.* 2005), principalmente em um domínio tão ameaçado quanto o Cerrado.

As matas de galeria constituem o componente florestal mais representativo e diverso no Cerrado, constituídas por matas estreitas permanentes ou semidecíduas (Redford & Fonseca, 1986; Felfili, 1997; Johnson *et al.*, 1999). Estas podem funcionar como corredores ecológicos, ligando diferentes manchas de habitats favoráveis, além de servirem como área de vida para espécies endêmicas dependentes de ambientes florestais (Redford & Fonseca, 1986; Meave *et al.*, 1991; Felfili, 1995; Johnson *et al.*, 1999). Em anfíbios anuros, a utilização da vegetação ripária como área de vida e reprodução é bem documentada (e.g. Lugli & Haddad, 2006a, b; Brasileiro *et al.,* 2005; Barros, 2011; Nali & Prado, 2012; Nali *et al.,* 2015). Esses organismos apresentam alto

risco de dessecação, e como consequência uma baixa capacidade de dispersão, impedindo seu movimento migratório por grandes distâncias (Rothermel & Semlitsch, 2002; Smith & Green, 2005; Wells, 2007; Titon Junior & Gomes, 2015), Assim, muitas espécies de anfíbios apresentam alta fidelidade ao sítio reprodutivo, principalmente em espécies que se reproduzem em corpos de água permanentes (Blaustein *et al.,* 1994; Cushman, 2006; Peterman *et al.,* 2014). Portanto, a configuração da paisagem que conecta as populações deve facilitar essa movimentação limitada (Holderegger & Wagner, 2008; Spear & Storfer, 2008), mantendo uma viabilidade ecológica e evolutiva das populações, já que o isolamento entre elas pode limitar o fluxo gênico (e.g. Spear & Storfer, 2008; Dixo *et al.*, 2009; Richmond *et al.*, 2009), maximizando os efeitos negativos da endogamia (Freeland, 2005; Allendorf & Luikart, 2007).

Nesta abordagem, a genética da paisagem tem contribuído bastante para compreender os processos de migração e fluxo gênico de diferentes animais (e.g. Arnaud, 2003; Holzhauer *et al.*, 2006; Sork & Smouse, 2006), incluindo os anfíbios (Austin *et al.*, 2004; Zamudio & Wieczorek, 2007; Angelone & Holderegger, 2009; Chan & Zamudio, 2009). A genética de paisagem é uma área de pesquisa atual e crescente, definida como a combinação da genética de populações com a ecologia de paisagens (Manel *et al.*, 2003). Seu objetivo é compreender a interação entre as variáveis da paisagem (com sua composição, configuração espacial e dinâmica) e os processos evolutivos populacionais, como fluxo gênico entre subpopulações (i.e. dispersão), deriva genética e seleção (Manel *et al.*, 2003; Holderegger & Wagner, 2006). A genética de paisagem permite estudar a estruturação genética entre populações ao longo de sua distribuição geográfica, estabelecendo barreiras ao fluxo gênico como estradas (Marsh *et al.*, 2005), rios (Angelone *et al.*, 2011), topografia (Funk *et al.*, 2005), gradientes de umidade (Manel *et al.*, 2003), cobertura vegetal (Dixo *et al.,* 2009), entre outros. Dentre os marcadores moleculares mais utilizados como ferramenta, destacam-se os microssatélites, inclusive em estudos com populações de anuros (e.g. Angelone & Holderegger, 2009; Blouin *et al.*, 2010; Schoville *et al.*, 2011). A alta variabilidade destes marcadores é útil para inferir diferentes processos demográficos e evolutivos, como (1) a detecção de impactos sobre as populações decorrentes de ação antrópica (Pearse & Crandall, 2004), (2) a evolução neutra de aspectos fenotípicos (Boul *et al.,* 2007; Funk *et al.,* 2009) e (3) eventos de hibridação interespecífica (Johnson et al 2015; Malukiewicz *et al.,* 2015).

Uma aplicação prática da análise de variação populacional é a detecção de áreas prioritárias para conservação, levando em conta não apenas a riqueza ou abundância das espécies, mas também a integridade genética das populações (Angermeier & Karr, 1996). Esta informação não é útil apenas às espécies atualmente ameaçadas de extinção, mas também para espécies endêmicas com distribuição restrita, cujas subpopulações podem estar isoladas em fragmentos, comprometendo a qualidade genética e o tamanho efetivo da população (e.g. Funk *et al.*, 2009). Ao amostrar tanto áreas antropizadas (e.g. fragmentos de matas em fazendas, com estradas próximas, culturas e pastagens), quanto áreas preservadas (e.g. unidades de conservação), pode-se comparar a integridade genética de populações com diferentes níveis de perturbação, verificando assim se (1) as áreas de proteção existentes são adequadas para a manutenção genética da espécie, e/ou (2) as áreas desprotegidas precisam ser incorporadas como unidade de conservação (e.g. Dixo *et al.*, 2009). Ações conservacionistas serão geralmente necessárias em populações isoladas e desprotegidas, pois são nessas áreas que se espera uma maior perda da diversidade genética devido aos efeitos negativos da endogamia, levando a uma maior probabilidade de extinção (Brown, 1995; Lande, 1999; mas veja Zamudio *et al.*, 2009). No entanto, interações com outras variáveis espaciais, como topografia, são importantes para gerar conclusões mais precisas no contexto da genética da paisagem (Manel *et al.,* 2003).

Além da estrutura genética, variações morfológicas e comportamentais tem sido utilizadas no contexto de diversificação populacional de anuros. Dentre as características morfológicas, alguns exemplos são o tamanho e forma do corpo, evidenciado por caracteres morfométricos (Lougheed *et al.,* 2006; Silva *et al.,* 2008; Amézquita *et al.,* 2009; Warwick *et al.,* 2015). Aspectos da variação morfológica sempre foram incorporados no campo da taxonomia, e mais recentemente combinados com diversificação genética em estudos de evolução para elucidar, por exemplo, a presença de espécies crípticas e diversificação de linhagens (Brusa *et al.,* 2013; Warwick *et al.,* 2015; Ortega-Andrade *et al.,* 2015; Clemente-Carvalho *et al.,* 2016). Na área comportamental, a análise de parâmetros acústicos das vocalizações dos machos de anuros tem revelado que sinais acústicos variam entre as populações distribuídas na paisagem (Ryan & Wilczynski,1988; Castellano *et al.*, 2002; Pröhl *et al.*, 2007; Funk *et al.*, 2009). Os motivos da variabilidade acústica interpopulacional pode estar associada com (1) a diferenciação genética, inferindo uma evolução neutra (Velázquez *et al.,* 2013), (2)

características da paisagem, como adaptação acústica de parâmetros do canto a ambientes com diferentes coberturas vegetais (hipótese da adaptação acústica; Morton, 1975; Ey & Fischer, 2009; Erdtmann & Lima, 2013), ou (3) a seleção sexual, como decorrência da escolha de fêmeas (seleção intersexual) e competição entre machos (seleção intrasexual; Boul *et al.,* 2007; Funk *et al.,* 2009). Além disso, existe normalmente uma associação entre morfologia e cantos em anuros, decorrente de modificações físicas do aparato vocal (McClelland *et al.,* 1996; Nali & Prado, 2014a). Portanto, estudos combinando a morfologia, bioacústica e genótipos de indivíduos ao longo de sua distribuição geográfica permitem um entendimento amplo sobre os padrões e processos evolutivos que levam à diversificação observada (Lougheed *et al.,* 2006; Brusa *et al.,* 2013).

Embora a diversificação populacional remeta a estudos intraespecíficos, a análise de variação genética, morfológica e comportamental pode ser empregada em espécies filogeneticamente próximas ao longo de áreas geográficas onde há simpatria. Interferências reprodutivas interespecíficas podem resultar em hibridação, definida como a reprodução entre membros de populações geneticamente distintas (Barton & Hewitt, 1985). Antigamente, a hibridação era considerada um fenômeno raro e desimportante na evolução biológica, devido a efeitos geralmente deletérios presentes nos híbridos, reduzindo seu *fitness* (Dobzhansky, 1940; Mayr, 1963; Wagner-Jr, 1969; Haddad *et al.,* 1990). Porém, a visão contemporânea é a de que a hibridação é um processo mais comum do que se imaginava (Abbott *et al.,* 2013), podendo ocorrer de diferentes formas (Allendorf *et al.,* 2001), e sendo responsável pela evolução e especiação em diferentes grupos animais e vegetais (Bogart, 1980; Bullini & Nascetti, 1990; Rieseberg & Wendel, 1993; Vanhaecke *et al.,* 2012; Abbott *et al.,* 2013). Além disso, a dinâmica entre características positivas e negativas da presença de híbridos em diferentes sistemas é complexa, já que ela pode resultar de interações naturais ou decorrentes de mudanças ambientais (Allendorf *et al.,* 2001; Detwiler *et al.,* 2005; Seehausen *et al.,* 2008). Em anuros, estudos de hibridação são aparentemente enviesados para espécies de reprodução explosiva, onde há geralmente pouca oportunidade de escolha das fêmeas e não há defesa de territórios pelos machos, facilitando a formação de acasalamentos heteroespecíficos (Brown, 1977; Sullivan, 1986; Haddad *et al.,* 1990; Ficetola & De Bernardi, 2005; Pearl *et al.,* 2005; Wells, 2007). No entanto, utilizando um conjunto apropriado de marcadores moleculares em espécies

simpátricas, e incorporando na análise caracteres morfológicos e acústicos, pode-se verificar a presença, a frequência, e também os possíveis mecanismos de hibridação até mesmo em anuros com complexos sistemas de reprodução, o que é atualmente pouco conhecido (Haddad *et al.,* 1994).

A espécie *Bokermannohyla ibitiguara* (Cardoso, 1983) é um hilídeo endêmico do Cerrado brasileiro, restrito à Serra da Canastra e áreas montanhosas adjacentes, no estado de Minas Gerais (Cardoso, 1983; Haddad *et al.,* 1988; Nali & Prado, 2012). É uma espécie abundante, que se reproduz ao longo de riachos com mata ciliar, e variação de caracteres morfológicos e acústicos dentro de uma mesma área já foi observada (Nali & Prado, 2014a; R. C. Nali, obs. pess.; Turin *et al.,* submetido). Sua reprodução é prolongada (outubro a maio) e mediada por seleção intra e intersexual; a corte é complexa e consiste de estímulos acústicos e táteis, e há emissão diferentes tipos de canto usados para atração das fêmeas e defesa de território contra machos intrusos, que podem resultar em combates físicos (Nali & Prado, 2012; 2014a; 2014b). A presença de machos satélites também foi observada, uma tática em que machos oportunistas não vocalizam e tentam interceptar a fêmea que foi atraída por machos vocalizadores residentes (Nali & Prado, 2012). A espécie ocorre tanto em áreas antropizadas dentro de propriedades rurais (Cardoso, 1983; Nali & Prado, 2012) quanto no Parque Nacional da Serra da Canastra (PNSC; Haddad *et al.,* 1988; Barros, 2011). O PNSC é constituído em sua maioria por um platô com baixa complexidade topográfica, com área de 700km^2, o "Chapadão da Canastra", e foi estabelecido em 1972 (Medeiros & Fiedler, 2004; MMA/IBAMA, 2005). Uma área maior foi recentemente legalizada, mas é composta ainda, em sua maioria, por áreas rurais não desapropriadas (MMA/IBAMA, 2005; May *et al.,* 2009). No Chapadão da Canastra, é conhecida a a simpatria de *B. ibitiguara* com uma espécie que possui canto, morfologia, habitat e padrão reprodutivo semelhante, apesar de estar atualmente incluída em um grupo taxonômico distinto (*Bokermannohyla sazimai*; C. F. B. Haddad, com. pess.; Cardoso & Andrade, 1982; Faivovichi *et al.,* 2005; Carvalho & Giaretta, 2013).

Desta forma, utilizamos a espécie *Bokermannohyla ibitiguara* como modelo para estudo de diversificação populacional nos contextos de conservação, evolução de caracteres fenotípicos e hibridação. A presente tese foi dividida em três capítulos e um apêndice. Neste último, já publicado (Nali *et al.,* 2014; apêndice A), caracterizamos e otimizamos 22 microssatélites para a espécie focal, e testamos a amplificação cruzada

em três outras espécies do mesmo gênero que ocorrem no Cerrado e/ou na Mata Atlântica brasileira (*Bokermannohyla hylax*, *B. circumdata* e *B. alvarengai*). No primeiro capítulo, em um contexto de conservação, comparamos a estrutura genética de populações de localidades dentro e fora do Parque Nacional da Serra da Canastra, e analisamos os papéis da topografia, modificação de habitat, cobertura de mata ciliar e distância geográfica na diversificação e integridade genética da espécie focal. No segundo capítulo, comparamos a variação morfológica e acústica entre as populações, e os mecanismos responsáveis pela diversificação acústica observada. Especificamente, avaliamos a influência da seleção sexual, da adaptação dos cantos ao ambiente (hipótese da adaptação acústica) e da diferenciação genética na diversificação acústica. No terceiro capítulo, estudamos o contexto de hibridação entre a espécie focal e a espécie *B. sazimai,* as quais ocorrem em simpatria na região do PNSC. Para tanto, além dos dados genéticos, incorporamos também dados de morfologia e vocalizações de ambas as espécies e dos híbridos.

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CHAPTER I:

TOPOGRAPHY, MORE THAN LAND COVER, EXPLAINS GENETIC DIVERSIFICATION OF A NEOTROPICAL SAVANNA TREEFROG

Renato C. Nali, C. Guilherme Becker, Kelly R. Zamudio & Cynthia P. A. Prado

TOPOGRAPHY, MORE THAN LAND COVER, EXPLAINS GENETIC DIVERSIFICATION OF A NEOTROPICAL SAVANNA TREEFROG

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ABSTRACT

Effective conservation policies rely extensively on information about population genetic structure and the suitability/connectivity of remnants of natural habitat. Geographic distance normally results in differentiation, yet natural or human-mediated landscape features may represent additional ecological constraints to gene flow. The study of the interactions among these variables in heterogeneous landscapes can uncover the relative contributions of different barriers to gene flow and its consequences for animal conservation. We characterized genetic structure and differentiation among 12 populations of a stream-dwelling treefrog from a mountainous savanna in Brazil, and tested hypotheses by quantifying the roles of geographic distance, topographic complexity, and land cover, as well as protection status. Four genetic clusters roughly separated populations within and outside a national Park. All localities significantly deviated from panmixia, and most showed significant genetic differentiation, except for those within the Park, which also had higher levels of allelic richness and heterozygosity. Genetic differentiation across populations in this landscape was primarily explained by topographic complexity. Within-population genetic measures were best explained by models including elevation and topographic complexity, and not habitat loss or gallery forest cover. Our results underscore that topography is a strong environmental factor in

shaping genetic structure among amphibian populations, and differences observed in populations within and outside the Park may rely on the degree of topographic relief. Therefore, we provide evidence that effective conservation strategies for endangered amphibians should take into account not only habitat disturbance, but also topographic complexity, which is key for the threatened fauna of the Brazilian Cerrado.

Keywords: Anura, isolation-by-resistance, landscape genetics, *Bokermannohyla ibitiguara*, microsatellites, biodiversity

INTRODUCTION

Effective conservation actions rely on information about population genetic diversity, structure, and connectivity (Moritz 1994; Frankham 2005; Allendorf & Luikart 2007). Population genetic data provide estimates of individual migration across distributional ranges, suitability of habitat patches, and the factors that have shaped such diversification, and these data in turn can be used to mitigate potential negative effects that may arise from isolation (Habel et al. 2015). The higher the functional connectivity, i.e., the degree to which individuals disperse throughout the landscape, more likely it is to preclude limited gene flow with consequent isolation, endogamy and inbreeding of populations (Spear & Storfer 2008; Dixo *et al*. 2009; Murphy *et al*. 2010). In other words, biological integrity of populations will rely on (1) the suitability of the habitat in the matrix between populations, and whether they promote natural levels of dispersal and (2) the quality of the natural habitat itself, given that persistence of individuals and their successful reproduction are critical for population diversity (Angermeier & Karr, 1996; Peterman *et al*. 2013; Coster *et al*. 2015).

Different features of the landscape itself can either promote or hinder gene flow, resulting in diversification of phylogenetic lineages and population structure even in pristine habitats (Funk et al. 2005; Dixo *et al*. 2009; Titus *et al*., 2014). Beyond geographic distance (Wright 1943), two of these naturally selected features are topography, represented by elevation and topographic complexity/relief, and habitat selection, represented by types of vegetation cover and microhabitats utilized by different species. In this case, environments with more forest cover and topographic complexity may yield more diverging lineages and deeper phylogeographic signature

(Guarnizo & Cannatella 2013; Rodríguez et al. 2015). On top of that, the distribution of lineages are further complicated when secondary, non-natural changes take place in a short period of time. One clear example is human-mediated habitat loss and fragmentation, which has devastated many ecoregions over the last decades (Machado *et al*. 2004; Dixo *et al.* 2009; Laurence 2010). The complex interactions between natural features vs. rapid anthropogenic changes will likely dictate species persistence or extinction following the logic of population connectivity vs. isolation.

Organisms are usually not homogeneously distributed throughout their geographic range, but rather live in patches of suitable habitat within a matrix (Levins 1969; Smith & Green 2005). If human activity changes mostly the matrix, but still permits successful dispersal events of individuals, populations will still persist by a sustained gene flow, resulting in large randomly mating populations (Galbusera et al. 2004). If on the other hand the human changes on the matrix are so deep and rapid that gene flow is largely compromised, or the topography itself limits dispersal independent of the matrix, then functional connectivity will be just as deeply impaired, evidencing the deleterious effects of isolated populations (Dixo et al. 2009; Guarnizo & Cannatella 2013). In addition, because reproductive success of residents will determine the amount of individuals recruited for dispersal, habitat quality itself is a factor intrinsic to functional connectivity, which has been usually overlooked compared with studies looking at the matrix itself (Ovaskainen & Hanski 2004; Garroway *et al*. 2008; Peterman *et al*. 2013; Coster *et al*. 2015). Thus, studies of focal organisms distributed in heterogeneous habitats and landscapes are key to examine the relative roles of natural and anthropogenic barriers to gene flow, how they are mediated in habitat specialist species, and the consequences for conservation.

Landscape genetic studies on temperate systems by far outnumber those of tropical areas (Manel & Holderegger 2013). The Neotropical Cerrado is the second largest South American morphoclimatic domain, with an original area of approximately 2.5 million km^2 (Silva *et al*. 2006). It is the most species-rich savanna in the world, and considered one of the 35 worldwide hotspots of biodiversity (Mittermeier *et al.* 2004). The original landscape includes seasonal savannas, grasslands and gallery/riparian forests, the latter being closed vegetation cover alongside streams that best represent the forest component within this formation (Meave *et al*., 1991; Felfili, 1995). Gallery forests are then crucial as they provide suitable habitats for forest-dependent species in

the Cerrado (Johnson *et al*. 1999; Nali & Prado 2012). Understanding habitat connectivity and gene flow is particularly important in extremely threatened ecoregions; by 2004, approximately 55% of the Cerrado had already been cleared or transformed for human uses (Machado *et al*. 2004), and only 2.2% of the Cerrado is legally protected (Klink & Machado 2005). Only by assessing the contemporary status of genetic differentiation across a species' range (Bergsten *et al.* 2012) can we determine the suitability of current conservation policies, or correctly establish new protected areas to avoid genetic isolation among populations.

Due to characteristics such as high desiccation risks, limited mobility across the environment, and site fidelity, amphibians are excellent taxa for studies of mechanisms shaping fine-scale population divergences across heterogeneous landscapes (Blaustein *et al*. 1994; Rothermel & Semlitsch 2002; Smith & Green 2005; Cushman 2006). The world's largest diversity of frog species is concentrated in the Neotropics, and ca. 1,000 species occur in Brazil alone (Segalla *et al*. 2014; Frost 2016). However, very few studies have investigated the processes leading to genetic differentiation in frogs living in naturally open habitats (Maciel *et al*. 2010; Prado *et al*. 2012), let alone those from mountainous landscapes (Eterovick *et al*. 2016). *Bokermannohyla ibitiguara* (Cardoso 1983) is a stream-dwelling treefrog endemic to the Cerrado, and known only from the Serra da Canastra mountain range, southeastern Brazil (Haddad *et al*. 1988; Nali & Prado 2012). This species is commonly associated with gallery forests from preserved (Serra da Canastra National Park, SCNP; Haddad *et al*. 1988) and modified surrounding Cerrado (Nali & Prado 2012), occurring throughout a topographically complex landscape, but its real population status and genetic diversity remains unknown (Caramaschi & Eterovick 2004). Considering the distribution of this forest-dependent species throughout an open landscape with variable fragmentation and topographic relief, we tested the hypotheses that: (1) individuals will be genetically differentiated among localities within this open matrix; (2) gene flow, when present, will be facilitated in areas of topographic homogeneity; and (3) genetic diversity will be higher and differentiation lower in populations within the SCNP due to the protected status and/or the topographically more homogeneous landscape. Combined, our results elucidate processes leading to population diversification of an endemic treefrog and provide novel information for the management and conservation of open savanna ecosystems.

MATERIALS AND METHODS

Study site

The Serra da Canastra mountain range is a markedly seasonal region in southeastern Brazil, state of Minas Gerais, with a hot and rainy summer (October to March) and a dry winter (April to September; Queirolo & Motta-Junior 2007). The region was originally covered by the Cerrado vegetation, patches of semideciduous forest, gallery forests in valleys alongside various streams, and grassland fields at higher elevations of up to 1,500 m (Dietz, 1984). Much of the original vegetation, however, suffered severe anthropogenic modification during the last decades and has been converted into agricultural/pasture lands (May *et al*., 2009). The Serra da Canastra National Park (SCNP) was established in 1972 and consists mostly of a flat plateau with an area of ~700 km^2 (the "Chapadão da Canastra", MMA/IBAMA 2005; Medeiros & Fiedler 2004). A larger area was much later legally included as part of the Park, but is still mostly composed by privately-owned farmlands distributed throughout a more topographically complex region (the"Chapadão da Babilônia" and adjacent lands; MMA/IBAMA 2005; May *et al*. 2009; the Instituto Chico Mendes de Conservação da Biodiversidade - ICMBio); thus, even though we sampled populations from both original and novel Park areas (see below), we considered as within PNSC only those from the original area.

Individual sampling

During three rainy seasons Oct/2010 – Mar/2011, Oct/2012 – Mar/2013, and Oct/2013 – Mar/2014 we sampled frogs in 12 streams: six located within and six located outside SCNP boundaries (supplemental table 1, figure 1). We collected 273 tissue samples from adults (liver, leg muscle or toe clip) and tadpoles (tail clip), preserved in 99.5% EtOH. We euthanatized individuals with a10% lidocaine applied to the ventral region of each frog. Voucher specimens were fixed in formalin 10%, and preserved in ethanol 70% (McDiarmid 1994). Tadpoles of *B. ibitiguara* do not form schools (Cardoso 1983; R. C. Nali, pers. obs.), and we collected tadpoles of different body sizes throughout the streams, reducing the probability of collecting tadpoles from a single clutch. Voucher specimens and tissues were deposited at the Coleção de Anfíbios Célio F. B. Haddad, Universidade Estadual Paulista, Rio Claro, SP, Brazil (supplemental table 1).

Laboratory protocols and microsatellite data

All individuals were genotyped at 21 microsatellite loci previously developed and optimized for the focal species (Nali *et al*. 2014). We extracted whole genomic DNA from collected individuals with DNeasy extraction columns (Qiagen, Valencia, CA, USA), following manufacturer's protocols. PCR profiles followed those in Nali *et al.* (2014). Each forward primer contained a 20 bp tag on the 5' end 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 four 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 Genomics facility of the Cornell Biotechnology Center.

We used GENEMARKER v. 2.4.0 (SoftGenetics LLC, State College, PA) to analyze genotype profiles for individuals. We re-genotyped 64 individuals/locus on average to calculate a genotyping error rate, defined as the percentage of the number of individuals genotyped differently for at least one allele, divided by the number of individuals re-genotyped. We used MICROCHECKER v. 2.2.3 (Van Oosterhout *et al*. 2004) to check for evidence of null alleles for each population. We tested for Hardy-Weinberg equilibrium for each population using GENEPOP 4.0.9 software (Rousset 2008), assessing statistical significance through Monte Carlo Markov Chains (MCMC) with 10,000 dememorization steps followed by 100 batches of 5,000 iterations each (Guo & Thompson 1992). Because we had evidence for null alleles at loci Bi1, Bi1122, Bi1521 and Bi3629 in more than 7 populations, we ran the HWE analyses again without these loci. We also tested for linkage disequilibrium across individuals and all pairs of loci using GENEPOP.

Population genetic analyses

We calculated pairwise relatedness among individuals in GENEALEX using the r_{qg} coefficient (Queller & Goodnight 1989), and then permuted genotypes from all populations 9999 times and derived upper and lower 95% confidence intervals to obtain a range of r_{qg} expected if mating was random across all populations. We bootstrapped confidence intervals 9999 times to obtain estimates of mean relatedness within populations. Finally, we used this analysis to visually confirm if our sampling, based on different proportions of adults and tadpoles collected per stream

(supplemental table 1), could bias our analyses of local population structure, if for example more tadpoles sampled resulted in higher relatedness values.

We used FSTAT 1.2 (Goudet, 1995) to calculate overall *F*-statistics (Wright 1969): F_{IT} (inbreeding coefficient of individual relative to the total population), F_{IS} (inbreeding coefficient of an individual relative to the sampling locality), and F_{ST} (effect of sampling locality compared to the total population). We also calculated F_{IS} per sampling locality. Pairwise F_{ST} was calculated as $F_{ST}/(1 - F_{ST})$ and used as a measure of genetic differentiation between sampling localities (Rousset, 1997). Statistical significance of this measure was assessed by using 9999 permutations in GENALEX v. 6.5 (Peakall & Smouse 2012). Following Narum (2006), we reported statistical significance for 66 comparisons using Bonferroni correction (P = 0.00076), and also the B-Y method (P = 0.01037), which is less conservative and more appropriate in conservation genetics (Benjamini & Yekutieli 2001).

To analyze genetic discontinuities without considering subpopulations *a priori*, we ran a Bayesian analysis using STRUCTURE v. 2.3.4 with the Markov Chain Monte Carlo method (Pritchard *et al*. 2000; Falush *et al*. 2003), verifying the real number of genetic clusters (K) and genetic discontinuities between collected individuals. We accessed K values from 1 to 13 using 25 MCMC runs with 200,000 burn-ins followed by 1 million steps, considering the admixture model and correlated allele frequencies. Presence of genetic structure was assumed when different plots of delta-K provided evidence of a clear peak for K values in STRUCTURE HARVESTER (Earl & vonHoldt 2011). The cluster data were permutated for the selected K value using CLUMPP (Jakobsson & Rosenberg 2007), and bar plots visualized in Excel. Because we had four loci with possibility of null alleles (Bi1, Bi1122, Bi1521 and Bi3629) for more than 7 populations, we ran STRUCTURE again excluding these loci to check for possible differences in clustering patterns.

Landscape genetic analyses

To determine the roles of geographic distance, topographic complexity, and land cover on genetic differentiation across our sampling locations, we calculated three connectivity indices: Euclidean distance (minimum straight-line distance between each pair of populations) and two surface resistance indices: topographic resistance and land cover resistance. We used the digital elevation model from the Shuttle Radar

Topography Mission (SRTM) to generate a topographic complexity raster using the raster calculator feature in ArcGIS 9.3.1 (ESRI 2009), where each elevation pixel (30 m resolution) was assigned the variance of the eight neighboring pixels (Huaxing 2008). This metric provides significant information on habitat heterogeneity and microclimate turnover. We characterized land cover suitability according to known habitat requirements for amphibians based on the national land cover database (1:50,000 scale shapefiles; CSR/IBAMA 2014). We coded low suitability for non-natural vegetation and high suitability for natural vegetation cover. We calculated topographic resistance and land cover resistance among pairwise populations by employing our topographic complexity and land cover suitability rasters as resistance grids using CIRCUITSCAPE v.3.3 (McRae & Shah 2009). We employed a cell connection scheme connecting each node to four neighbors. Surface resistance weights all possible paths between pairs of sampling locations and produces a summary connectivity raster. We then used simple and partial Mantel tests (Mantel, 1967) to correlate Euclidean distance and both surface resistance indices with pairwise multi-locus F_{ST} values using PASSAGE v. 2.0 (Rosenberg & Anderson 2011).

We analyzed the impact of landscape variables on within-population genetic parameters: allelic richness (AR), private allelic richness (PAR), observed heterozygosity (H_O), expected heterozygosity (H_E), number of alleles (NA), effective number of alleles (ENA), and inbreeding coefficient (F_{IS}). We obtained AR and PAR in HP-RARE (Kalinowski, 2005); NA, ENA, H_O, and He in GENALEX; F_{IS} in FSTAT. We calculated average topographic resistance and percent modified land cover (habitat loss) within a radius of 700 m around each sampling location. We used high resolution satellite images CNES/Astrium satellites in 2013 or 2014 (pixel resolution = 0.35 m) to quantify the remaining gallery forests (%) within a radius of 500 m around each sampling location using ArcGIS 9.3.1. (ESRI 2009). Average elevation per locality was based on several GPS records of collected individuals. We used a General Linear Model approach (GLM - standard least squares) running all possible models including explanatory landscape variables (topographic resistance, elevation, percent habitat loss, and percent gallery forest), including their one-level interactions, and genetic parameters as response variables in turn. We ranked models based on Akaike Information Criterion (AICc) and reported the most parsimonious significant model for each run.

Comparisons among populations inside vs. outside SCNP

Because we expected differences in connectivity, and consequently, gene flow in populations within SCNP vs. outside SCNP, we used a Tukey's Test (Tukey-Kramer Honestly Significant Difference) to compare the average pairwise F_{ST} values categorized in three groups: Within/Within SCNP, Within/Outside SCNP, Outside/Outside SCNP. In addition, to test for statistical difference (two-sided P) in pairwise F_{ST} values from populations within vs. outside SCNP, we used a non-parametric analysis with 10,000 permutations implemented in FSTAT.

Genetic measures of diversity were compared between localities within vs. outside SCNP using one-way ANOVA (AR, PAR, H_O, H_E, NA, ENA and F_{IS}). To provide additional evidence, we conducted the non-parametric permutation analysis implemented in FSTAT (see above) to compare these two groups for H_O, H_E, and AR.

Because of the configuration of our populations, where protected ones are located in flat highlands (SCNP) while the others are included in topographically more complex regions at lower elevations, we employed structural equation models to provide information about the relative strength of direct and indirect effects of topographic complexity, elevation and habitat loss on allelic richness.

RESULTS

Microsatellite data

Our 21 markers were highly polymorphic (11 to 58 alleles; average 20.8 ± 2.4). We had an error rate of 3.5% in genotyping, ranging from 0 to 7.5% across loci. All loci showed evidence for null alleles in MICROCHECKER when considering all individuals together. When divided by population, loci Bi1, Bi1122, Bi1521 and Bi3629 had evidence of null alleles for more than 7 populations. None of our populations were under Hardy-Weinberg Equilibrium for the full data set. When excluding the four loci subject to null alleles for over 7 populations, only populations MP3 and R25 were under HWE (Bonferroni P = 0.0029). We found linkage disequilibrium for only 10 pairs out of 210 comparisons (Bonferroni P = 0.00024) across all loci and individuals (Bi1/Bi94, Bi179/Bi1050, Bi94/Bi1397, Bi94/Bi3003, Bi1397/Bi3202, Bi1050/Bi3370, Bi1122/Bi3370, Bi2312/Bi3370, Bi94/Bi3629, Bi1/Bi4144).

Population genetic analyses

Individuals of every sampling locality significantly differed from panmixia (all *P* values < 0.01; figure 2), i.e., reproduction is not random across all sites. Populations with higher relatedness values (R1, R2, R3, and PRT) formed separate clusters (see below). One could expect increasing relatedness values for populations with increasing tadpoles sampled (white < gray < black diamonds; figure 2), but we detected variation regardless of the predominant sample type. Thus, the population differences in relatedness are likely due to the genetic structure and isolation of our populations themselves rather than an artifact of sampling.

The 12 populations of *B. ibitiguara* in this study had an average F_{IT} of 0.233 ± 0.034, average F_{ST} of 0.064 ± 0.007, and average F_{IS} of 0.18 ± 0.035 (mean ± SE). Within-population inbreeding was high, ranging from 0.127 to 0.244. The analysis of pairwise F_{ST} differentiation yielded significant results for 56 out of our 66 pairs of populations (Table 1).

In our STRUCTURE analysis, delta K showed a clear peak for 4 clusters, with an average coefficient of membership (percentage of individual assignment) of 91.6% (figure 1). Excluding the four loci with possibility of null alleles, we had very similar results, indicating K = 4 and average coefficient of membership = 90% (complete results not shown). The only difference was found for the population GLG, which was included together with the PRT genetic cluster (coefficient of membership = 59%), instead of the previous cluster found with the full dataset (coefficient of membership = 34%). However, because this was the population with more admixture independent of the datasets used, we decided to use all 21 microsatellite markers for downstream analyses.

Landscape genetic analyses

Euclidean distances among sampling localities were positively correlated with pairwise F_{ST} values (Mantel: $R = 0.52$, one-tailed $P < 0.001$). Reconstruction of surface resistance for topographic complexity showed high connectivity across some sampling localities, mainly within SCNP (figure 1). We found a highly significant effect of topographic resistance on F_{ST} values across *B. ibitiguara* populations (Mantel: $R = 0.66$; one-tailed $P < 0.01$), even when Euclidean distance was controlled for (partial Mantel: $R = 0.47$, one-tailed $P < 0.05$). Landcover resistance did not predict F_{ST} values across our sampled populations when Euclidean distances were held constant (partial Mantel: $R = 0.16$, one-

tailed $P = 0.26$).

Landscape variables significantly explained four of the within-population genetic parameters (H_O, H_E, ENA and AR). The most parsimonious models included topographic complexity and elevation as explanatory variables for AR, H_E, H_O, and ENA (Table 2). Habitat loss and percent gallery forests were not included in any of the most parsimonious models (supplemental table 3).

Comparisons among populations within vs. outside SCNP

We found significant differences among pairwise F_{ST} of populations within SCNP only, within vs. outside SCNP, and outside SCNP only (ANOVA; $F_{2,63} = 23.333$; $P < 0.0001$; figure 4). Populations within SCNP showed the lowest levels of population differentiation (average $F_{ST} \pm SE = 0.014 \pm 0.004$), and populations outside SCNP showed the highest divergences (0.083 ± 0.005). Within vs. outside SCNP comparisons showed intermediate levels of differentiation (0.054 ± 0.008; figure 3). Likewise, the permutation analysis showed that F_{ST} was significantly higher in populations outside the SCNP when compared to those within the park boundaries ($P = 0.01$). Populations outside SCNP showed significantly less AR, H_O and H_E for at least one statistical test (Table 3). Moreover, populations outside SCNP showed numerical expected tendencies for most of the remaining parameters: less NA, less ENA, and higher F_{IS}.

Our structural equation model confirmed that areas of high elevation harbor anuran populations with higher AR, which coincides with our populations within SCNP. Lower elevation sites are on average topographically complex, and this complexity was in turn linked to lower allelic richness, which coincides with our non-protected populations. Elevation was also negatively associated with deforestation, but the amount of natural vegetation cover did not significantly predict AR (figure 3).

DISCUSSION

In this study we uncovered the contemporary scenario of genetic structure in a Brazilian Cerrado treefrog, and how landscape variables might have contributed to the observed pattern. Our focal species provided insight on the direct and indirect effects of habitat loss, gallery forests, topography, and geographic distance to spatial connectivity and genetic integrity of populations. Employing many polymorphic microsatellites, we

showed that frog populations distributed in patches of gallery forests throughout an open environment are overall genetically differentiated; however, gene flow can still be maintained via likely dispersal across areas of decreased topographic complexity, which in this case coincide with the protected Park.

Genetic differentiation among populations

Our first hypothesis predicted that populations would primarily show genetic differentiation, which was at least partially corroborated. Most of the studied population pairs showed significant pairwise F_{ST} differentiation (56 out of 66 pairs). Although overall average F_{ST} was not particularly high (0.06; range = 0 to 0.13), significant differentiation is not negligible if F_{ST} is as small as 0.05 or even less (Wright 1978). Together with the consistent deviations of each sampling locality from a large, panmictic population (figure 2), and a higher overall inbreeding coefficient (F_{IS} = 0.18), we showed that *B. ibitiguara* populations are overall genetically differentiated among streams, which helps explain the lack of Hardy-Weinberg Equilibrium (Nei 1977; Austin *et al*. 2011). This was expected due to the mentioned amphibian limited mobility, physiological constraints that confine adults to moist environments, and extreme site fidelity, which can preclude dispersion and limit gene flow (Blaustein *et al*. 1994; Cushman 2006; Peterman *et al*. 2014). The Brazilian Cerrado is predominantly an open environment dominated by a mosaic of grasslands and shrubs, where streams with gallery forests are sparsely distributed (Silva *et al*. 2006; Meave *et al*. 1991). In this landscape, forest-dependent animals may use those gallery forests for reproduction and refugia, instead of migrating throughout grasslands (Redford & Fonseca 1986; Johnson *et al*. 1999). In fact, phylogeographic structure of frog lineages are correlated with higher forest levels and microhabitat selection when compared to species that live and breed in open habitats (Rodríguez *et al.* 2015), which could be the case of *B. ibitiguara*, a forest-dependent species within an open habitat.

Reproductive characteristics can contribute to genetic differentiation in frogs (Funk *et al*. 2009). In *B. ibitiguara*, individuals reproduce for many months during the rainy season (Nali & Prado 2012). Males spend energy defending oviposition territories to obtain females, which are the limiting resource; females, on the other hand, feed and produce eggs to ensure maximum reproductive output, sometimes depositing more than one clutch during a season (Wells 2007; Nali & Prado 2012; R. C. Nali, pers. obs.). It is

therefore unlikely that adults of this species would undertake major migration events while breeding. In the remaining non-reproductive months, however, the environment is much drier and desiccation is likely (Smith & Green 2005; Titon Junior & Gomes 2015). As a result, genetic differentiation will accumulate with time in different streams that become more isolated in the matrix of open environment. In *B. ibitiguara*, the natal area of juveniles is also a potential breeding site and genetic differentiation among streams may be caused by a higher phylopatry when compared to species that require migration from foraging areas to breeding sites, such as pond-breeding amphibians (Gamble *et al*. 2007; Semtlisch 2008; Coster *et al*. 2015). Site fidelity was also consistent with our significant within-population relatedness values. One could argue that tadpoles could migrate downstream and reach different streams facing less risks than post-metamorphs (Eterovick *et al*. 2009; Lawson 2013). Streams inhabited by *B. ibitiguara* are typically narrow, shallow, and normally partially obstructed (e.g. by fallen trees), which could hamper tadpole dispersal far enough to promote genetic differentiation among patches in a landscape scale. Although detailed characterizations of tadpole dispersal capabilities are sorely needed (Wahbe & Bunnell 2001), mounting evidence suggests that migration/dispersal events of amphibians are primarily made by adults and/or juveniles instead of tadpoles (Cushman 2006; Semtlisch 2008), which is also corroborated by stream fishes that present restricted movement during most of their lives (Rodríguez 2002).

Landscape features and facilitated gene flow

Despite our evidence for low gene flow in our focal species, not all populations were genetically differentiated; most of the comparisons among populations within SCNP showed non-significant parwise F_{ST} values, even with large areas of open grasslands surrounding the sampled gallery forests (figure 1, table 1). Thus, SCNP itself seems to be essential in maintaining the gene flow of *B. ibitiguara* populations, showing a highly connected landscape through a flat plateau even across large geographic distances (figure 1A). If post-metamorphs/adults promote population admixture, dispersal throughout the open grasslands in between streams is possible, resulting in less genetic differentiation and clustering among habitat patches (Eterovick *et al*. 2009). What landscape feature could then facilitate dispersal in this hazardous environment, especially for a forest-dependent species?

Geographic distance restricts individual dispersal and consequently gene flow in different taxa, but other landscape features can act as barriers, precluding isolation-by-distance or resulting in a non-stationary pattern of IBD (Murphy *et al*. 2010; Duforet-Frebourg & Blum 2014; Marschalek & Berres 2014; Wright *et al*. 2015). In our second hypothesis, we predicted that existent gene flow would be facilitated by topographic complexity. We did find a significant IBD pattern in *B. ibitiguara*; however, topographic complexity was a more important restrictor for gene flow regardless of geographic distance, whereas land cover was not an explanatory variable when controlling for distance. Data for different taxa show that topographic complexity prevents gene flow among populations, given that larger slopes may represent steeper terrains that are costly for dispersal (Pérez-Espona *et al*. 2008; Rodríguez *et al.* 2015; Guarnizo *et al*. 2016). More than a barrier to gene flow, we found here that topographic complexity and/or elevation – and not land cover – explained four of our genetic measures of diversity within populations (table 2); also, only topography was directly associated with AR in our path analysis (figure 3). A similar effect was found in another mountain frog, in which differences in elevations explained H_E and AR (Funk *et al*. 2005). Local factors are important to consider when studying genetic differentiation, because they affect onsite reproductive output, which in turn will influence how juveniles and future adults might be recruited to promote dispersal events (Peterman *et al*. 2013; Coster *et al*. 2015). Ours is one of the few studies that explicitly show that topography influences not only the connectivity in-between habitats, but also the maintenance of genetic integrity within-habitat.

Our third hypothesis predicted that the SCNP promotes less genetic differentiation and more diversity. The Bayesian clustering approach uncovered four genetic demes (figure 1) with a rough separation of the populations within SCNP (red) from those outside the park (blue, purple, green, and red [with admixture, see discussion below]). Populations within SCNP were genetically less differentiated than those outside the SCNP, and pairwise comparisons between those two sets of localities showed intermediate values (figure 4). Moreover, AR, H_O and H_E of populations within SCNP were statistically higher than those from outside (table 3), and, as mentioned, most within-population measures had an effect of topography rather than land cover. Specifically, our path analysis showed that elevation and topographic complexity correlated with AR, whereas habitat loss did not, despite influences of topography on

habitat loss. Moreover, sites at increased elevation show less topographic complexity, and these areas coincide with the area of the SCNP (figure 3). Our combined results indicate that the occurrence of a dominant single cluster within SCNP occurs due to the topographic homogeneity of SCNP at higher elevations (>1,200 m, mostly Chapadão da Canastra), compared to the localities outside the SCNP, which are found across the rest of the complex landscape at lower elevations (<1,100 m; figure 1), and not because of protection status, which only partially corroborates our third hypothesis. In another study with a tropical stream-breeder frog, Zancolli *et al*. (2014) found a different pattern; in the African mountain species *Amietia wittei*, facilitated gene flow was associated with topographic complexity, so that non-physical and cryptic barriers such as local environmental conditions could possibly be responsible for differentiation instead of topography. *Bokermannohyla ibitiguara* occurs up to approximately 1,500m, while the African frog occurs from 1,700 to 3,500 m, with no high-altitude flat plateau. Thus, frogs with similar life histories may still present different dispersal capabilities and adaptations depending on factors such as altitude range and configuration of highlands. Landscape genetic studies with tropical species are far fewer when compared to temperate ones, and topography has not been explicitly considered for the few Brazilian frogs investigated (e.g. Telles *et al*. 2007; Prado *et al*. 2012; Manel & Holderegger 2013; Eterovick *et al*. 2016). Comprehension of the factors leading to diversification of stream-breeding frogs will only become feasible with the increase of published research with landscape genetics in the tropics.

While topography was important in shaping genetic structure of *B. ibitiguara*, we still found some inconsistencies. First, we showed genetic differentiation and clustering among populations R1, R2, and R3, and these populations are not in an area of particularly rough terrain. Second, populations CAL and GLG, despite genetic differentiation and admixture in the latter, belong to the same cluster as other populations within SCNP, even with no obvious topographic connectivity with populations within the SCNP and occurring at lower elevations (figure 1). The first could be explained by pre-mating barriers due to population call differentiation, which prevents the formation of inter-population mating pairs (Narins & Smith 1986; Lüddecke & Sánchez 2002; Sullivan-Beckers & Cocroft 2010); however, despite the complex communication in this species (Nali *et al*. 2014), ongoing analyses suggest that pre-mating barriers are unlikely (Chapter 2). Both inconsistencies could be then at least

partially explained by the locality of each population relative to the species' known range. Populations R1, R2 and R3 are found on the outer edge of the range, which normally have lower population sizes than core populations (Anderson & Danielson 1997), decreased individual genetic diversity, and increased isolation from other populations (Eckert *et al*. 2008; Lawson 2013; Marschalek & Berres 2014). Conversely, populations CAL and GLG are much more centrally located, and may have retained ancestral polymorphism due to sufficient population sizes (Zancolli *et al.* 2014). Populations closer to the core generally have higher genetic diversity that stem from increased gene flow relative to peripheral populations (Frankham 1996). Non-sampled populations likely exist in streams between Chapadão da Babilônia and Chapadão da Canastra (SCNP), and wetlands/swamps could serve as stepping stones for dispersal using less complex pathways relative to topography (Coster *et al*. 2015). Even so, the GLG population has a stronger admixture with the also peripheral PRT (Figure 1), once again highlighting the role of a less complex topography in promoting dispersal events of individuals. The Cerrado is a complex mosaic of different types of vegetation (Silva *et al*. 2006; Carvalho *et al*. 2009) and preferred areas for dispersing animals and prospective corridors are not entirely captured by binary classifications such as forest vs. non-forest or disturbed vs. natural area (this study; Dixo *et al*. 2009). Fine-scale environmental/landscape data might further explain inconsistencies observed here, but we are still limited by the lack of detailed land cover maps and comparable studies for most of the Cerrado.

Conservation implications

Amphibians are among the most endangered vertebrates (Wake & Vredenburg 2008), and a myriad of mechanisms lead to loss of genetic diversity and potential species declines (e.g. inbreeding depression, increased susceptibility to diseases, changes in temperature dynamics via global warming, invasive species, habitat changes, etc.; Blaustein & Kiesecker 2002; Becker *et al*. 2007; Savage *et al*. 2015). Comprehensive studies accounting for genetic integrity and factors responsible for diversification of frog populations in different regions are necessary to prevent further extinctions, due to increased effects of endogamy and inbreeding in isolated populations (Frankham 2005; Spear & Storfer 2008; Dixo *et al*. 2009). Over the last decades we have learned from landscape genetics that conservation efforts should rely on variables other than

geographic distance to promote genetic integrity of populations. Indeed, landscape genetic studies have informed on effective conservation goals and the design of reserve networks that enable gene flow among remnant populations and facilitate connectivity between terrestrial habitats and aquatic breeding sites (Becker *et al*. 2007; Keller *et al*. 2015). The establishment of the SCNP in 1972 was key for the conservation of *B. ibitiguara* because it facilitates dispersal through areas of low topographic complexity. As a consequence, the gene pool of a large, dominant cluster was successfully preserved, with higher genetic integrity of populations within the park boundaries.

This study brings novel information for the conservation of Cerrado species at both local and broad scales. Locally, we argue that the choice of preserved habitat patches relative to topography may determine the isolation or connectivity of remnants. In a farmland, topographically complex regions are normally difficult to use for cattle ranching and agriculture, so the mandatory conservation area is primarily chosen in those complex regions whenever possible (Brasil 2000; 2012). This reveals a paradox, because the preservation of such areas include populations with lower connectivity, as observed here for *B. ibitiguara*, instead of guaranteeing dispersal and gene flow. At a broader scale, one can think of major Brazilian Cerrado formations with rough topography. How efficient is the small percentage of protected areas of the Brazilian Cerrado (~2%) if topographic homogeneity is to be considered crucial for the genetic integrity of populations? For instance, the Chapada dos Guimarães, in Central Brazil, and the Espinhaço mountain range, in Northeast and Southeast Brazil, are two topographically complex regions where conservation should be prioritized (Werneck 2011). The first, encompassed by a National Park, also comprises a large plateau similar to the Serra da Canastra, where its historical uplift may have led to the evolution of a cryptic treefrog lineage (Prado *et al*. 2012). The second is the only Brazilian cordillera (~1000 km in extension), where many conservation units are distributed throughout areas of rough relief. A recent study of *Bokermannohyla saxicola* in the Espinhaço mountain range showed that human occupancy across the landscape reduces heterozygosity of populations (Eterovick *et al*. 2016), but topographic complexity could be an important underlying mechanism, although it was not explicitly analyzed. Our study underscores that our knowledge on diversification of Cerrado frogs is limited, therefore more research accounting for topographic barriers to dispersal is needed to

encompass different mechanisms leading to both speciation and the conservation of threatened species.

Despite limited research, it is now clear that topographic relief has shaped population diversification in this biodiversity hotspot in past times of little or no human influence (Prado *et al*. 2012; Guarnizo *et al*. 2016). In the last decades, however, accelerated habitat loss in this diverse savanna may have disrupted population connectivity to a point where increased topographic complexity potentiates isolation and genetic depression of populations. We highlight that topographic complexity can be used as a proxy for habitat connectivity, informing effective conservation strategies and the design of protected areas in the wild. The persistence of various subpopulations across a gradient of topographic configurations is important, as adaptation to different environments may offer resistance/resilience to stochastic events such as bottlenecks, especially for endangered animal lineages living in geographic formations under intense human modification (Peterson *et al*. 1998; Becker *et al*. 2007). Thus, the proper surveillance of the proposed expanded SCNP is desired to encompass this heterogeneity of environments, but in light of a changing world and the imminent biodiversity crisis, preserving many populations with facilitated gene flow is also desirable in terms of conservation. As a consequence, this may require safeguarding topographically homogeneous lands to maintain the genetic integrity and connectivity of the remaining amphibian populations.

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TABLES AND FIGURES

Table 1. Analysis of genetic differentiation (pairwise F_{ST}) among 12 sampling localities of *Bokermannohyla ibitiguara* within the state of Minas Gerais, Brazil. Two negative F_{ST} values were converted to zero (NSF–RC: -0.003 and NSF–RJ: -0.001). Comparisons among localities within SCNP (Serra da Canastra National Park) are in bold.

	CAL	GLG	PRT	R1	R2	R3	CM3	MP3	NSF	R25	RC	RJ
CAL	0											
GLG	0.027*	0										
PRT	0.038*	0.036*	0									
R1	0.121*	0.114*	0.133*	0								
R2	0.092*	0.089*	0.121*	0.080*	0							
R3	0.067*	0.068*	0.086*	0.098*	0.074*	0						
CM3	0.028*	0.028*	0.046*	0.093*	0.069*	0.051*	**0**					
MP3	0.032*	0.039*	0.064*	0.114*	0.087*	0.074*	**0.050***	**0**				
NSF	0.010†	0.020*	0.028*	0.099*	0.084*	0.055*	**0.002**	**0.029***	**0**			
R25	0.020*	0.024*	0.038*	0.091*	0.068*	0.054*	**0.003**	**0.041***	**0.003**	**0**		
RC	0.016*	0.029*	0.043*	0.098*	0.075*	0.058*	**0.013**	**0.030***	**0.000**	**0.005**	**0**	
RJ	0.018*	0.018*	0.039*	0.097*	0.080*	0.057*	**0.005**	**0.030***	**0.000**	**0.004**	**0.001**	**0**

* Significant under Bonferroni $P = 0.00076$; † Significant under B-Y method $P = 0.01037$

Table 2. Significant most parsimonious models explaining intra-population genetic indices of *Bokermannohyla ibitiguara* from 12 localities within the state of Minas Gerais, Brazil. Non-significant models were omitted.

Term	Beta	R^2	t	P
Allelic richness				
Topographic complexity	-0.524	-	-2.66	0.026
Elevation	0.464	-	2.36	0.042
** Whole model: $F[2,9]=13.54$; $P<0.01$; $R^2 = 0.75$*				
Effective number of alleles				
Topographic complexity	-0.593	0.35	-2.33	0.042
Expected heterozygosity				
Topographic complexity	-0.629	0.39	-2.56	0.028
Observed heterozygosity				
Elevation	0.612	0.37	2.45	0.034

Table 3. Comparisons of genetic measures between six populations of *Bokermannohyla ibitiguara* within the SCNP (Serra da Canastra National Park, state of Minas Gerais, Brazil) vs. six populations outside the SCNP, as determined across 21 microsatellites. Averages are reported for each category. Asterisks mark significant values at $P \leq 0.05$.

Genetic parameter	Within SCNP	Outside SCNP	One-way ANOVA *P*	Permutation test *P*
Allelic richness	7.293	6.317	0.027*	0.023*
Private allelic richness	0.395	0.373	0.756	–
Observed heterozygosity	0.663	0.620	0.050*	0.031*
Expected heterozygosity	0.770	0.748	0.220	0.044*
Inbreeding coefficient	0.170	0.191	0.286	0.379
Number of alleles	9.413	8.651	0.403	–
Effective number of alleles	5.642	4.957	0.151	–

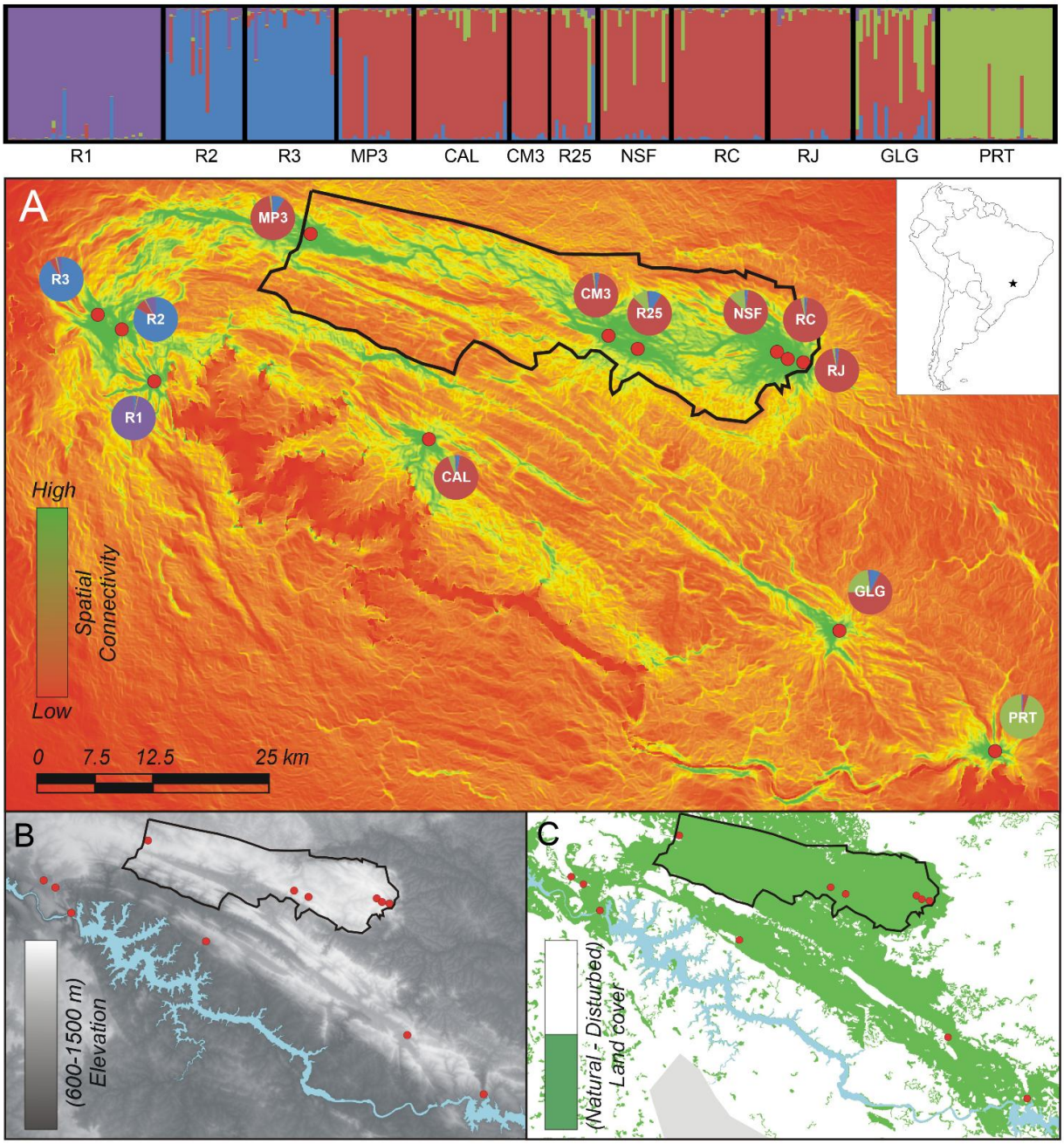

Figure 1. Structure plot (above) and assignments of 4 genetic clusters found for the 12 sampling localities (acronyms, see supplemental table 1) of *Bokermannohyla ibitiguara* based on 21 microsatellites. Spatial connectivity (A) based on topographic resistance obtained from a Digital Elevation Model (B). Land cover suitability map (C) is also shown. The outlines represent the Park boundaries and the star on the inset map is the general locality in southeastern Brazil.

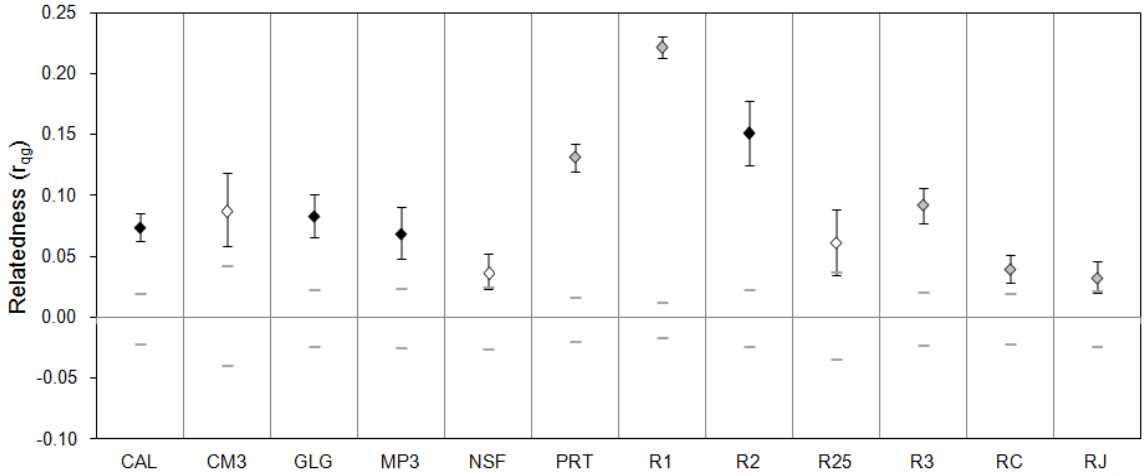

Figure 2. Average within-population relatedness for the 12 sampling sites of *Bokermannohyla ibitiguara* within the state of Minas Gerais, Brazil. Horizontal gray bars indicate the 95% confidence intervals that enclose relatedness values expected for panmitic populations. All populations significantly differed from panmixia. White diamonds mark populations with the fewest tadpole sampling (20 to 37%), gray diamonds mark populations with intermediate tadpole sampling (52 to 75%), and black diamonds mark populations with highest tadpole sampling (76 to 92%; see supplemental table 1).

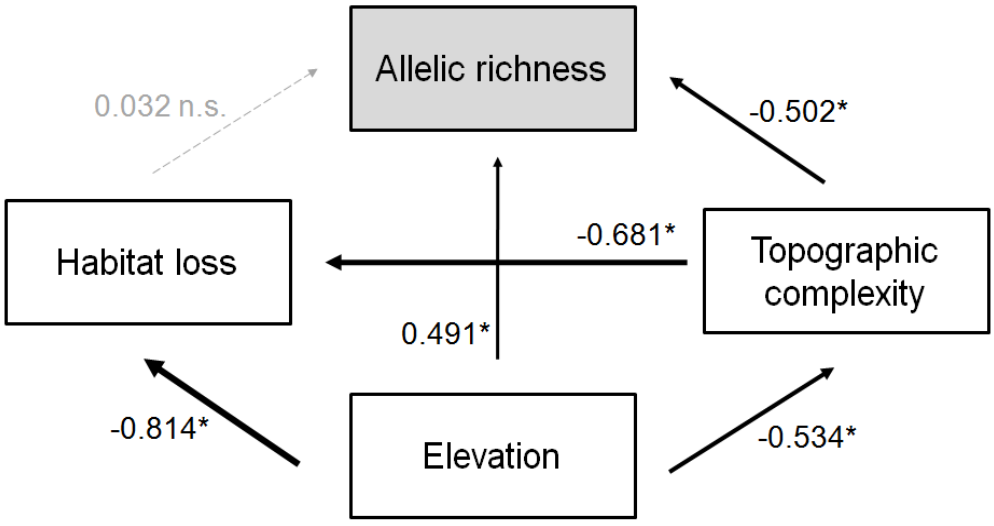

Figure 3. Path analysis showing the causal relationships among landscape variables and within-population allelic richness of *Bokermannohyla ibitiguara*. The relative magnitude of each effect is indicated by the thickness of each arrow. The grey/dashed arrow shows a non-significant result, and the asterisks mark significant results.

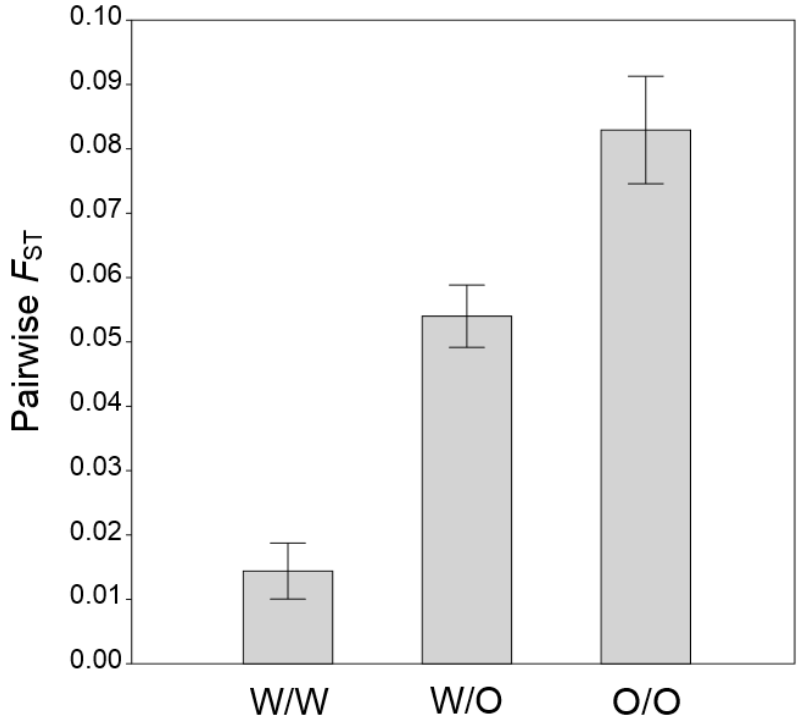

Figure 4. Means and standard errors of genetic differentiation values between pairs of populations of *Bokermannohyla ibitiguara* within the Serra da Canastra National Park only (W/W), within vs. outside the Park (W/O), and outside the Park only (O/O), state of Minas Gerais, Brazil. The three groups were statistically different (all $P < 0.01$).

Supplemental Table 1. Information on 12 sampling streams of *Bokermannohyla ibitiguara*, Serra da Canastra mountain range, state of Minas Gerais, Brazil (see figure 1).

Locality name (acronym)	Within PNSC?	Latitude (°S)	Longitude (°W)	Elevation (m)	Gallery forest (%)	*N* adult samples	*N* tadpole samples	Tadpoles (%)
Córrego Água Limpa (CAL)	No	20.3315	46.7972	810	28.18	2	23	92
Capão de Mata 3 (CM3)	Yes	20.2273	46.6164	1,339	3.11	8	2	20
Gruta do Lobo Guará (GLG)	No	20.5242	46.3838	1,064	7.91	4	18	82
Mata da Portaria 3 (MP3)	Yes	20.1246	46.9164	1,226	3.46	5	15	75
Nascente do São Francisco (NSF)	Yes	20.2434	46.4466	1,353	1.12	15	4	21
Pousada do Rio Turvo (PRT)	No	20.6458	46.2269	779	8.04	9	22	71
Riacho 1 (R1)	No	20.2732	47.0739	737	29.08	20	22	52
Riacho 2 (R2)	No	20.2208	47.1066	875	16.69	5	16	76
Riacho km 25 (R25)	Yes	20.2404	46.5868	1,290	2.54	8	4	33
Riacho 3 (R3)	No	20.2062	47.1309	836	23.65	8	16	67
Riacho do Campo (RC)	Yes	20.2507	46.4357	1,393	5.56	11	14	56
Retiro do Jaguarê (RJ)	Yes	20.2540	46.4203	1,304	5.46	7	15	68

Average straight-line distance among localities: 44.8 ± 26.4 km; range = 1.4 - 106 km

Supplemental Table 2. Tissue accession numbers (CFBH-T) of *Bokermannohyla ibitiguara* individuals used in this study, separated by locality. Tissues were deposited at Coleção de Anfíbios Célio F. B. Haddad, Rio Claro, São Paulo, Brazil. When available, the collected specimen (CFBH) is stated in parentheses next to each tissue number.

Locality	Vouchers
CAL	18246 (40595), 18247-18255, 18256 (40596), 18257, 18259-18271
CM3	17300 (40566), 17301 (35867), 17305, 17308 (35868), 17309 (35869), 17310 (35870), 17321 (35871), 17322 (35872), 17323 (35873), 17324
GLG	18140 (40582), 18141-18151, 18152 (40583), 18153 (40584), 18154 (40585), 18155-18161
MP3	18112 (40577), 18113-18118, 18119 (40578), 18120 (40579), 18121 (40580), 18122 (40581), 18123-18131
NSF	17238 (35859), 17239 (35860), 17240 (35861), 17245 (35862), 17246 (35863), 17253 (35864), 17254 (35865), 17255 (35866), 17256 (40564), 17257-17261, 17275, 17276, 17277 (40565), 17278, 17279
PRT	18215 (40586), 18216 (40587), 18217 (40588), 18218 (40589), 18219 (40590), 18220 (40591), 18221 (40592), 18222 (40593), 18223-18244, 18245 (40594)
R1	17338 (31751), 17339 (31752), 17340-17345, 17348 (31755), 17349 (31756), 17350 (31757), 17351 (31758), 17352 (31759), 17353 (31760), 17354 (31761), 17355 (31762), 17356 (31763), 17357 (31764), 17358 (31765), 17360 (31767), 17361 (31768), 17363 (31770), 17364 (31771), 18069-18084, 18211 (40622), 18212 (40623), 18213 (40624)
R2	18100-18107, 18108 (40573), 18109 (40574), 18110 (40575), 18111 (40576), 18132-18139, 18201
R25	17306 (40562), 17307 (40563), 17316 (40567), 17325 (40568), 17326 (40569), 17327 (40570), 18200 (40617), 18202 (40618), 18203-18206
R3	17334 (31747), 17335 (31748), 17336 (31749), 17337 (31750), 17346 (31753), 17347 (31754), 17359 (31766), 17362 (31769), 18085-18099, 18214 (40548)
RC	17241 (40549), 17247-17249, 17262 (40556), 17263 (40557), 17264 (40558), 17265, 17280 (40559), 17281-17290, 17298 (40560), 17299 (40561), 17317 (40571), 17318-17320
RJ	17242 (40550), 17243 (40551), 17244 (40552), 17250 (40553), 17251 (40554), 17252, 17266 (40555), 17267-17274, 17291-17297

Supplemental Table 3. Linear regression models of four landscape variables vs. seven genetic measures of 12 *Bokermannohyla ibitiguara* populations within the state of Minas Gerais, Brazil. AICc = Akaike Information Criterion. Bold values = statistically equivalent models according to delta AIC.

Effective number of alleles			Inbreeding coefficient		
Variables	AICc	Δ AIC	Variables	AICc	Δ AIC
Topographic complexity	31.799	**0**	Elevation	-42.42	**0**
Elevation	32.53	**0.731**	Habitat loss (%)	-42.22	**0.2**
Gallery forest (%)	34.887	3.088	Topographic complexity	-41.235	**1.185**
Topographic complexity, Elevation	34.916	3.117	Gallery forest (%)	-41.117	**1.303**
Habitat loss (%), Elevation	35.626	3.827	Elevation, Gallery forest (%)	-40.087	2.333
Allelic richness			Private allele richness		
Variables	AICc	Δ AIC	Variables	AICc	Δ AIC
Topographic complexity, Elevation	24.845	**0**	Topographic complexity	-13.589	**0**
Topographic complexity	25.904	**1.059**	Habitat loss (%)	-12.269	**1.32**
Elevation	27.099	2.254	Gallery forest (%)	-10.73	2.859
Topographic complexity, Gallery forest (%)	28.107	3.262	Elevation	-10.51	3.079
Topographic complexity Habitat loss (%)	28.357	3.512	Topographic complexity, Habitat loss (%)	-10.094	3.495
Expected heterozygosity			Number of alleles		
Variables	AICc	Δ AIC	Variables	AICc	Δ AIC
Topographic complexity	-47.773	**0**	Topographic complexity	48.086	**0**
Elevation	-44.343	3.43	Elevation	49.858	**1.772**
Topographic complexity, Elevation	-43.376	4.397	Gallery forest (%)	50.74	2.654
Topo. Complex, Habitat loss (%)	-43.099	4.674	Habitat loss (%)	51.56	3.474
Gallery forest (%)	-43.001	4.772	Topographic complexity, Elevation	51.56	3.474
Observed heterozygosity					
Variables	AICc	Δ AIC			
Elevation	-41.163	**0**			
Topo. Complex	-38.116	3.047			
Elevation, Gallery forest (%)	-37.992	3.171			
Gallery forest (%)	-37.219	3.944			
Elevation, Habitat loss (%)	-37.111	4.052			

CHAPTER II:

ENVIRONMENT, GENETICS OR SEXUAL SELECTION? PHENOTYPIC DIFFERENTIATION IN A NEOTROPICAL TREEFROG

Renato C. Nali, Kelly R. Zamudio & Cynthia P. A. Prado

ENVIRONMENT, GENETICS OR SEXUAL SELECTION? PHENOTYPIC DIFFERENTIATION IN A NEOTROPICAL TREEFROG

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ABSTRACT

Variation among animal populations is common, yet studies rarely combine various traits and mechanisms to address adaptive and neutral drivers of differentiation. We compared morphological and acoustic variation of a Neotropical tree frog with complex reproductive behaviors across its distribution range. We then examined the roles of sexual selection, genetic drift, and acoustic adaptation to forested habitats (Acoustic Adaptation Hypothesis; AAH) on call differentiation. Calls varied more than morphology, suggesting stronger selective pressures on this behavioral phenotype. Acoustic traits associated with individual discrimination or female attraction were significantly different among populations. Genetic differentiation did not explain acoustic population distances. Likewise, forest cover did not explain acoustic differences according to AAH predictions. Call traits that varied in contrast with AAH indicated a role of male competition associated with likely increased density. Thus, individual recognition and female preferences may have shaped call differentiation in this species, while intermale competition appears as an underlying mechanism (inter and intrasexual selection). Female choosiness and male territoriality observed for this species support our results. This study shows that a multi-trait and multi-mechanism approach can elucidate intricate processes leading to behavioral differences, and that species with complex reproductive behaviors are particularly interesting models.

Keywords: Sexual selection, Amphibia, acoustic adaptation hypothesis, morphometry, bioacoustics, genetic differentiation

INTRODUCTION

Evolution can be understood as micro-evolutionary ongoing mechanisms based on populations as the unit of evolutionary change, which can be accounted for successful speciation events or otherwise possible extinction of species over time (Crow & Kimura 1970; Futuyma 2005; Wagner 2016). Diversification events in different organisms may occur upon reproductive displacement due to sexually selected divergent traits during mate recognition, or vicariant events in which discontinuities of the environment preclude gene flow and the subsequent genetic differences will create unique lineages (Gerhardt 1999; Manel et al. 2003; Funk et al. 2005). As the association among traits are complex, population divergence should be studied in a multi-character framework, composed of genotypes and phenotypic/behavioral traits, subject to different selective mechanisms. These mechanisms can in turn be adaptive, such as sexual and ecological selection, or neutral, such as genetic differentiation due to genetic drift and isolation (Wilkins et al. 2013).

Geographic variation among animal populations is a common pattern across taxa (Talal et al. 2015; Mitani et al. 1999; Elmer et al. 2010; Brusa et al. 2013). Genetic differentiation normally accumulates with geographic distance and other landscape variables that limit gene flow (Manel et al., 2003; chapter 1), potentially increasing variation in correlated phenotypes in populations scattered throughout a landscape. One of the most conspicuous phenotype that has been classically acknowledged in different animal populations is morphology. Morphological variation has always been reported in the field of taxonomy, and more recently combined with genetic variation to uncover, for example, undetected cryptic species and diversification of lineages (e.g., Elmer et al. 2010; Havermans et al. 2013; Warwick et al. 2015; Ortega-Andrade et al. 2015). However, few studies have combined population genetics, morphological variation, as well as behavioral phenotypes to study the evolution of animal lineages (Lougheed et al. 2006; Funk et al. 2011; Talal et al. 2015).

Acoustic communication has always interested behavioral ecologists, as it is conspicuous in a broad range of taxa, such as birds, mammals, insects, frogs and fishes (Bradbury and Vehrencamp 2011). Animal communication mediates virtually every aspect of an organism's life history, from interespecific interactions, such as predator-prey recognition and interactions and selective pressures imposed by acoustic partitioning (Ryan et al. 1982; Sueur 2002; Moreno-Gómez et al. 2013), to intraspecific ones. The latter consists of same-sex interactions, normally competition among males for territories and/or females, and opposite-sex interactions, usually females choosing males among the available ones in the population (Ryan 1990, Gerhardt 1994). Discrimination mechanisms among species and within populations will rely on the reception and interpretation of acoustic signals (Wilkins et al. 2013), and successful communication will reverberate on the ecology and natural history of a species (Endler 1992). Ultimately, understanding how and why call characteristics vary among populations means clarifying the scenario in which individuals have shaped their social interactions to ensure greater reproductive success, with direct consequences on the evolutionary history of lineages.

In many organisms, certain morphological and call traits are intimately related; for example, individuals and species with larger body sizes emit low-pitched calls, which have been attributed to physical aspects of the vocal apparatus (Ryan and Brenowitz 1985; McClelland et al. 1996; Barclay et al. 1999; Nali and Prado 2014a). Depending on the mating system, females can use acoustic clues to select larger males, due to their higher ability to defend a territory with important resources for reproduction (e.g., food, breeding sites). Alternatively, females may prefer males with similar body sizes to increase fertilization success during spawning, mainly in animals with external fertilization and cloacae juxtaposition (Bastos & Haddad 1996; Lu et al. 2010). The combination of acoustic and morphological characteristics is crucial to infer mechanisms of adaptive evolution within and among populations (Lougheed et al. 2006). These intertwined factors demonstrate that not only multiple-traits, but also multiple possible mechanisms shaping variation, are crucial when dealing with natural history, behavior and evolution of lineages (e.g. González et al. 2011; Warwick et al. 2015).

A well-studied adaptive mechanism shaping call differentiation is sexual selection, to which frogs have been a good model (Gerhardt 1994; Ryan and Rand 2003a; Lemmon 2009). Acoustic selection can either be stabilizing, in which females

prefer values toward the mean to avoid heterospecific mating, or directional, in which females prefer extreme values that indicate high-quality males (Ryan 1990). In addition, emission of certain variables and parts of the call might evolve to repeal other males, also with direct consequences on male reproductive success (Endler 1992; Nali and Prado 2014a; Reichert 2014). Therefore, sexual selection could operate differently and generate call differentiation among populations. On the other hand, non-adaptive evolution due to genetic isolation and drift can also lead to call differentiation. As mentioned above, population demographic history and landscape barriers can accumulate genetic differences, which in turn can shape call traits, with a diminished or absent effect of sexual selection (Velásquez et al. 2013). In this case, neutral processes, as opposed to selective mechanisms, would be responsible for most of the call variation, or at least contribute effectively to shape the acoustic window that could then be subject to selection (i.e., the available space for signals to evolve in a specific taxon in a specific locality; Wilkins et al. 2013).

Despite the efforts to investigate call differentiation based on sexual selection, and to a less extent based on genetic isolation, significantly less attention has been given to the role of ecological/environmental adaptive mechanisms (see Wilkins et al. 2013). One of these mechanisms proposed is the Acoustic Adaptation Hypothesis (AAH; Morton 1975; Hansen 1979), which states that acoustic signals are adapted to maximize transmission success by avoiding degradation and attenuation, as production and reception of signals are equally important to initiate receiver's behavioral responses (Endler 1992). Acoustic communication across an obstructed environment, such as a forested area, may impose certain selective pressures on temporal and spectral parameters. Calls with lower frequencies would evolve in more closed environments, due to physical properties that warrant more efficient transmission across the vegetation (Morton 1975). Calls would have longer duration because longer signals in an obstructed environment would both increase the probability of its detection, and create reverberations that enhance propagation distance (Slabbekoorn et al. 2002; Nemeth et al. 2006). Consequently, the repetition rate of notes would be lower to avoid overlapping of the reverberating signals, which could disturb the receiver's detection. The AAH has been investigated mostly for birds and mammals, to which there is controversial, yet stronger support (Boncoraglio & Saino 2007; Ey and Fischer 2009). Significantly less studies are known for frogs, and yet with very inconsistent results (reviews in Ey and

Fischer 2009; Erdtmann and Lima 2013). Yet, a recent study evidenced that vegetation cover can indeed explain a good part of call variation (Ziegler et al. 2011). Studies with other species of frogs with quantitative environmental characterization are much welcomed to understand the possible role of the environment in calling behavior (Erdtmann and Lima 2013), preferably if various mechanisms can be combined.

Our focal species is the Brazilian Cerrado frog *Bokermannohyla ibitiguara* (Anura, Hylidae), which inhabits streams covered by riparian forest. This is a territorial treefrog with a complex courtship (Nali and Prado 2012), complex calling behavior (Cardoso 1983; Nali & Prado 2014a), and individual call variation (Turin et al. submitted). Populations are genetically differentiated among most breeding sites, which has been attributed mostly to topographic complexity as an effective physical barrier to migration (Chapter 1), but possible pre-mating barriers due to phenotypic differences are unknown. We used this species as a model to investigate (1) whether calls and morphology are significantly variable phenotypes; (2) whether call variation is more pronounced than morphological variation and (3) whether neutral processes (genetic drift/isolation) and/or adaptive mechanisms (sexual selection and environment adaptation) shaped call differentiation in this species. We draw attention to the intertwined relationships among the analyzed mechanisms for call variation, and finish with perspectives for these important types of studies.

MATERIALS AND METHODS

Study site and individual sampling

The Serra da Canastra mountain range is located in southeastern 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). The area was originally covered by the Cerrado vegetation, patches of semideciduous forest, gallery forests placed in valleys alongside streams, and grassland fields at higher elevations, reaching up to 1,500 m (Dietz, 1984).

We sampled 12 streams throughout the area in the rainy months (October-March) from 2010 to 2015 (figure 1). Individuals were found by active search at the breeding sites guided by male vocalizations. We recorded calls at approximately 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. We measured air temperature for each recording with an analogical thermometer. Most males were then collected for morphological measurements, euthanatized by spraying a solution of lidocaine 10% in the gular region, fixed in formalin 10% and preserved in ethanol 70% (McDiarmid, 1994). Unvouchered individuals were identified by unique natural marks (Nali & Prado 2014a) or marked with the toe-clipping method (Chapter 1). Collected specimens were deposited at the Coleção de Anfíbios Célio F. B. Haddad, Department of Zoology, Universidade Estadual Paulista "Júlio de Mesquita Filho", Rio Claro, SP, Brazil (supplemental table 2).

Morphological and call variables' measures

To avoid biases, a single researcher (RCN) measured the voucher specimens and analyzed the calls. A Mitutoyo digital caliper was used to measure 12 morphometric traits to the nearest 0.01 mm for each collected specimen (supplemental figure 1): 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 randomly measured (independent of population), and every measure was taken from the same side across specimens.

All calls were analyzed 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. Males of *B. ibitiguara* emit calls with long notes, and short notes always in sequences (Cardoso 1983; Nali and Prado 2014a). Thus, we measured 14 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 short notes' sequence (SNseq min freq); 9) dominant frequency of the short notes' sequence (SNseq dom freq), 10) duration of the short notes' sequence (SNseq dur); 11) number of long notes per minute (LN/min); 12) number of short notes per minute (SN/min); 13) number of short notes' sequences 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 five long notes, five short notes and up to five sequences of short notes per individual, and calculated the mean for each variable. When few individuals called from inside cavities and/or at water level, only temporal variables were measured, due to physical differences in spectral parameters in those situations (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. These quality control procedures yielded ca. 8.4% of missing data, and as a result we had slightly different sample sizes across our analyses. Specifically in the Canonical Variate Analysis (see below), in which missing data of a single variable results in individual exclusion, we completed the few blanks with average values per stream, following our prediction of acoustic differences among streams.

We calculated Person's product-moment correlations between air temperature and all 14 call parameters to remove the influence of this variable. Temperatures were then adjusted to the average temperature of 20.67°C (range = 17 - 25; SD = 2.16; N = 69) based on the overall regression coefficients for each parameter (e.g. Pröhl et al. 2007). To remove body size effect, we did the same for correlations with male snout-vent length (SVL); significant call parameters were adjusted to the average SVL of 39.95 mm (range = 33.3 - 49.15 mm; SD = 3.6; N = 82). We used this fully adjusted acoustic dataset in subsequent analyses.

Genetic distances and genetic cluster assignment

To assess population genetic distances in this species, we used a previously developed genetic distance matrix (Chapter 1). The matrix was obtained for the exact same 12 populations, in which 273 individuals (including most of those used in this study) were genotyped at 21 microsatellite markers developed for the species (Nali et al. 2014; Chapter 1). The genotypes were then used to establish pairwise F_{ST} values ($F_{ST}/[1 - F_{ST}]$) between pairs of populations, one of the most used measures of genetic distance (Rousset, 1997). Likewise, each population was classified as belonging to one of the four genetic clusters assigned previously under a Bayesian analysis for the same 273 individuals on the software STRUCTURE (Pritchard et al. 2000; Chapter 1; supplemental

table 1; figure 1): Sacramento 1, Sacramento 2, SCNP and Capitólio. In this analysis, delta K showed a clear peak for these 4 clusters, with high average coefficient of membership (percentage of individual assignment = 91.6%; Chapter 1). Detailed procedures on laboratory protocols, software parameters, quality control of data and interpretations can be found in Chapter 1.

Morphological and acoustic variation: relative contributions, relationship and basis for sexual selection

We used two complementary classifications when analyzing acoustic and morphological variation: by populations (12 streams) and by genetic clusters (4 clusters; Chapter 1). Dividing by populations allowed us to see potential selective mechanisms at the breeding sites; dividing by genetic clusters helped us explore the variation in genetically differentiated groups. First, we used Kruskal-Wallis to test for statistical differences in morphometric and call variables among populations and clusters, using the software STATISTICA (StatSoft, 2011). We also calculated coefficients of variation in percentage (CV = standard deviation/mean*100) for each morphometric and acoustic variable. Only males were considered in this analysis, as they are the targets of sexual selection. CVs among individuals from the same population/cluster were calculated by obtaining the average and standard deviation (SD) for each population. CVs among populations/clusters were then calculated from grand averages and SDs, i.e., based on averages of each population/cluster. Simple comparisons among the coefficients of variation informed whether each trait varied more among or inside populations/clusters. Additionally, we ran a Mann-Whitney test to see if CVs of acoustic and morphological traits were higher inside each genetic cluster than among clusters; the population approach in this case was not used due to extreme discrepancies in sample sizes.

We ran different analyses to compare morphological and call variation in this species. First, we used a Mann-Whitney test with CVs of variables that significantly differed among populations under Kruskal-Wallis to check weather among-population variation was higher for calls compared to morphology. We used Canonical Variate Analyses (CVA) with log-transformed morphological and acoustic variables in STATISTICA, again dividing by either populations or clusters and pooling all morphological or acoustic variables together. We then used Wilks' lambda tests to see if

individuals were significantly discriminated, and also looked at the percentage of individuals that were correctly classified for each morphological and acoustic analysis. A lack of discrimination and low percentages of correct classifications mean less differentiation according to each dataset. The first two Canonical roots of each analysis were plotted, with points classified by populations/clusters, using the software PAST (Hammer et al. 2001). From the CVA analyses, we also calculated morphological and acoustic distance matrices among populations (squared Mahalanobis distances). These matrices were then correlated using Mantel tests (Mantel 1967) to check for an association between morphological and call differentiation in this species.

Neutral evolution on call differentiation

To test for correlations between genetic vs. call distances, i.e., possible neutral evolution on calls, we first calculated individual Mahalanobis distances for acoustic variables that were significantly different among populations in the Kruskal-Wallis tests (e.g. Funk et al. 2009). We then used Mantel tests to correlate the genetic distance matrix vs. the distances of each significant acoustic parameters. Additionally, we used a squared Mahalanobis matrix of acoustic distances among populations calculated from our CVA, and ran the Mantel correlation between genetic distance vs. global acoustic distance. Geographic distance was held constant in these analyses due to a significant relationship with acoustic distances (see Results). In this species, topography, and not geographic distance, best explains genetic differentiation (Chapter 1). A correlation between call distance and geographic distance is thus less informative than the genetic distance itself on the neutral evolution of calls in this species; nonetheless, we also reported the results without controlling for geographic distance. We obtained simple Mahalanobis distances and ran Mantel tests using PASSAGE v. 2.0 (Rosenberg and Anderson 2011).

Environmental influence on call differentiation

We employed a quantitative approach to test if call differentiation among populations could result from acoustic adaptation to different forest covers. High resolution satellite images from each population were extracted from the software Google Earth Pro. The images originated from CNES/Astrium satellites (pixel resolution = 0.35 m), during 2013 or 2014, years that coincide with most of our fieldwork, and were visualized from ca. 1.5 km altitude. Each picture was georeferenced and processed in ArcGIS 9.3.1 (ESRI 2009).

We drew a circle with a 500 m radius around the centroid of each population, and classified the gallery forests therein manually under visual supervision. We then calculated the percentage of forested area per locality (relative to the total circle area) and used it as a proxy, so that higher percentages mean a more closed environment. Finally, we conducted linear regression analyses between the percentage of forested area in each population vs. ten different call variables. Following the general predictions of the AAH, the call variables used in this analysis were: minimum frequencies of long notes, short notes, and short notes' sequences; dominant frequencies of long notes, short notes, and short notes' sequences; duration of long notes, short notes, and short notes' sequences, and note repetition rate. The latter was obtained by combining the mean number of long and short notes per minute for each stream.

RESULTS

Individual sampling and dataset correction

We recorded 82 males, and measured 87 collected male specimens. Air temperature was significantly correlated with 12 out of the 14 call variables, so the dataset was corrected accordingly. Male size (SVL) was correlated with 7 call variables, and we proceeded likewise. Corrected call variables can be assessed in supplemental table 3.

Morphological and acoustic variation

Summaries of our means, standard deviations and CVs for morphological and acoustic variables can be found on supplemental tables 3 to 6. Eight call parameters (the six spectral parameters plus SNseq dur and LN/min) were significantly different among populations; among clusters, the results were similar, with differences in some parameters. For morphology, all 14 parameters were different among 12 populations, but only tympanum diameter was different among the four genetic clusters.

CVs of most call variables were numerically higher on average inside each population than among populations, except SNseq min freq, SNseq dom freq, and SNseq dur. All call variables showed, on average, numerically higher CVs inside each cluster than among clusters, with a significant difference ($U = 265$, $P < 0.05$). All CVs of morphometric variables were numerically higher on average inside populations, instead

of among populations. The same was observed when considering the genetic clusters, with a significant difference (U = 13, $P < 0.0001$).

CVs for call variables that were significantly different among populations ranged from 5.06 % to 29.46% (average 11.2%; N = 8; supplemental table 3), and those for morphometric variables ranged from 4.76% to 9.92% (average 6.11%; N = 12; supplemental table 4). Acoustic CVs were significantly higher than morphological CVs (U = 25; $P < 0.05$), meaning that calls varied more than morphology across populations. Overall discrimination of individuals was significant (all $P < 0.001$; Wilks' lambdas: call [cluster] = 0.25; call [population] = 0.016; morphology [cluster] = 0.344; morphology [population] = 0.026), but scatterplots of the first two roots of our Canonical Variate Analyses showed morphological and acoustic overlapping (figure 2). This overlapping was less evident for calls; for instance, the Capitólio cluster separated completely in calls, but not in morphology; the Sacramento 1 cluster completely overlapped with others in morphology, but not in calls (figure 2A-B). All populations tended to overlap towards the center for morphology, which was not observed for calls (figure 2C-D). Accordingly, individuals were classified correctly in the discriminant analysis more for calls (clusters = 77.2%; populations = 75.6%) than for morphology (clusters = 73.5%; populations = 65.5%). These differences agree with our partial Mantel test (controlling for geographic distance), which showed no significant correlation between morphological distance and acoustic distance among populations (table 1). This result remained non-significant even without controlling for geographic distance ($R = 0.29$; $P = 0.11$).

Neutral evolution and call differentiation

Supplemental tables 7 to 10 contain, respectively, the matrices for genetic, morphological, acoustic and geographic distances among populations. Our partial Mantel tests with genetic distance vs. single call parameters' distances were all non-significant (table 2). This result is in agreement with our partial Mantel test between global acoustic distance vs. genetic distance, which yielded a non-significant result (table 1). This remained non-significant without controlling for geographic distance (global Mantel $R = 0.07$; $P = 0.36$).

Environmental adaptation and call differentiation

Percentage of gallery forest varied from 1.12% to 29.08% among our 12 populations (supplemental table 1). Our regression analyses between the percentages of gallery forest vs. 10 call variables yielded non-significant results for all but two parameters; increases in forested areas represented increases in SN min freq and SNseq min freq (table 3).

DISCUSSION

Morphology, calls and sexual selection

In this multi-trait and multi-mechanism study of a Neotropical frog, our results showed a significant morphological and call variation across its distribution range; however, calls varied much more than morphology. Looking at the coefficients of variation (CV) for each acoustic and morphological variable, while morphology was relatively conserved among and inside populations and clusters, call characteristics showed more pronounced variation. Among-populations CVs of call variables were also statistically higher than those of morphological variables. When comparing morphological vs. acoustic differentiation among the genetic clusters (i.e., differentiated lineages), nine call variables were significantly different, whereas only one tympanum diameter was morphologically different, an attribute clearly related with acoustic communication (Narins et al. 2007). These first results suggest that directional selective pressures on call parameters would be stronger compared to morphology, and one can observe that directly when analyzing variation on call variables and higher CVs, or indirectly, when looking at the one differentiated morphological variable related to acoustic communication, and lower CVs.

Analyses of combined variables also confirmed the differences between call and morphology. First, morphological distances among populations were not correlated to call distances (table 1). Second, in our Canonical Variate Analysis, overlapping was more evident in morphological traits than call traits, with a slightly more accurate individual classification for calls (figure 2). Other frog species show more intense call variation among lineages when compared to morphology, including some in the Hylidae family (e.g. Lougheed et al. 2006; Funk et al. 2011). It is predicted that sexual selection on traits responsible for mate recognition may precede selection on other phenotypes, such as

morphology; this behavioral mechanism could then drive population differentiation and even speciation at a higher evolutionary rate (Hoskin et al. 2005; Lougheed et al. 2006; González et al. 2011). Given that female frogs are well-known to recognize and select males based on call properties (Gerhardt 1994; Murphy and Gerhardt 2002; Baugh and Ryan 2010), this difference was expected and suggests sexual selection shaping call variation. Specifically for *B. ibitiguara*, the operational sex-ratio is highly male-biased (OSR; the relative number of reproductive males to females during a breeding event; Nali and Prado 2012; R. C. Nali, pers. obs.). When a single female frog arrives at a breeding site with many active calling males, it can potentially choose among them (e.g. Murphy and Gerhardt 2002; Schwartz et al. 2004), which was observed for this species (Nali & Prado 2012). Calls can be perceived and discriminated at a great distance, whereas choosing morphological traits directly (e.g. male size) would require a much closer contact. Thus, even if females ultimately seek larger males that best defend their territories (Nali & Prado 2012), or males with similar sizes to increase fertilization success (size-assortative mating; e.g., Bastos & Haddad 1996), selecting calls may be a first filter that highly increases female efficiency in selecting partners.

While variability is crucial for selection, variable and differentiated call traits alone are not sufficient to show the role of sexual selection mechanisms, also because call traits could simply be more plastic than morphology. However, there is evidence for a link between those traits and female preferences and/or male competition in this species. For example, LN/minute was a significantly different trait across populations; long notes of the advertisement call are believed to play a role in female attraction (Nali and Prado 2014a), further supported because the courtship call (the call directed specifically to females), consists of long notes only (R. C. Nali, pers. obs.). Increasing the emission of long notes may have an effect in female attraction and thus indicates sexual selection. Female frogs may indeed prefer calls with higher repetition rates (Sullivan 1983; Schwartz 1986; Forester & Czarnowsky 1985). Moreover, regarding the remaining variables significantly different among populations in this study, duration of the short notes' sequence, dominant frequencies and call duration are among those that contribute mostly to individual discrimination in this species (Turin et al., submitted). Thus, females may rely on those variables to select potential mates, while males could use these signals to recognize conspecific competitors (Bee et al. 2001). According to Endler (1992), the degree of intermale competition and territoriality will affect the

structure of calling signals sent by reproductive males, and at the same time, the mating success of these males can affect the evolution of calls, especially in species with complex courtships. On top of that, the occurrence of alternative mating strategies like satellite males (opportunistic males that do not call and instead try to intercept females) could alter the distribution of different calls at a breeding site, because satellites can mate without being acoustically selected by the female (Forester & Czarnowsky 1985; Wells 2007). The above factors have been observed for the studied species (male territoriality, complex courtship and satellite males; Nali & Prado 2012, 2014a, b), and may differ among breeding sites, as they are social-dependent strategies, promoting call differentiation.

Our data point to sexual selection on call characteristics which may shape call differentiation over time. However, even with call differences more prominent than morphology, and the corresponding relationships with female preferences and individual recognition, are there real pre-mating barriers across the geographic range of the species? Our results point to a negative answer. All morphological and all but three acoustic CVs were numerically higher inside populations than among populations; all CVs were higher inside clusters than among clusters. Individuals from different populations and clusters do not occupy the exact same space in our multivariate analyses, but we did not find clear gaps among populations/clusters either (figure 2). If available males at a single breeding site (or at breeding sites within a genetic cluster) show more variation in morphology and call traits, with overlapping values in the CVA, there is little reason to assume that females might reject outside males based on these phenotypes. In other words, our results do not meet the criteria that among-population differences should be greater than within-population differences, so that significant pre-mating barriers should occur (Gerhardt 1999, 2013). Thus, despite our evidence for sexual selection, our results do not support behavioral pre-mating barriers in this species, especially for calls, which are a crucial part of the mate recognition systems in frogs. We confirm here that genetic divergences in this species may rely on the individual ability to disperse across breeding habitats (Chapter 1), and not on prevented mating between a male that successfully dispersed and a local female that did not prefer/recognize the male. Playback experiments with females would be useful to confirm such assumptions (Ryan and Rand 2003a,b; Velásquez et al. 2015).

Neutral evolution and ecological adaptation of calls

Even with evidences for sexual selection shaping call variation in this species, we went further to test for other possible intertwined mechanisms: neutral evolution of calls (due to isolation and genetic drift) and ecological adaptation of call signals (the AAH). Our partial Mantel test among genetic and global acoustic population distances yielded a non-significant result; accordingly, distances of the eight call parameters that significantly differed among populations were also unrelated to genetic distance. First, these results corroborate a lack of behavioral pre-mating barriers among different populations, as discussed above, given that the evolution of call differentiation and genetic differentiation are at least to some extent decoupled in this species. Moreover, the lack of correlation between genetic and acoustic distances again points to selective mechanisms on call differentiation, possibly related to non-random mating, rather than genetic drift and landscape isolation effects (e.g. Boul et al. 2007). Our results differ from those of another Neotropical frog (*Pleurodema thaul*), in which call and genetic distances were correlated, so call variation was attributed to genetic drift and isolation (Velásquez et al. 2013). Further playback experiments demonstrated that despite the influence of inter-male interactions on the signal evolution, lack of female preferences suggested a minor role of intersexual selection (Velásquez et al. 2014, 2015). The difference between this species and *B. ibitiguara* may thus rely on strong sexual selection pressures on both sexes of the latter, i.e., evolution of choosy females consistent with complex mating behaviors and requirement of good oviposition sites (Nali & Prado 2012; this study), combined with strong male-male competition and complexity of aggressive signals (Nali and Prado 2012, 2014a, b).

Individuals of *B. ibitiguara* are adapted to streams along gallery forests where oviposition sites have been observed (Nali and Prado 2012; R. C. Nali, pers. obs.); thus, males are more likely to call from forested areas, which could have imposed ecological pressures on call evolution. However, we found no evidence for the Acoustic Adaptation Hypothesis in this species, at least regarding dominant frequencies, minimum frequencies, durations and repetition rate of calls. Eight acoustic variables did not correlate with percent of gallery forest, and in the two significant correlations, the direction was the opposite than that predicted by the AAH (see discussion below). One could argue that behavioral plasticity of males was responsible for this lack of association; by measuring microhabitat of vocalization in a very fine-way, one study has

found that male frogs are capable of adapting to surrounding environment, with direct consequences on call trait variation (Ziegler et al. 2011). As explained in the Methods section, males of *B. ibitiguara* that call from inside cavities or at water level may emit calls with lower frequencies (R. C. Nali, pers. obs.), but frequencies of these few males were excluded from the analyses. Classifying areas of forest vs. non-forest for each locality is valid because environmental selection on call traits might be stronger in forests, but relaxed or minimal in open habitats (Ryan et al. 1990), and it has yielded answers on the AAH in different animals (Mitani et al.1999; Boncoraglio & Saino 2007). Furthermore, our quantitative assessment of gallery forest percentages was a step further compared to broad qualitative classifications commonly used for frogs (review in Erdtmann and Lima 2013). The bottom line is, by employing a quantitative analysis we found that environment may have a minor role in shaping call diversification in *B. ibitiguara*, as previously found for some frog species (Castellano et al. 2003; Penna and Moreno-Gómez 2015).

Interestingly, contrary to the AAH, individuals that inhabit localities with less gallery forest emitted short notes and short notes' sequences with lower minimum frequencies. As these results are based on correlations, other factors could interweave the associations here found, as normally observed for deviations from the AAH (Kalko 1995; Sugiura et al. 2006; Ey and Fischer 2009). Short notes convey a territorial/aggressive message in this species, such that males emit these notes and even lower their frequencies (i.e. the aggressive call) to repel intruders and avoid physical combats. (Nali and Prado 2014a). Since competition for breeding sites would be higher in less forested areas (likely increased male density), emiting calls with lower frequencies would be an advantage for competing males, given that lower frequencies are correlated with larger body sizes (Nali and Prado 2014a; Reichert 2014). In other words, less gallery forests would represent more intense intermale competition for the available territories, which in turn could select males that emit short notes with lower frequencies, as found here. Yet, it does not explain the lack of association between percent of gallery forests and dominant frequencies of short notes and short notes' sequences. This could be caused by an increased role of female selection on dominant frequencies, even for short notes (the territorial part of the call), as dominant frequencies of short and long notes of the advertisement calls are correlated (Nali and Prado 2014a). Ecological adaptation, intrasexual selection and intersexual selection are

tangled mechanisms that might shape call differentiation altogether (Wilkins et al. 2013). Not every call variable is efficient to elicit female responses and/or serve as individual recognition, so while some call variables could be sexually selected, others may have relaxed or different sexual selection patterns (Ryan 1990; Gerhardt 1991). However, we suggest that our significant correlations between two call variables vs. forest percentages did not imply direct causation, but they are rather mediated by underlying male-male competition (intrasexual selection).

Conclusions and perspectives

Studies that ally genotypes and phenotypes, and employ methods to evaluate multiple mechanisms underlying population differentiation are crucial in the fields of behavior, ecology and evolution (this study; Wilkins et al. 2013; Warwick et al. 2015). This is particularly interesting in organisms with resource defense/lek-like mating systems, in which the occurrence of complex calls and elaborated reproductive behaviors favor the evolution of intra and intersexual selection components (this study; Murphy & Gerhardt 2002; Brusa et al. 2013). We showed that acoustic variation is more pronounced than that of morphology in *B. ibitiguara*, likely due to the importance of call recognition and selection by females (intersexual selection) and male-male competition (intrasexual selection) during breeding events along a stream. Sexual selection is likely the strongest mechanism shaping call variation in *B. ibitiguara*, as we found no evidence for the acoustic adaptation hypothesis or neutral evolution of calls by genetic drift. This was expected due to the complex courtship and other reproductive behaviors in this species; under a highly male-biased operational sex-ratio, choosy females might be favored, and male competition is increased (Murphy and Gerhardt 2002; Meuche et al. 2013; Nali and Prado 2012; R. C. Nali, pers. obs.).

Morphologically conserved organisms can accumulate enough genetic divergences that warrant a new species status, and our results reinforce the necessity of incorporating call differences for frogs, as a way of suggesting population diversification and even speciation (Padial et al. 2008; Funk et al. 2011; Prado et al. 2012; Guarnizo et al. 2016). Specifically for the analysis on call differentiation, we recommend, after descriptive analysis of variation, conducting playback experiments with females to assess preferences for specific, highly variable signals (intersexual selection; e.g. Ryan and Rand 2003a). Likewise, playback experiments with males can inform of aggressive

responses to specific signals (intrasexual selection) that may also shape call differentiation (Endler 1992; Reichert and Gerhardt 2013; Nali & Prado 2014a). Finally, recording individuals from different sites and measuring the surrounding vegetation at local and spatial scales can aid in the analysis of ecological determinants on call differentiation (this study; Ziegler et al. 2011; Goutte et al. 2016), mainly if allied to focal observations of intermale interactions and/or courtship behaviors to remove the effects of sexual selection mechanisms. While conducting so many procedures altogether is certainly a laborious task (Gerhardt 2013), mounting work for each system will gradually allow behavioral ecologists to elucidate the complex and poorly understood associations among the surrounding environment, call properties and sexual selection throughout the animal kingdom (Ey and Fischer 2009; Erdtmann and Lima 2013; Wilkins et al. 2013).

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TABLES AND FIGURES

Table 1. Results of Mantel and partial Mantel tests to investigate correlations among genetic, acoustic and morphological distances in populations of *Bokermannohyla ibitiguara*, state of Minas Gerais, Brazil. Geographic distances were held constant (partial Mantel) in analyses containing geographic-dependent variables.

Distance matrix 1	Distance matrix 2	Controlled matrix	*R*	*P*	*N* populations
Geographic	Genetic	_	0.52	**<0.001**	12
	Acoustic	_	0.46	**<0.01**	11
	Morphological	_	0.24	0.09	12
Genetic	Morphological	(Geographic)	-0.12	0.38	12
	Acoustic	(Geographic)	-0.21	0.28	11
Acoustic	Morphological	(Geographic)	0.20	0.21	11

Table 2. Results of the partial Mantel tests (controlling for geographic distance) among genetic distances vs. Mahalanobis distances in eight call variables of *Bokermannohyla ibitiguara*.

Call variable	*R*	*P*	*N* populations
LN dom freq	0.013	0.39	11
LN min freq	-0.081	0.47	11
SN min freq	-0.281	0.09	11
SN dom freq	-0.037	0.49	11
SNseq min freq	-0.204	0.28	11
SNseq dom freq	-0.087	0.34	11
SNseq dur	-0.061	0.46	12
LN/min	0.045	0.36	12

Population GLG was excluded from some analyses due to a single individual with measured call frequencies.

Table 3. Simple regression analyses between percentages of gallery forest vs. 10 call variables across *Bokermannohyla ibitiguara* populations, state of Minas Gerais, Brazil. Statistically significant results in bold.

Call variable	F	Regression coefficient	R^2	P	N
LN min freq	2.21	2.8645	0.197	0.171	11
LN dom freq	2.23	4.0578	0.198	0.170	11
SN min freq	5.87	3.8538	0.395	**0.038**	11
SN dom freq	3.55	5.1927	0.283	0.092	11
SNseq min freq	6.69	4.2410	0.427	**0.029**	11
SNseq dom freq	2.65	5.0008	0.228	0.138	11
LN dur	0.08	-0.0005	0.007	0.789	12
SN dur	0.87	-0.0002	0.080	0.373	12
SNseq dur	0.01	0.0007	0.001	0.937	12
Note repetition rate	2.54	0.3154	0.202	0.142	12

Population GLG was excluded from some analyses due to a single individual with measured call frequencies.

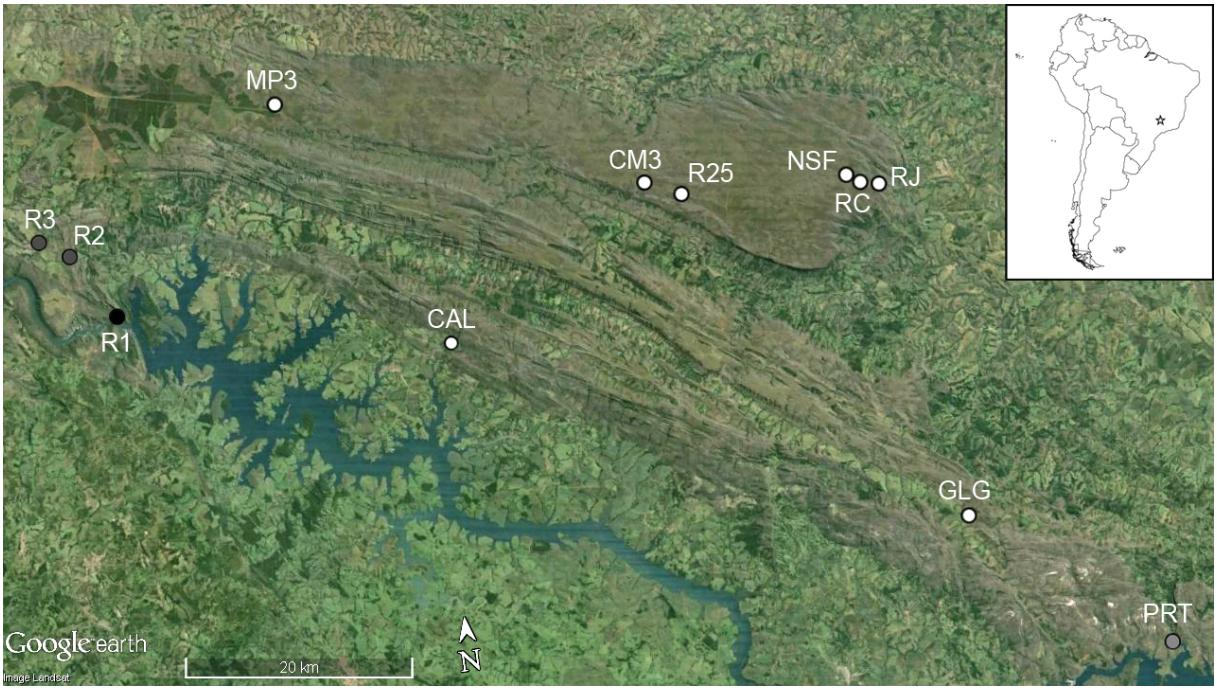


Figure 1. Sampling localities (streams) of *Bokermannohyla ibitiguara* in Southeastern Brazil (star in detail), across the Serra da Canastra mountain range. Points are colored according to different genetic clusters under a previous Bayesian analysis (white, light gray, dark gray and black; see text for details).

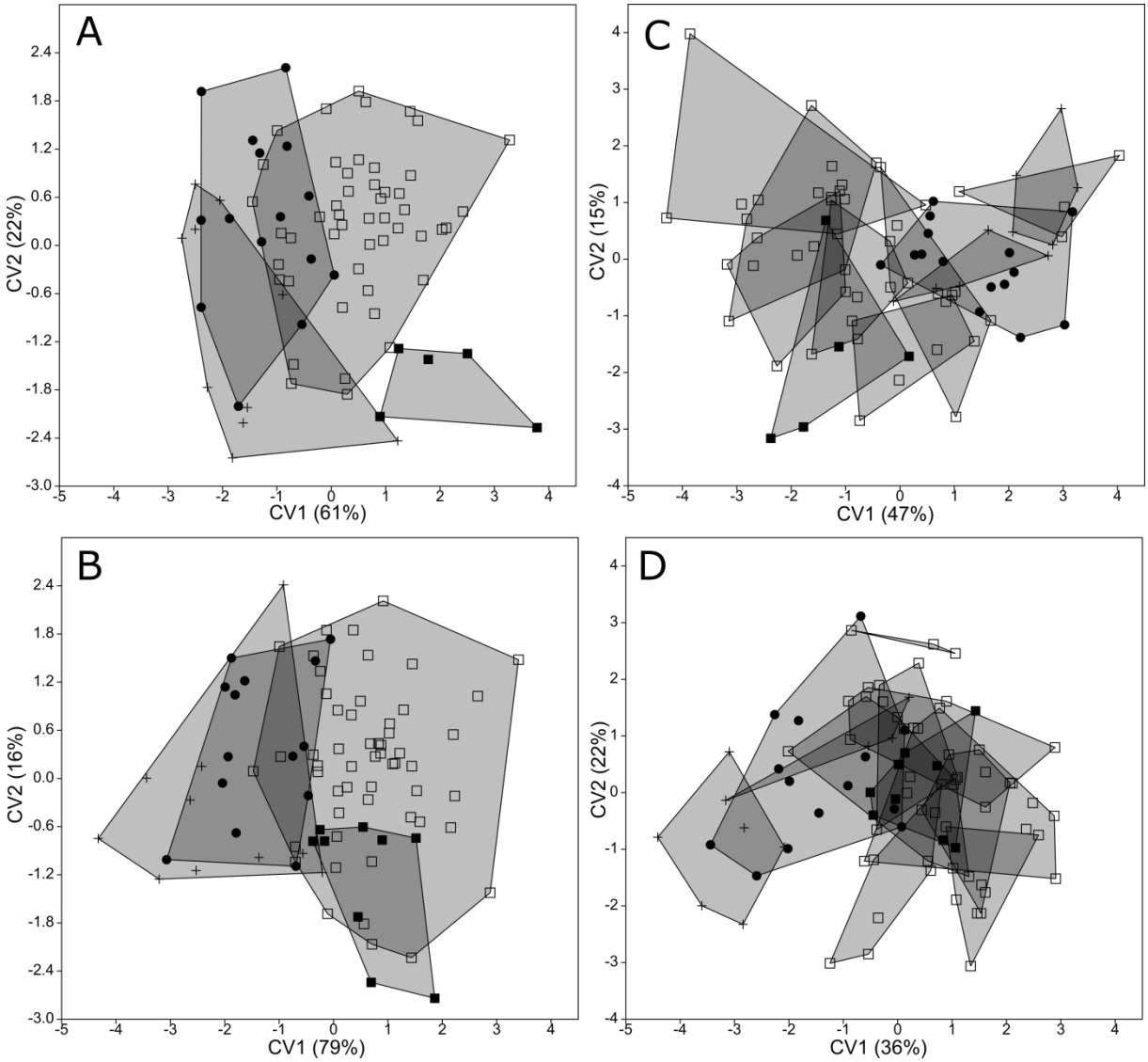


Figure 2. Graphical representation of acoustic (A and C) and morphological (B and D) variation in *Bokermannohyla ibitiguara*, in which each convex hull represents a genetic cluster (A and B) or a population (C and D) within the state of Minas Gerais, Brazil. Scatter plots were built upon the first and second loading roots of our Canonical Variate Analyses, with respective percentages of explanation. The four genetic clusters are Sacramento 1 (dots), Sacramento 2 (plus signals), SCNP (empty squares) and Capitólio (filled squares); the populations followed the same symbols according to the respective assigned genetic cluster (supplemental table 1).

SUPPLEMENTAL MATERIAL

Supplemental Table 1. Information on the localities and sample sizes for recorded males and collected specimens of *Bokermannohyla ibitiguara*, state of Minas Gerais, Brazil.

Locality name (acronym)	Genetic cluster	Latitude (°S)	Longitude (°W)	*N* specimens	*N* males recorded	Gallery forest (%)
Pousada do Rio Turvo (PRT)	Capitólio	20.6458	46.2269	9	6	8.04
Riacho 1 (R1)	Sacramento 1	20.2732	47.0739	14	15	29.08
Riacho 2 (R2)	Sacramento 2	20.2208	47.1066	4	5	16.69
Riacho 3 (R3)	Sacramento 2	20.2062	47.1309	6	5	23.65
Capão de Mata 3 (CM3)	SCNP	20.2273	46.6164	7	7	3.11
Córrego Água Limpa (CAL)	SCNP	20.3315	46.7972	7	7	28.18
Gruta do Lobo Guará (GLG)	SCNP	20.5242	46.3838	3	3	7.91
Mata da Portaria 3 (MP3)	SCNP	20.1246	46.9164	5	4	3.46
Nascente do São Francisco (NSF)	SCNP	20.2434	46.4466	10	12	1.12
Retiro do Jaguarê (RJ)	SCNP	20.2540	46.4203	6	6	5.46
Riacho do Campo (RC)	SCNP	20.2507	46.4357	8	7	5.56
Riacho km 25 (R25)	SCNP	20.2404	46.5868	8	5	2.54

Supplemental Table 2. Collected specimens of *Bokermannohyla ibitiguara* used in this study (separated by locality, see supplemental table 1), all deposited at Coleção de Anfíbios Célio F. B. Haddad, Rio Claro, São Paulo, Brazil. Specimens in bold were not recorded.

Locality	Voucher numbers (CFBH)
CAL	40595-40596, 40625, 40626, 40628-40630
CM3	35867-35873
GLG	40582, 40583, 40585
MP3	**40577**, 40578-40581
NSF	35859-35866, 40564, **40565**
PRT	40586-40591, **40592-40594**
R1	**31751**, **31752**, **31755-31763**, **40622-40624**
R2	**40573-40576**
R25	40562, 40563, 40567, 40568, **40569**, 40570, **40617**, **40618**
R3	**31747-31750**, **31753**, **31754**
RC	40549, 40556, 40557, **40558-40561**, 40571
RJ	40550-40553, **40554**, 40555

Supplemental Table 3. Descriptive data on 14 call variables (see text for full names and units) in 12 populations of *Bokermannohyla ibitiguara* analyzed in this study. The maximum number of individuals recorded is shown next to each population acronym, in parentheses. Mean values and standard deviations (SD; in parentheses) are shown for each population. Kruskal-Wallis was used to test for statistical differences among populations (*P* values in bold). The NA represents missing data, in which case the population was excluded in the statistical test. Coefficients of variation among populations (CV) were calculated with the grand means and SDs for each variable. The variables were corrected for temperature (T) and/or male size (S) when appropriate (see text for details).

Population	LN min freq^{T,S}	LN dom freq^{T,S}	LN dur^{T,S}	LN pulses^{T}	SN min freq^{S}	SN dom freq^{T,S}	SN dur^{T}
CAL (7)	829.75 (158.18)	1838.78 (180.9)	0.94 (0.19)	28.26 (6.56)	820.01 (134.54)	1870.86 (197.04)	0.0649 (0.0074)
CM3 (7)	803.11 (82.03)	1845.53 (93.68)	0.83 (0.11)	23.43 (5.67)	763.06 (114.98)	1861.6 (74.65)	0.0688 (0.0122)
GLG (3)	NA	NA	0.76 (0.07)	25.39 (5.66)	NA	NA	0.0932 (0.024)
MP3 (4)	890.45 (81.54)	2035.12 (62.41)	0.91 (0.21)	23.33 (4.42)	782.72 (105.44)	2021.32 (56.49)	0.0702 (0.0056)
NSF (12)	794.13 (47.35)	1815.23 (120.25)	0.84 (0.11)	29.46 (4.02)	679.83 (59.3)	1794.5 (94.81)	0.0666 (0.0123)
PRT (6)	754.14 (81.49)	1861.67 (132.13)	0.99 (0.22)	31.89 (4.43)	719.31 (97.31)	1848.16 (104.85)	0.0668 (0.0091)
R1 (15)	883.47 (64.46)	1994.37 (90.02)	0.81 (0.07)	25.65 (6.91)	819.73 (57.12)	1999.06 (83.82)	0.0634 (0.0084)
R2 (5)	863.52 (44.26)	1931.88 (20.47)	0.86 (0.05)	21.1 (8.56)	817.72 (53.85)	1934.43 (35.82)	0.066 (0.0039)
R25 (5)	823.63 (118.59)	1804.96 (159.37)	0.87 (0.14)	29.75 (7.55)	718.14 (129.87)	1753.25 (204.64)	0.0703 (0.0115)
R3 (5)	991.08 (51.29)	2082.71 (56.76)	0.79 (0.1)	23.49 (3.76)	851.73 (112.27)	2091.21 (61.19)	0.0615 (0.0141)
RC (7)	770.31 (59.17)	1796.76 (56.42)	0.88 (0.1)	26.17 (4.34)	676.26 (51.94)	1799.68 (76.13)	0.059 (0.0046)
RJ (6)	912.56 (75.28)	1921.16 (177.89)	0.87 (0.16)	27.13 (2.77)	858.16 (62.11)	1936.52 (182.05)	0.0665 (0.0106)
P	**0.0017**	**0.0004**	0.4378	0.0916	**0.0014**	**0.0001**	0.3271
Grand mean	840.27	1896.87	0.86	26.25	756.61	1896.83	0.0681
Grand SD	70.36	95.95	0.06	3.18	85.75	101.62	0.0086
CV (%)	8.37	5.06	7.47	12.10	11.33	5.36	12.60

Supplemental Table 3. (Continued).

Population	SNseq min freq[S]	SNseq dom freq[T,S]	SNseq dur[T]	LN/ min[T]	SN/ min[T]	SNseq/min[T,S]	SN/seq[T]
CAL (7)	827.91 (132.64)	1875.88 (171.92)	1.38 (0.22)	18.45 (10.88)	28.72 (17.04)	4.54 (1.89)	6.77 (1.05)
CM3 (7)	787.64 (104.36)	1878.99 (89.52)	1.31 (0.38)	19.58 (11.69)	19.91 (9.08)	3.51 (1.77)	5.9 (1.53)
GLG (3)	NA	NA	0.98 (0.22)	25.3 (3.61)	12.77 (2.65)	2.37 (0)	5.33 (1.32)
MP3 (4)	800.98 (111.31)	2053.59 (56.46)	2.09 (0.3)	25.33 (11.1)	30.42 (9.72)	4.51 (1)	7.77 (3.37)
NSF (12)	698.51 (51.74)	1800.77 (95.39)	1.19 (0.21)	19.79 (9.5)	26.93 (10.24)	4.39 (1.94)	6.37 (1.23)
PRT (6)	735.41 (97.34)	1860.64 (106.16)	1.41 (0.13)	12.99 (8.98)	18.68 (12.16)	2.56 (1.72)	7.08 (0.6)
R1 (15)	840.32 (54.48)	1996.8 (88.2)	1.21 (0.22)	27.34 (10.18)	28.21 (14.27)	5.44 (2.95)	5.53 (1.3)
R2 (5)	840.68 (46.64)	1923.22 (47.72)	1.53 (0.29)	28.3 (16.31)	23.92 (11.94)	3.21 (1.06)	7.13 (2.12)
R25 (5)	715.57 (127.83)	1738.23 (187.87)	1.21 (0.14)	21.3 (10.89)	19.77 (8.29)	3.37 (1.41)	5.87 (0.6)
R3 (5)	899.53 (66.64)	2098.91 (56.67)	1.4 (0.35)	32.55 (20.27)	17.66 (10.31)	3.13 (1.41)	5.7 (1.36)
RC (7)	697.84 (49.65)	1777.94 (52.23)	1.14 (0.39)	15.23 (3.11)	30.27 (18.32)	4.63 (1.98)	6.08 (1.54)
RJ (6)	862.54 (69.06)	1945.15 (170.2)	1.11 (0.17)	12.35 (4.82)	32.25 (11.78)	5.38 (2.13)	5.99 (0.6)
P	**0.0004**	**0.0001**	**0.0055**	**0.0304**	0.2057	0.1139	0.2728
Grand mean	774.71	1894.23	1.33	21.54	24.13	3.92	6.29
Grand SD	88.76	113.79	0.28	6.35	6.23	1.03	0.74
CV (%)	11.46	6.01	21.28	29.46	25.80	26.37	11.80

Supplemental Table 4. Descriptive data on 12 morphometric variables (mm; see text for full names) in 12 populations of *Bokermannohyla ibitiguara* analyzed in this study, with respective number of measured specimens next to each population acronym, in parentheses. Mean values and standard deviations (SD; in parentheses) are shown for each population. Kruskal-Wallis was used to test for statistical differences among populations (*P* values in bold). Coefficients of variation among populations (CV) were calculated with the grand means and SDs for each variable.

Population	SVL	HL	HW	ED	TD	END
CAL (7)	37.23 (2.31)	13.89 (0.76)	14.04 (0.92)	4.11 (0.24)	2.57 (0.15)	3.31 (0.2)
CM3 (7)	38.49 (5.24)	14.26 (1.54)	14.29 (1.8)	4.12 (0.61)	3.06 (0.58)	3.38 (0.52)
GLG (3)	38.73 (1.57)	14.07 (0.56)	14.33 (0.65)	3.72 (0.19)	2.6 (0.06)	3.51 (0.22)
MP3 (5)	43.14 (2.77)	15.69 (0.98)	15.56 (0.85)	4.7 (0.21)	2.98 (0.23)	3.63 (0.11)
NSF (10)	40.95 (4.04)	14.97 (1.75)	14.92 (1.6)	4.24 (0.54)	2.99 (0.53)	3.75 (0.45)
PRT (9)	38.94 (3.76)	14.29 (1.13)	14.52 (1.01)	4.16 (0.39)	2.69 (0.25)	3.62 (0.39)
R1 (14)	39.76 (2.97)	14.53 (1.09)	14.51 (1.15)	4.45 (0.39)	2.63 (0.28)	3.51 (0.34)
R2 (4)	41.94 (1.39)	15.29 (0.31)	15.57 (0.69)	4.55 (0.17)	2.73 (0.26)	3.66 (0.19)
R25 (8)	38.52 (3.61)	14.67 (1.18)	14.46 (1.33)	4.24 (0.38)	2.9 (0.37)	3.39 (0.49)
R3 (6)	36.05 (3)	13.08 (0.53)	12.82 (0.64)	4.07 (0.31)	2.1 (0.16)	3.15 (0.17)
RC (8)	40.99 (4.48)	15.44 (1.6)	15.24 (1.47)	4.71 (0.56)	2.99 (0.5)	3.63 (0.47)
RJ (6)	35.19 (1.46)	12.98 (0.49)	13.17 (0.43)	4.14 (0.4)	2.58 (0.2)	3.16 (0.15)
P	**0.005**	**0.0014**	**0.0018**	**0.0081**	**0.0016**	**0.0199**
Grand mean	39.16	14.43	14.45	4.27	2.73	3.47
Grand SD	2.35	0.86	0.84	0.29	0.27	0.20
CV (%)	6.00	5.94	5.84	6.70	9.92	5.72

Supplemental Table 4. (Continued).

Population	IND	FaT	FaL	HaL	FoL	TiL
CAL (7)	3.62 (0.32)	5.05 (0.47)	7.05 (0.49)	12.2 (0.53)	27 (1.4)	19.42 (0.76)
CM3 (7)	3.49 (0.42)	4.87 (0.42)	7.46 (0.86)	11.81 (1.69)	27 (3.53)	19.42 (2.38)
GLG (3)	3.62 (0.05)	5.05 (0.36)	7.15 (0.19)	12.5 (0.43)	27.58 (0.94)	19.45 (0.65)
MP3 (5)	3.74 (0.16)	5.5 (0.21)	7.88 (0.31)	13.66 (0.62)	30.12 (1.61)	21.29 (1.38)
NSF (10)	3.65 (0.35)	5.49 (0.71)	7.5 (0.87)	12.75 (1.31)	28.46 (2.64)	20.41 (1.84)
PRT (9)	3.58 (0.28)	5.15 (0.36)	7.23 (0.61)	12.24 (1.08)	26.95 (2.2)	19.45 (1.58)
R1 (14)	3.67 (0.31)	5.11 (0.46)	7.29 (0.62)	12.65 (0.88)	28.86 (2.06)	20.4 (1.43)
R2 (4)	3.83 (0.25)	5.35 (0.38)	7.49 (0.4)	13.12 (0.47)	29.5 (0.53)	20.5 (0.88)
R25 (8)	3.56 (0.3)	5.14 (0.5)	7.36 (0.68)	12.26 (1.18)	27.36 (2.45)	19.48 (1.69)
R3 (6)	3.28 (0.19)	4.46 (0.31)	6.6 (0.47)	11.08 (0.51)	26.08 (1.36)	18.67 (1.39)
RC (8)	3.78 (0.44)	5.48 (0.55)	8.03 (0.79)	12.57 (1.5)	28.44 (2.96)	20.7 (2.2)
RJ (6)	3.23 (0.16)	4.82 (0.37)	6.69 (0.52)	10.94 (0.69)	24.85 (1.36)	17.91 (0.9)
P	**0.0353**	**0.0058**	**0.0049**	**0.0006**	**0.003**	**0.0151**
Grand mean	3.59	5.12	7.31	12.31	27.68	19.76
Grand SD	0.18	0.31	0.42	0.77	1.48	0.94
CV (%)	5.12	6.07	5.71	6.27	5.33	4.76

Supplemental Table 5. Descriptive data on 14 call variables (see text for full names and units) in 4 genetic clusters of *Bokermannohyla ibitiguara* analyzed in this study. The maximum number of individuals recorded is shown in parentheses. Mean values and standard deviations (SD; in parentheses) are shown for each population. Kruskal-Wallis was used to test for statistical differences among populations (*P* values in bold). Coefficients of variation among populations (CV) were calculated with the grand means and SDs for each variable. The variables were corrected for temperature (T) and/or male size (S) when appropriate (see text for details).

Genetic Cluster	LN min freq^{T,S}	LN dom freq^{T,S}	LN dur^{T,S}	LN pulses^{T}	SN min freq^{S}	SN dom freq^{T,S}	SN dur^{T}
Capitólio	883.47	1994.37	0.81	25.65	819.73	1999.06	0.0634
(6)	(64.46)	(90.02)	(0.07)	(6.91)	(57.12)	(83.82)	(0.0084)
SCNP	927.3	2007.3	0.83	22.29	834.72	2012.82	0.0638
(51)	(81)	(89.09)	(0.08)	(6.36)	(84.93)	(95.2)	(0.01)
Sacramento 1	820.1	1855.09	0.87	27.14	743.66	1852.12	0.0679
(15)	(92.15)	(136.7)	(0.14)	(5.33)	(111.32)	(146.95)	(0.0127)
Sacramento 2	754.14	1861.67	0.99	31.89	719.31	1848.16	0.0668
(10)	(81.49)	(132.13)	(0.22)	(4.43)	(97.31)	(104.85)	(0.0091)
P	**0.001**	**0.0004**	0.1799	**0.0245**	**0.0094**	**0.0002**	0.68
Grand mean	846.25	1929.61	0.88	26.74	779.36	1928.04	0.0655
Grand SD	75.55	82.46	0.08	3.98	56.49	90.14	0.0022
CV (%)	8.93	4.27	9.34	14.90	7.25	4.68	3.41

Supplemental Table 5. (Continued).

Genetic Cluster	SNseq min freq^{S}	SNseq dom freq^{T,S}	SNseq dur^{T}	LN/min^{T}	SN/min^{T}	SNseq/min^{T,S}	SN/seq^{T}
Capitólio	840.32	1996.8	1.21	27.34	28.21	5.44	5.53
(6)	(54.48)	(88.2)	(0.22)	(10.18)	(14.27)	(2.95)	(1.3)
SCNP	870.11	2011.07	1.46	30.43	20.79	3.17	6.42
(51)	(62.47)	(104.95)	(0.31)	(17.49)	(11.03)	(1.18)	(1.84)
Sacramento 1	757.77	1854.95	1.28	18.95	26.11	4.24	6.28
(15)	(107.43)	(147.47)	(0.37)	(9.14)	(12.7)	(1.83)	(1.49)
Sacramento 2	735.41	1860.64	1.41	12.99	18.68	2.56	7.08
(10)	(97.34)	(106.16)	(0.13)	(8.98)	(12.16)	(1.72)	(0.6)
P	**0.0017**	**0.0006**	0.0769	**0.0057**	0.3153	**0.0487**	0.0863
Grand mean	800.90	1930.86	1.34	22.43	23.45	3.85	6.33
Grand SD	64.53	84.61	0.12	7.94	4.46	1.26	0.63
CV (%)	8.06	4.38	8.61	35.42	19.01	32.84	10.00

Supplemental Table 6. Descriptive data on 12 morphometric variables (mm; see text for full names) in 4 genetic clusters of *Bokermannohyla ibitiguara* analyzed in this study, with respective number of measured specimens next to each population acronym, in parentheses. Mean values and standard deviations (SD; in parentheses) are shown for each population. Kruskal-Wallis was used to test for statistical differences among populations (*P* values in bold). Coefficients of variation among populations (CV) were calculated with the grand means and SDs for each variable.

Genetic Cluster	SVL	HL	HW	ED	TD	END
Capitólio (9)	38.94 (3.76)	14.29 (1.13)	14.52 (1.01)	4.16 (0.39)	2.69 (0.25)	3.62 (0.39)
SCNP (54)	39.23 (4.09)	14.56 (1.45)	14.53 (1.4)	4.28 (0.5)	2.86 (0.43)	3.48 (0.42)
Sacramento 1 (14)	39.76 (2.97)	14.53 (1.09)	14.51 (1.15)	4.45 (0.39)	2.63 (0.28)	3.51 (0.34)
Sacramento 2 (10)	38.41 (3.86)	13.97 (1.22)	13.92 (1.55)	4.26 (0.35)	2.35 (0.38)	3.35 (0.32)
P	0.75	0.66	0.54	0.31	**0.0057**	0.4596
Grand mean	39.08	14.34	14.37	4.29	2.63	3.49
Grand SD	0.57	0.27	0.30	0.12	0.21	0.11
CV (%)	1.45	1.91	2.08	2.77	8.12	3.19

Supplemental Table 6. (Continued).

Genetic Cluster	IND	FaT	FaL	HaL	FoL	TiL
Capitólio (9)	3.58 (0.28)	5.15 (0.36)	7.23 (0.61)	12.24 (1.08)	26.95 (2.2)	19.45 (1.58)
SCNP (54)	3.59 (0.35)	5.2 (0.54)	7.42 (0.76)	12.33 (1.29)	27.62 (2.64)	19.81 (1.85)
Sacramento 1 (14)	3.67 (0.31)	5.11 (0.46)	7.29 (0.62)	12.65 (0.88)	28.86 (2.06)	20.4 (1.43)
Sacramento 2 (10)	3.5 (0.35)	4.81 (0.56)	6.95 (0.62)	11.9 (1.15)	27.45 (2.06)	19.4 (1.49)
P	0.6758	0.2142	0.347	0.4381	0.1951	0.3888
Grand mean	3.58	5.07	7.23	12.28	27.72	19.76
Grand SD	0.07	0.17	0.20	0.31	0.81	0.46
CV (%)	1.97	3.43	2.73	2.52	2.92	2.33

Supplemental Table 7. Genetic distance matrix (pairwise F_{ST}) among 12 *Bokermannohyla ibitiguara* populations (see supplemental table 1), based on 21 microsatellite genotyping (see Chapter 1 for details).

	CAL	CM3	GLG	MP3	NSF	PRT	R1	R2	R25	R3	RC	RJ
CAL	0											
CM3	0.028	0										
GLG	0.027	0.028	0									
MP3	0.032	0.050	0.039	0								
NSF	0.010	0.002	0.020	0.029	0							
PRT	0.038	0.046	0.036	0.064	0.028	0						
R1	0.121	0.093	0.114	0.114	0.099	0.133	0					
R2	0.092	0.069	0.089	0.087	0.084	0.121	0.080	0				
R25	0.020	0.003	0.024	0.041	0.003	0.038	0.091	0.068	0			
R3	0.067	0.051	0.068	0.074	0.055	0.086	0.098	0.074	0.054	0		
RC	0.016	0.013	0.029	0.030	0.000	0.043	0.098	0.075	0.005	0.058	0	
RJ	0.018	0.005	0.018	0.030	0.000	0.039	0.097	0.080	0.004	0.057	0.001	0

Supplemental Table 8. Morphological distance matrix (squared Mahalanobis distances) among 12 *Bokermannohyla ibitiguara* populations (see supplemental table 1), based on 12 morphometrical traits.

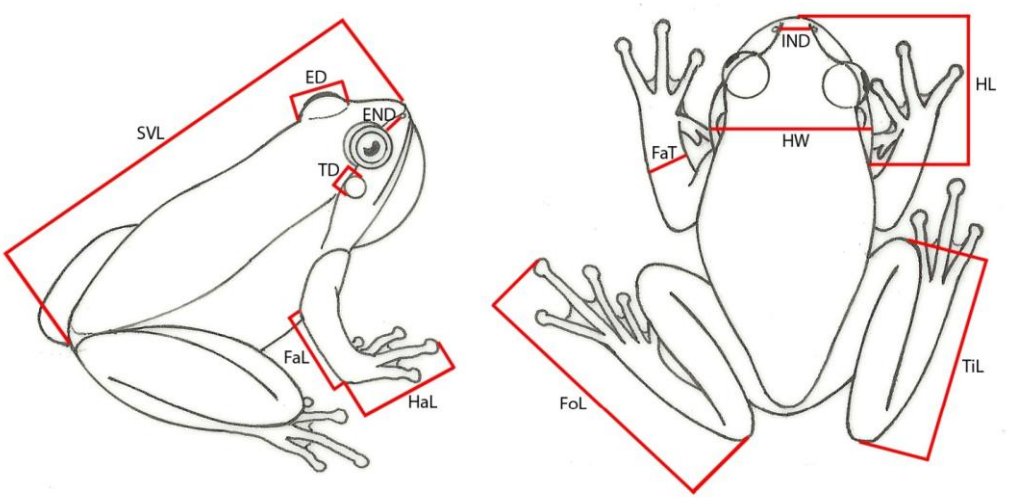
	CAL	CM3	GLG	MP3	NSF	PRT	R1	R2	R25	R3	RC	RJ
CAL	0											
CM3	10.744	0										
GLG	9.727	13.466	0									
MP3	8.638	11.312	14.106	0								
NSF	7.832	7.798	7.767	7.168	0							
PRT	4.324	8.922	9.674	9.608	3.347	0						
R1	5.201	12.659	12.518	6.049	7.299	6.924	0					
R2	7.198	14.422	10.818	5.385	9.023	7.133	3.238	0				
R25	4.962	3.005	11.664	5.655	6.146	5.595	8.034	8.277	0			
R3	17.381	25.042	26.676	21.558	20.143	16.564	7.052	13.781	22.018	0		
RC	9.234	9.561	24.384	12.084	12.265	7.851	13.508	14.190	5.620	24.801	0	
RJ	9.597	10.561	24.767	14.314	11.054	7.002	9.232	14.265	9.010	13.815	10.536	0

Supplemental Table 9. Acoustic distance matrix (squared Mahalanobis distances) among 11 *Bokermannohyla ibitiguara* populations (see supplemental table 1), based on 14 call traits.

	CAL	CM3	MP3	NSF	PRT	R1	R2	R25	R3	RC	RJ
CAL	0										
CM3	5.734	0									
MP3	17.337	16.538	0								
NSF	11.404	8.715	25.304	0							
PRT	9.131	10.465	29.952	11.889	0						
R1	5.937	4.652	12.896	11.943	16.204	0					
R2	8.348	5.150	15.242	15.454	16.228	5.461	0				
R25	18.972	17.993	33.157	6.126	15.737	19.812	19.095	0			
R3	19.260	11.680	12.988	22.806	28.562	8.529	10.394	31.348	0		
RC	11.516	10.073	34.977	4.160	9.655	13.750	13.395	7.427	27.869	0	
RJ	7.300	8.893	25.959	7.298	10.081	9.086	12.435	12.322	19.120	8.717	0

Supplemental Table 10. Geographic distance matrix (Euclidean distances in kilometers) among 12 *Bokermannohyla ibitiguara* populations (see supplemental table 1).

	CAL	CM3	GLG	MP3	NSF	PRT	R1	R2	R25	R3	RC	RJ
CAL	0											
CM3	22.13	0										
GLG	48.10	40.96	0									
MP3	26.16	33.33	71.12	0								
NSF	37.85	17.81	31.90	50.78	0							
PRT	68.92	61.75	21.21	92.32	50.26	0						
R1	29.58	48.00	77.14	23.31	65.52	97.48	0					
R2	34.54	51.15	82.55	22.55	68.90	103.13	6.75	0				
R25	24.17	3.42	37.99	36.73	14.63	58.64	50.95	54.28	0			
R3	37.50	53.73	85.53	24.16	71.52	106.13	9.53	3.01	56.90	0		
RC	38.76	19.04	30.89	52.09	1.40	49.03	66.62	70.07	15.81	72.71	0	
RJ	40.24	20.68	30.28	53.74	2.99	48.00	68.21	71.70	17.44	74.33	1.65	0



Supplemental Figure 1. Representation of the 12 morphometric variables measured in this study (see text for full names).

CHAPTER III:

HYBRIDIZATION DESPITE ELABORATE REPRODUCTIVE BEHAVIORS AND FEMALE CHOICE IN NEOTROPICAL TREEFROGS

Renato C. Nali, Kelly R. Zamudio & Cynthia P. A. Prado

HYBRIDIZATION DESPITE ELABORATE REPRODUCTIVE BEHAVIORS AND FEMALE CHOICE IN NEOTROPICAL TREEFROGS

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ABSTRACT

Hybridization is an important process for diversification, as the reproductive interactions among individuals from genetically distinct populations may alter the evolutionary history of lineages in multiple ways. In frogs, heterospecific matings typically occur in species with explosive breeding patterns, and that breed in dense aggregates with few opportunities for mate selection. Using highly variable microsatellites and acoustic and morphological data sets, we examined the degree and consequences of hybridization between two prolonged-breeding congener frogs (*Bokermannohyla sazimai* and *B. ibitiguara*) from the Brazilian savanna. Our genetic analyses confirmed the identity of both parental species, and nearly 10% of individuals in our sample were hybrids. Hybrids showed overlapping but intermediate values of genetic dissimilarities compared to individuals of the parental species. Morphological and call analyses revealed that hybrids typically showed variable, but not necessarily intermediate phenotypes, including two hybrids with a different call type from parentals. *Bokermannohyla ibitiguara* has territorial males, elaborate courtship behaviors, choosy females, as well as opportunistic mating strategies. Our study reveals that, contrary to expectation, animals with long breeding seasons and complex reproductive behaviors may still erroneously mate and hybridize. We discuss the possible directionality of the hybrids between our focal species and the importance of

investigating hybridization in frogs and other taxa that typically show elaborate reproduction and female choice.

Keywords: Anura, bioacoustics, Cophomantinae, microsatellites, morphology, Neotropics.

INTRODUCTION

Hybridization events in nature are important for the process of diversification, as the reproductive interactions between two species can alter the evolutionary history of animal and plant lineages in multiple ways (Coyne & Orr 2004; Abbott et al. 2013). Hybridization, defined as reproductive events between members of genetically distinct populations (Barton & Hewitt, 1985), has classically been interpreted as a promoter of fitness loss when compared to conspecific mating events, as it often results in offspring with low fertility and/or viability (Dobzhansky 1940; Mayr 1963; Wagner 1969; Haddad et al. 1990). However, more recent studies show that natural hybridization can be a creative process, promoting adaptive introgression, adaptive genetic variation, and speciation (Allendorf et al. 2001; Burke & Arnold 2001; Abbott et al. 2013). We now recognize that hybrids can fall along a fitness continuum depending on the study system, yet the basis for increased hybrid fitness is not very clear (Burke & Arnold 2001). Likewise, the dynamics of positive and negative consequences of hybridization is complex, because hybrids can arise either naturally or as a result of anthropogenic environmental change (Allendorf et al. 2001; Detwiler et al. 2005; Seehausen et al. 2008). Therefore, the persistent reproductive interactions between sympatric species are meaningful in the study of evolution and conservation (Gröning & Hochkirch 2008; Abbot et al. 2013).

Hybrids have been characterized in a broad range of taxa (e.g. Rieseberg & Wendel 1993; Vanhaecke et al. 2012; Johnson et al. 2015; Malukiewicz et al. 2015): overall, 10 to 30% of all multicellular organisms are assumed to hybridize regularly at different rates (Mallet 2005). An important step in the study of this widespread phenomenon is to go beyond natural observations of hybridization and genetically characterize the degree to which hybrids occur in natural populations. Increased availability of molecular resources has enabled molecular assessments of hybridization

even with non-model species (Sequeira et al. 2011; Abbott et al. 2013; Pereyra et al. 2016). In addition, because genetic incompatibility and morphological/behavioral incompatibilities seem to have different evolution rates (Grant & Grant 1997; Lougheed et al. 2006), the combined analysis of phenotypes and genetics is crucial to understand mechanisms of heterospecific reproductive interactions that lead to hybridization.

Often, differences between species' phenotypes, when properly recognized, constitute good pre-mating barriers (Coyne & Orr 2004). However, hybridization arises when incomplete mating recognition occurs among closely-related species (Spencer et al. 1986; Schaefer and Ruxton 2015), leading to heterospecific mating. Different contexts of breeding may contribute to the occurrence of heterospecific matings and thus hybridization. For instance, frog species show various mating strategies along a continuum of extremely explosive to extremely prolonged breeding seasons, in which female selection, individual recognition and male territoriality tend to progressively increase from the former to the latter (Wells 1977; 2007). To that sense, frogs are known to erroneously mate mostly in situations of explosive breeding; males and females form dense aggregates, males actively search and grab females indiscriminately and/or dislocate other males already in amplexus, leaving little opportunity for female choice (Brown 1977; Grossenbacher 1977; Ficetola & De Bernardi 2005; Pearl et al. 2005). This fact is particularly well-documented for species of toads (family Bufonidae), which typically have explosive breeding pattern (Sullivan 1986; Haddad et al. 1990; Wells 1977, 2007; Bezerra & Cascon 2011). In contrast, hybridization seems less common in species with prolonged breeding seasons and complex courtships and calls, where female choice and male territoriality are generally strong mechanisms to enhance fitness (Wells 1977, 2007). In those systems, females may rely on morphological cues and calls to select potential partners; calls are among the most important signals used for mating recognition in frogs (Lougheed et al. 2006), and call characteristics are in turn often related to morphological characters such as body size (McClelland et al. 1996; Morais et al. 2012; Nali & Prado 2014a).

Neotropical frogs are underrepresented in the study of genetic hybridization, and even fewer studies have specifically employed a multi-character framework, including genotypes, morphology, and calls (e.g. Sequeira et al. 2011; Vargas-Salinas & Amézquita 2013; Pereyra et al. 2016). Here, we investigated hybridization between two frog species that co-occur in the Brazilian savanna in southeastern Brazil. The first,

Bokermannohyla ibitiguara, is a prolonged breeder, with complex courtship, territorial/aggressive males, choosy females, and calling behaviors that last for many months (Nali & Prado 2012; 2014a; 2014b). The second, *Bokermannohyla sazimai*, is also a prolonged breeder, although details of the reproductive system have not been thoroughly explored (Cardoso & Andrade 1982; Haddad et al. 1988). Both species emit advertisement calls that are similar in general structure, i.e., a complex call with long and short multipulsed notes (Carvalho et al. 2012; Nali & Prado 2014a). The two species are congeners but not sister taxa, and are thought to belong to two independent groups within the genus (Faivovich et al. 2005). Using highly variable microsatellite markers (Nali et al. 2014), data on morphology and calls, and natural history descriptions available for the species, we discuss possible mechanisms of reproductive interactions and test the hypothesis that failed mating recognition systems in parental species may have led to the formation of hybrids despite the elaborate breeding behavior.

MATERIALS AND METHODS

Study site

The Serra da Canastra mountain range is a markedly seasonal region in southeastern Brazil, originally covered by the Cerrado vegetation, patches of semideciduous forest, gallery forests alongside various streams, and grassland fields at higher elevations of up to 1,500 m (Dietz, 1984; Queirolo & Motta-Junior 2007). Our focal taxa co-occur and have been found breeding at the same streams in the region of the the Serra da Canastra National Park (SCNP; Haddad et al. 1988; C. F. B. Haddad, pers. comm.). This park consists mostly by a flat high-elevation plateau with an area of ~700 km^2 (the "Chapadão da Canastra", MMA/IBAMA 2005; Medeiros & Fiedler, 2004). *Bokermannohyla ibitiguara* is abundantly distributed in streams throughout the SCNP, forming a well-defined genetic cluster, likely due to facilitated dispersal, and it is known from additional close localities throughout the Serra da Canastra (Nali & Prado 2012; Chapter 1). *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 over 300 km north of the SCNP (figure 1; Carvalho & Giaretta 2013).

Genetic and individual sampling

During 2013 and 2014, we sampled eight streams in the SCNP (supplemental table 1; figure 1), and classified individuals as *B. sazimai* or *B. ibitiguara* according to the stream where they occurred and morphology. We collected tissue samples from adults (liver, muscle or toe clip) and tadpoles (tail clip), and preserved in absolute ethanol. Collected adults were euthanatized with a solution of lidocaine 10% spray, fixed in formalin 10% and preserved in ethanol 70% (McDiarmid 1994; ICMBio permits # 33735 and #19269-1). Tadpoles with different body sizes were collected from each stream, reducing the probability of collecting tadpoles from a single clutch (Chapter 1). Specimens and tissues were deposited in the Coleção de Anfíbios Célio F. B. Haddad, Department of Zoology, Universidade Estadual Paulista "Júlio de Mesquita Filho", Rio Claro, SP, Brazil.

We recorded male calls at approximately 1m from the individual, using a Marantz PMD-660 digital recorder and Sennheiser ME66 unidirectional microphone, at a 16-bit resolution and 44,100 Hz (chapter 2). Air temperature and hour of recording were registered.

Laboratory protocols and microsatellite data

All individuals were genotyped at 21 microsatellite loci previously developed and optimized for *B. ibitiguara* (Nali et al. 2014). All markers had sufficient amplification for *B. sazimai* under the same conditions. We extracted whole genomic DNA with DNeasy extraction columns (Qiagen, Valencia, CA, USA), following 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 four 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, Cornell University.

We used GENEMARKER v. 2.4.0 (SoftGenetics LLC, State College, PA) to analyze genotyping profiles of individuals. Each locus was then tested for Hardy-Weinberg Equilibrium separately for *B. ibitiguara* and *B. sazimai*, using ARLEQUIN v. 3.5 (Excoffier and Lischer, 2010). 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 individuals of each species separately and all pairs of loci using GENEPOP v. 4.0.9 (Rousset 2008), with 10,000 dememorization steps, and 100 batches with 5,000 iterations each.

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 (Johnson et al. 2015; Milián-García et al. 2015; Turchetto 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 assessed 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. Presence of genetic structure was assumed when different plots of delta-K provided evidence of a clear peak for K values, as obtained in STRUCTURE HARVESTER (Evanno et al. 2005; Earl & VonHoldt 2011). The cluster data were permutated for the selected K value using CLUMPAK with standard parameters (Kopelman et al. 2015), and bar plots visualized in Excel. We considered individuals with $0.1 < Q < 0.9$ as potential hybrids. Our 0.9 Q-score threshold was validated by simulating 120 genotypes (30 each F1, F2, *B. ibitiguara* backcross and *B. sazimai* backcross) using HYBRIDLAB (Nielsen et al. 2006). To determine individual and population-level hybrid composition, we 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). We ran the analysis with K = 2 and the aforementioned parameters.

We classified individuals in the STRUCTURE run with $Q > 0.9$ as pure *B. ibitiguara* or pure *B. sazimai* (e.g. Johnson et al. 2015), and used this classification as input for the software NEWHYBRIDS v. 1.1 (Anderson & Thompson 2002) to identify different hybrid classes in our sample. We performed 5 runs with 50,000 sweeps of burn-in and 200,000

following sweeps and averaged the probability of each individual of being a pure *B. ibitiguara*, pure *B. sazimai*, hybrid F1, hybrid F2, backcross *B. ibitiguara* or backcross *B. sazimai* (e.g. Johnson et al. 2015). Individuals with $P > 0.9$ to any of the categories were assigned as such.

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 the species and identify hybrids. This matrix was also used to create a Neighbor-Joining Tree in R (R Core Team 2014), using the package *ape* (Paradis et al. 2004).

Potential hybrids were checked for the presence of diagnostic alleles identified in the parental *B. sazimai* or *B. ibitiguara* individuals. Private alleles were defined as those present in one species only, with a frequency higher than 5% to prevent sampling and/or genotyping errors (e.g. 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 even with a frequency as low as 3.6%, due to the few hybrids found.

Genetic comparisons with other *Bokermannohyla*

To further investigate the genetic proximity between sympatric *B. sazimai* and *B. ibitiguara* relative to other Cerrado congeners from the two taxonomic groups, we ran an additional STRUCTURE analysis including three non-sympatric species and using genotypes of five microsatellites that successfully amplified across all five species (Bi179, Bi609, Bi639, Bi1397 and Bi3370; Nali et al. 2014). The additional tissues were obtained from the Coleção de Anfíbios Célio F. B. Haddad (supplemental table 5), and the species were *B. circumdata* (Bci; N = 15) and *B. hylax* (Bhy; N = 14), both from the same taxonomic group of *B. sazimai*, and *B. alvarengai* (Bal; N = 15), from the same group of *B. ibitiguara* (Faivovich et al. 2005). We assessed K values from 1 to 6 using the same parameters of our first STRUCTURE analysis. To visualize genetic dissimilarities among species, we also ran a PCoA with this dataset after calculating squared genetic distances between pairs of samples in GenAlex.

Analyzing phenotypes: morphology and calls

We incorporated data on morphology and calls of adults of both focal species to investigate possible similarities between them, which could support heterospecific mating leading to hybridization. We measured from each specimen 12 morphometric traits to the nearest 0.01mm using a digital caliper (supplemental figure 1): 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 randomly measured (independent of collection locality), and every measure was taken from the same side across specimens.

We analyzed the 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. 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 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 short notes' sequence (SNseq min freq); 9) dominant frequency of the short notes' sequence (SNseq dom freq), 10) duration of the short notes' sequence (SNseq dur); 11) number of long notes per minute (LN/min); 12) number of short notes per minute (SN/min); 13) number of short notes' sequences 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 five long notes, five short notes and up to five sequences of short notes per individual, and calculated the mean for each variable. To avoid potential biases, a single researcher (RCN) measured the voucher specimens and analyzed the calls.

Mean body sizes (SVL) of *B. ibitiguara*, *B. sazimai* and the recognized hybrids were compared with a Kruskal-Wallis test followed by post hoc comparisons of Student-Newman-Keuls. Number of long notes per minute was compared between *B. ibitiguara* and *B. sazimai* with a Mann-Whitney test, due to a possible role in female attraction (Nali and Prado 2014a, R. C. Nali, unpublished data). The 12 morphometric values, as well as

the call variables, were log-transformed and used in Principal Component Analyses in the software STATISTICA (StatSoft 2011). They were visualized in scatterplots using the first and second principal component axes, which explain most of the variation.

RESULTS

Genetic sampling and microsatellite data

We collected a total of 146 tissues from adults and tadpoles (supplemental tables 1 and 6). At first, they were identified as 108 *B. ibitiguara* and 38 *B. sazimai*, but after the genetic identification of hybrids, the sample was re-classified as 99 *B. ibitiguara*, 33 *B. sazimai* and 14 hybrids.

All loci were highly polymorphic across our samples (average = 19.2 alleles; range = 11 - 42). Most loci were under HWE for each species, except 7 in *B. ibitiguara* and 6 in *B. sazimai* (Bonferroni $P < 0.0024$). Only one 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.

Genetic analyses and identification of hybrids

The Bayesian assignment analysis including *B. ibitiguara* and *B. sazimai* revealed two major genetic clusters compatible with the two species and streams used for their *a priori* classification in this study (K = 2; figure 2). The lack of genetic structure among localities of each species was expected due to a single genetic cluster previously found for *B. ibitiguara* within the SCNP (Chapter 1). Ninety-nine individuals showed coefficients of membership (Q) > 0.9 to *B. ibitiguara*, and 33 to *B. sazimai*. However, 14 individuals showed intermediate coefficients ($0.1 < Q < 0.9$) that could represent hybrids (figure 2; table 1). The STRUCTURE analysis using the simulated genotypes in HYBRIDLAB showed that 53 of the 60 parental individuals were assigned Q > 0.9, and 59 of the 60 F1 + F2 hybrids were assigned $0.1 < Q < 0.9$. Moreover, average Q for parental *B. ibitiguara* and parental *B. sazimai* were above 0.9 (respectively 0.95 and 0.93); thus, the threshold value of 0.9 used here appropriately identified hybrids. Using NEWHYBRIDS, we found that 11 out of the above mentioned 14 individuals were F2 hybrids, i.e., their probability of being F2 hybrids were > 0.9 (average = 0.971; table 1).

The majority of individuals belonged to either pure *B. sazimai* or pure *B. ibitiguara*, similar to our STRUCTURE analysis.

The first and second axes of our PCoA explained ca. 9.1% of the genetic variation. We found some genetic overlap between parental species; the hybrids identified in the previous analyses occupied a central position in the overlap region of the PCoA (figure 3A). Our neighbor-joining tree also revealed a separation of *B. ibitiguara* clades from *B. sazimai* clades (figure 3B). Despite the observed separation, the two clades were separated by short branch lengths indicating genetic similarity between the species; the assigned hybrids were distributed throughout the tree (Figure 3B).

Our analyses of private alleles revealed that nine 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 loci that is diagnostic of *B. sazimai* (supplemental table 2). In our second approach, nine hybrids showed alleles absent from either *B. sazimai* or *B. ibitiguara* (10 different alleles across 10 loci; table 1; supplemental table 3).

Genetic comparisons with three other species of *Bokermannohyla*

The STRUCTURE analysis with a subset of five loci revealed two genetic clusters (K = 2), clearly separating a cluster formed by both *B. sazimai* and *B. ibitiguara* (average Q = 0.992) from the other cluster with three *Bokermannohyla* species (average Q = 0.989; supplemental figure 3). The first and second axes of our PCoA with five species explained ca. 17.3% of the genetic variation. While Bci, Bhy and Bal formed separate species with minimal overlapping, *B. sazimai* and *B. ibitiguara* overlap greatly with each other, reinforcing genetic similarities (figure 4).

Phenotypes: morphology and calls

We collected and measured morphometric characters of 16 adult specimens of *B. sazimai* (one female), 41 of *B. ibitiguara* (one female), and eight hybrids (no females). Four adult hybrids were morphologically more similar to *B. ibitiguara* by sight, and the other four, to *B. sazimai*, consistently with the streams where they were collected (supplemental figure 2). We recorded and measured calls of seven *B. sazimai* males, 38 *B. ibitiguara* males, and five hybrids. Two of the hybrids emitted a different type of short note, absent from both *B. ibitiguara* and *B. sazimai*, which sounded like whines with

irregular pulse structure (supplemental table 4; supplemental figure 4). However, for the Principal Component Analyses (PCA), missing data is not allowed for any variables, resulting in the exclusion of some individuals, and a reduced acoustic dataset of 29 *B. ibitiguara*, seven *B. sazimai*, and two hybrids.

Body sizes of *B. ibitiguara* were larger (mean SVL = 39.6 mm) compared to those of *B. sazimai* (31.8 mm), and body sizes of hybrids were intermediate (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*, but not significant for *B. sazimai* vs. hybrids (supplemental figure 5). Number of long notes per minute was significantly higher for *B. sazimai* (median = 50; N = 7) compared to *B. ibitiguara* (median = 15; N = 38; Mann-Whitney U = 34.5; P < 0.01). The first and second principal components of each PCA accounted for ca. 93% of the morphological variation (figure 5A), and ca. 63% of the acoustic variation (figure 5B). We observed a separation of *B. ibitiguara* and *B. sazimai* both due to morphological and call differentiation, and the hybrids were distributed across the phenotypic space of both parental species. Despite the separation, in our morphological PCA, the single female of *B. ibitiguara* fell closer to the males of *B. sazimai* instead of those of her own species (figure 5A).

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 a great geographic overlap (Cardoso & Andrade 1982; Haddad et al. 1988). The occurrence of hybrids was relatively uncommon, reaching 7.5 or 9.5% of the total individuals sampled, depending on the hybrid assignment method. Previous cytogenetic analyses of these species indicated possible hybridization events. First, both species have the same karyotypes, which seems to be highly conserved across genera of this subfamily (2N = 24; Catroli et al. 2011). Moreover, neither species present heteromorphic sex chromosomes (Catroli et al. 2011), which are hypothesized to be potential promoters of hybrid incompatibilities (Meiklejohn & Tao 2010; Austin et al. 2011; Johnson & Lachance 2012).

We found 14 hybrids using the Bayesian analysis, from which 11 were classified as F2 hybrids in NewHybrids (table 1). Due to higher recombination of parental and hybrid genomes, F2 hybrids are expected to be more variable than F1 hybrids. In our study, hybrids had genetic, morphological and acoustic characteristics that overlapped significantly with those of parental species, indicating high variability (figure 3; figure 5; supplemental figure 2). Plus, two hybrids emitted different call types from parental form individuals (supplemental figure 4; supplemental table 4). Testes of hybrids and parental individuals were similar in morphology and mass, so we are not sure about hybrid infertility at this level (R. C. Nali, pers. obs; Wünsch & Pfennig 2013). Moreover, Haldane's rule, which states that hybrids of the heterogametic sex are more likely to evolve infertility, also does not apply (Austin et al. 2011), as neither species have heteromorphic sexual chromosomes (see above). Outbreeding depression (i.e., when hybrid individuals have reduced fitness compared to the parentals) is less likely to occur when genetic divergence between species is not very pronounced (Allendorf et al. 2001). Moreover, the program NewHybrids can differentiate hybrid classes when using more than 20 non-diagnostic markers (Anderson & Thompson 2002), and this was the case with our data. Given the proximity between *B. sazimai* and *B. ibitiguara*, confirmed by comparisons with three other congeners (figure 4, supplemental figure 3), and the aforementioned reasons, the hybrids found here may be from a later generation and corroborate the sustained hybridization in this system. Even if the power to reliably detect hybrid classes is lower than expected, we nonetheless confirmed the presence of highly variable hybrids in this system.

The hybridization between *B. sazimai* and *B. ibitiguara* is probably homoploid, i.e., hybrids are generated with equal number of chromosomes, without ploidy changes (Abbott et al. 2013). Although cytogenetic studies will be required to confirm this, we found only two alleles for all hybrid individuals genotyped in our sample. In allopolyploid hybridization, i.e., those involving ploidy changes, genetic incompatibilities between parentals and hybrids tend to be stronger and often lead to speciation, such as in the Brazilian leaf-frog *Phyllomedusa tetraploidea* (Haddad et al. 1994; Abbott et al. 2013). Homoploid events are typically much more subtle, so that speciation is more likely to occur only when hybrids have the opportunity to evolve in a different habitat from the parentals, exploring a different niche (Burke & Arnold 2001; Barton 2001; Mallet 2007; Abbot et al. 2013). In our case, all hybrids occurred in the exact same

streams, as did both parental species, so that the hybridization between *B. ibitiguara* and *B. sazimai*, although natural and persistent, may not represent actual speciation and occurs sporadically throughout the sites. We also show that the level of genetic divergence between *B. sazimai* and *B. ibitiguara* from the SCNP is low, mostly when compared to other congeners. It is hypothesized that hybridization among genetically similar species may represent little genetic/phenotypic novelty in formed hybrids, precluding possible advantageous additive effects in these hybrids (Burke & Arnold 2001; Abbott et al. 2013). This is corroborated by our call and morphological traits, in which potential hybrids were scattered and did not represent a particular identity or intermediate values when compared to pure *B. ibitiguara* and *B. sazimai* (except those with a different call type; see discussion below).

Our analyses including three non-sympatric congeners provided information of a possible "outgroup" at the molecular level. Bal is presently included within the *B. pseudopseudis* group together with *B. ibitiguara*, whereas Bci and Bhy are in the *B. circumdata* group, together with *B. sazimai*, mostly based on morphology (Faivovich et al. 2005). Even so, with 5 cross-amplifying microsatellites, we found (1) *B. ibitiguara* and *B. sazimai* as a single, fully supported genetic cluster, apart from the other three species (supplemental figure 3), and (2) a genetic separation of Bhy, Bci and Bal, but a pronounced overlapping between *B. ibitiguara* and *B. sazimai* (figure 4). We confirm here that these species share an evolutionary history and some polymorphisms, but the relationships within the present taxonomic groups are obscured and likely complicated due to hybridization. Given that our microsatellite data do not seem to correspond with the present classification, we strongly suggest that the species' groups within the genus *Bokermannohyla* be reassessed with DNA sequencing. We highlight the importance of combining phenotypic and molecular data when dealing with species that overlap geographically, and recommend sampling regions of known sympatry among the analyzed species.

Mechanisms of hybridization and phenotypic comparisons

We characterized here a different pattern from that commonly observed in heterospecific reproduction in anurans. Typically, hybridization in frogs occurs among species that breed synchronously in large groups, also known as explosive breeders. In explosive breeders, the reproductive season is short, individuals form dense

aggregations of males and females after heavy rains, and there is little to no territorial defense or female selection (Wells 1977; 2007). In contrast, in prolonged breeders, females arrive asynchronously at breeding sites and choose from available calling males, which generally defend a territory (Wells 1977; 2007). Heterospecific matings are particularly well-documented worldwide for explosive breeders, i.e., with little or no opportunity for female choice, and in species with simple or lacking courtships (Sullivan 1986; Azevedo et al. 2003; Sequeira et al. 2011). Nonetheless, we found hybrids between prolonged-breeders in which mating is mediated by strong sexual selection for at least one of the species involved (Nali & Prado 2012).

Although little is known about the natural history of *B. sazimai* (Cardoso & Andrade 1982), *B. ibitiguara* is well-studied. *Bokermannohyla ibitiguara* has a complex courtship, involving acoustic and tactile stimuli between males and females, with inspection of nests by the latter at the breeding sites (Nali & Prado 2012). Males aggressively defend territories and oviposition sites through calls and physical combats (Nali & Prado 2012; 2014a; 2014b). Selection of males by females is strong in this species, likely leading to call differentiation (Chapter 2; Nali & Prado 2012; 2014a; R. C. Nali, pers. obs.). The natural history of this species strongly indicates that hybrids occur not due to simple misidentification with no opportunity of female choice, such as in "mating balls" of explosively-breeding species (Sequeira et al. 2011; Pereyra et al. 2016); rather, heterospecific mating must occur throughout the rainy season, when males call and defend territories to attract females, and somehow pass a highly selective behavioral filter. 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). Our results on the reproductive interference between these species are interesting, but exactly how the complex courtships of *B. ibitiguara* and likely of *B. sazimai* interact and lead to erroneous mating is a phenomenon that should be thoroughly investigated (Gröning & Hochkirch 2008).

A known mechanism of reproductive interference is heterospecific rivalry, in which males of a different species are mistaken for conspecifics and targeted with aggressive behaviors (Severinghaus et al. 1981; Jones et al. 1998; Schultz et al. 2001; Gröning & Hochkirch 2008). We have indeed observed that males of *B. sazimai* respond aggressively to broadcasted *B. ibitiguara* calls in close proximity (R. C. Nali, pers. obs.),

and this confusion of acoustic signals between these species might as well extend to females during selection of partners, as they might target heterospecific males for reproduction. In fish, it has been shown that sexual selection potentiates hybridization, as females may prefer males of the other species instead of their own, which is linked to the fact that heterospecific males and hybrids may have enhanced capabilities in defending territories, ultimately leading to different rates of reproductive success (Rosenfield & Kodric-Brown 2003). We cannot speculate that sexual selection actually reinforces hybridization between *B. sazimai* and *B. ibitiguara*; hybrids we found in this system were uncommon (ca. 10%) and species boundaries seem to persist between these sympatric species, regardless of hybridization. Nonetheless, our findings that even species with complex social interactions among males and females can naturally hybridize raise interesting questions about the roles of territorial and aggressive behaviors and hybridization in frogs.

As we observed (1) short notes in two hybrids that are different and absent in parental species, and (2) similar calls to the parental forms in two other hybrids, our data underscore that hybrid phenotypes are not always intermediate, but rather a mosaic of different and sometimes unique phenotypes (Grant 1981; Campton 1987; Allendorf et al. 2001). Although it is hypothesized that hybrid frogs should have different advertisement calls compared to parental species (Bogart 1980), sometimes parentals and hybrids have very similar calls (Haddad et al. 1994; Vargas-Salinas & Amézquita 2013). Here, two hybrids had different calls, yet the other two had calls similar to parental forms, corroborating that incomplete pre-zygotic mechanisms may be operating in this system. This poor differentiation in signals of hybrids may be due to the similarity of calls between the parental species. Although the calls of *B. sazimai* and *B. ibitiguara* can be separated statistically (figure 5B), call variation across the distributional range of *B. ibitiguara* is common (Chapter 2); this variance, and the similar structure of *B. sazimai* and *B. ibitiguara* calls (long notes followed by a sequence of short notes) may contribute to signal jamming (Gröning & Hochkirch 2008), causing females to poorly discern conspecific from heterospecific calls. In addition, long notes were emitted more by males of *B. sazimai* than *B. ibitiguara*; in the latter, emission of long notes increases when females are present, and the courtship call is composed only by long notes, indicating that long notes are more attractive to females (Nali & Prado 2014a; R. C. Nali, pers. obs.). Thus, combined with signal jamming, *B. ibitiguara* females

could be attracted to the higher repetition rate of long notes emitted by *B. sazimai* males. In summary, calling behavior and call characteristics in these species are not sufficiently distinct to prevent heterospecific mating.

Even if females are misguided towards heterospecific male calls, close-contact between the sexes could cause the courtship to terminate prematurely (Wong & Candolin 2005; Gröning & Hochkirch 2008). This may be particularly important when females are choosy and engage in complex courtships, as observed for *B. ibitiguara* (Nali & Prado 2012). Interestingly, the morphology of the single *B. ibitiguara* female in our dataset was closer to that of *B. sazimai* males compared to males of its own species (figure 5A, black asterisk). Size-assortative mating is known for frogs, where females seek males with similar sizes, which could assure better fertilization rates (Bastos & Haddad 1996; Wogel et al. 2005; Lu et al. 2010). Alternatively, the occurrence of satellite behavior, i.e., opportunistic males that do not participate in the courtship and steal females from a resident male, could result in heterospecific mating and hybridization. For instance, Taylor et al. (1996) suggested that satellite behaviors in hybridizing fishes were likely favored in the genetic group with smaller body sizes. Satellite behavior was described for *B. ibitiguara* and it is a common strategy in many prolonged-breeding frogs (Wells 2007; Nali & Prado 2012). Thus, the smaller size of *B. sazimai* males could reinforce heterospecific matings between *B. sazimai* males with *B. ibitiguara* females through this opportunistic behavior. Unfortunately, as females are difficult to find in the field (R. C. Nali, pers. obs.), we have collected only a single *B. ibitiguara* female within the SCNP. Nonetheless, we show that incorporating morphology in studies of hybridization is crucial and we should take into account differences between sexes and opportunistic mating tactics.

Although we found a few private alleles in hybrids, we did not observe an evidence of genetic erosion/disruption of parentals (Allendorf et al. 2001; Malukiewicz et al. 2015). Our results show that hybridization among closely-related frogs is not restricted to species with explosive breeding and naturally occurs even among species with complex courtships. Upon examination, more cases like this may be found in other members of the Cophomantinae subfamily, which have highly conserved karyotypes across genera (Catroli et al. 2011). The Cophomantinae is a large clade of treefrogs for which many complex courtships and male aggressive interactions have been described, and contains almost 30% of the known hylids (Duellman et al. 2016; Frost 2016).

Studying hybridization in this clade in a multi-character framework will help uncover the role of hybridization in the diversification of Neotropical treefrogs. Unfortunately, available molecular phylogenies include few species from this clade, which leads to a purely morphological characterization of taxonomic groups for all recognized taxa (Faivovich et al. 2005; Pyron & Wiens 2011; Duellman et al. 2016). Complete phylogenies built from molecular data will help confirm the clades within this genus and the occurrence of hybridization, with the extended possibility of using mitochondrial DNA to confirm the directionality of the hybrids found here and other frogs upon examination (e.g. Sequeira et al. 2011; Vanhaecke et al. 2012).

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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 hybrid private alleles (absent from Bsa and Bib) at the specified loci (specific alleles in supplemental tables 2 and 3). Individuals 17326, 18117 and 18172 were classified as hybrids by STRUCTURE only. The order of hybrids follow that of figure 2.

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

F1 and F2 = hybrid generations; Bx = Backcross; ^{h} homozygote at the specified locus.

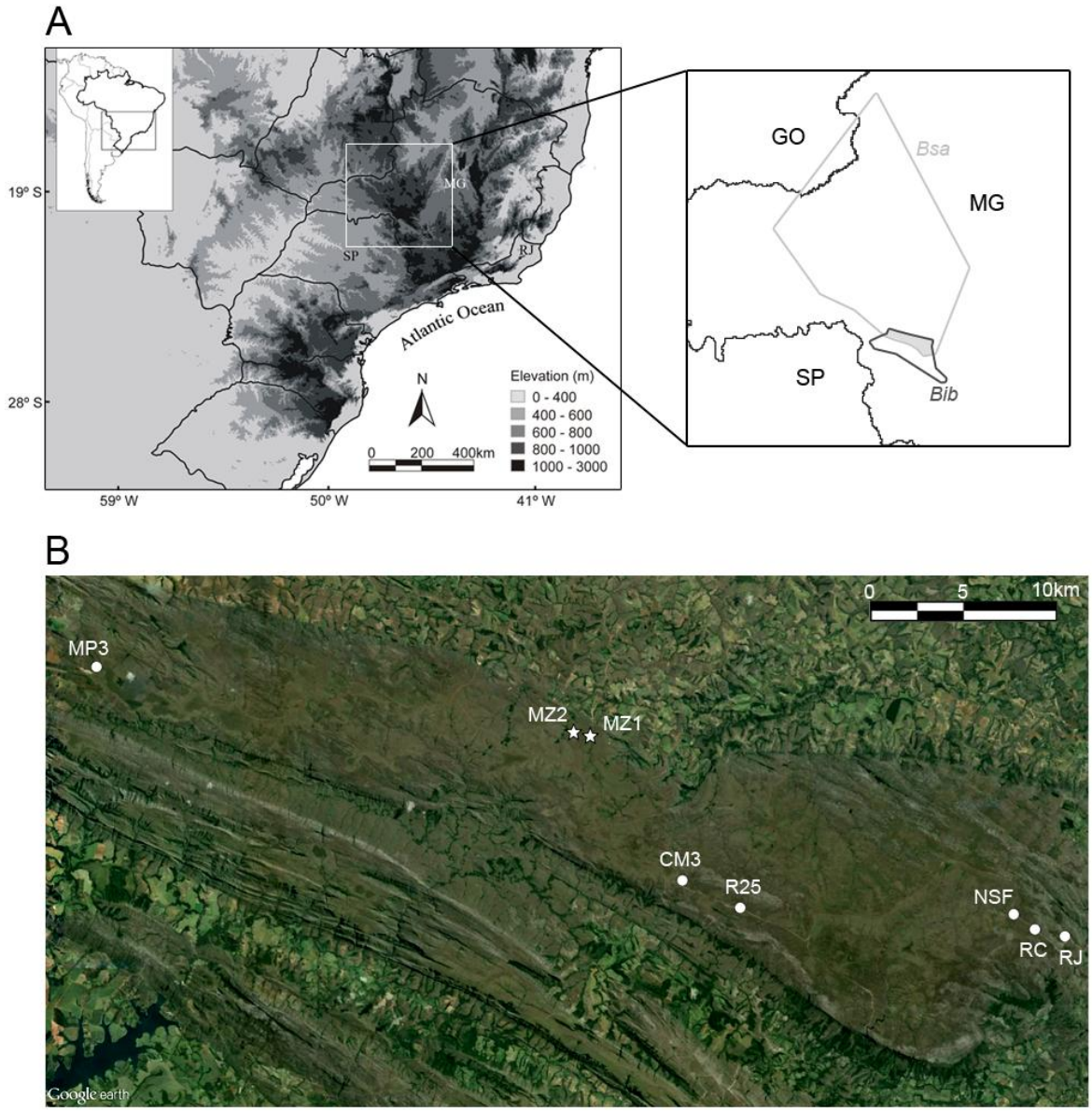


Figure 1. Geographic ranges and sampling sites of *Bokermannohyla ibitiguara* and *B. sazimai* in southeastern Brazil. A) General locality and known ranges of both species; *B. ibitiguara* (Bib) based on Haddad et al. (1988), Feio (2002), and personal observations; *B. sazimai* (Bsa) based on Carvalho & Giaretta (2013). The contact hybrid zone is marked in grey, located within the Serra da Canastra National Park. Acronyms are Brazilian states: GO = Goiás, MG = Minas Gerais and SP = São Paulo. B) Eight sampling localities of *B. ibitiguara* (dots) and *B. sazimai* (stars) within the hybrid zone (see supplemental table 1 for full names and coordinates). Satellite image obtained from Landsat and visualized on Google Earth.

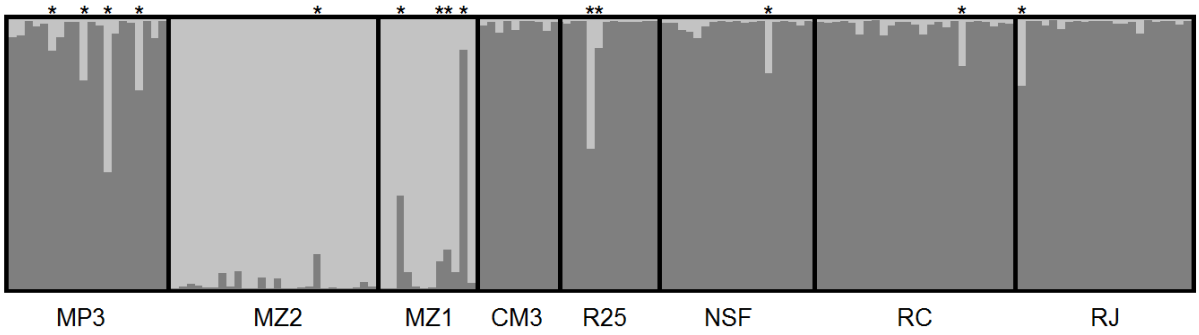


Figure 2. Result of our Bayesian analysis for eight sampled streams (fom west to east) within the Serra da Canastra National Park. We found two genetic clusters (K = 2) representing the two frog species (dark gray: *Bokermannohyla ibitiguara*; light gray: *B. sazimai*). Asterisks represent hybrid individuals with intermediate cluster assignments (0.1 < Q < 0.9).

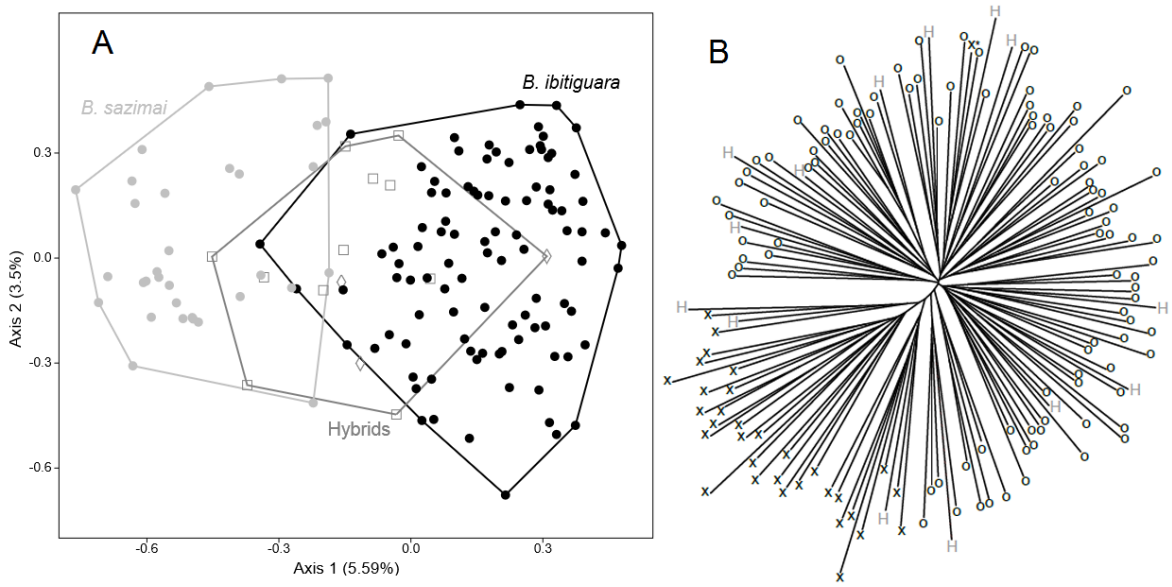


Figure 3. Genetic dissimilarities among *Bokermannohyla ibitiguara*, *B. sazimai* and identified hybrids, based on squared genetic distances between pairs of samples. (A) Principal Coordinates Analysis with convex hulls delimiting parental species and overlapping hybrids. The more conservative estimates 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.

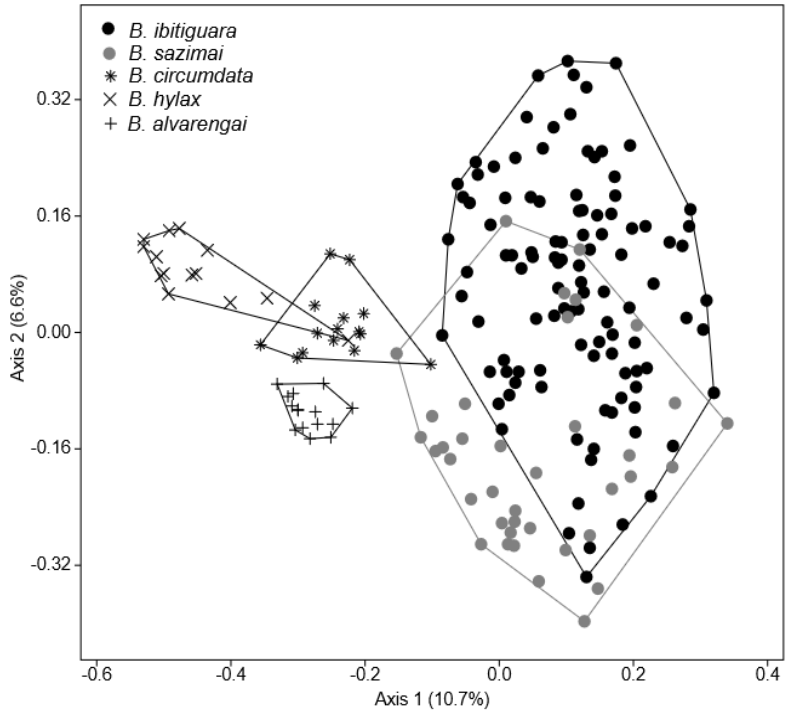


Figure 4. Genetic dissimilarities (Principal Coordinates Analysis) among two *Bokermannohyla* species from this study and three other non-sympatric congeners.

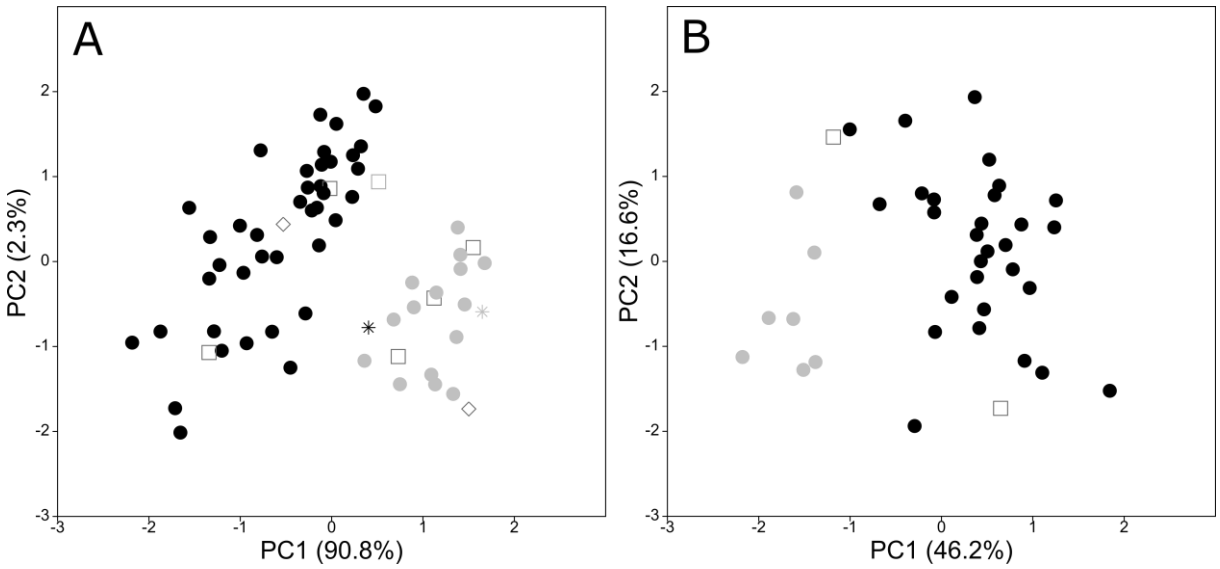


Figure 5. Principal Component Analyses with adults of *Bokermannohyla ibitiguara* (black dots), *B. sazimai* (gray dots), and putative hybrids (gray squares are conservative hybrids; gray diamonds are lenient hybrids), using (A) morphological variables, including two females (asterisks), and (B) acoustic variables.

SUPPLEMENTAL MATERIAL

Supplemental Table 1. Sampling localities within the hybrid zone of *Bokermannohyla ibitiguara* and *B. sazimai*, Serra da Canastra National Park, state of Minas Gerais, Brazil.

Locality acronym	Locality name	Latitude (°S)	Longitude (°W)	*N* adult samples	*N* tadpole samples
CM3	Capão de Mata 3	20.2273	46.6164	8	2
MP3	Mata da Portaria 3	20.1246	46.9164	5	15
NSF	Nascente do São Francisco	20.2434	46.4466	15	4
R25	Riacho km 25	20.2404	46.5868	8	4
RC	Riacho do Campo	20.2507	46.4357	11	14
RJ	Retiro do Jaguarê	20.2540	46.4203	7	15
MZ1	Mata do Zagaia 1	20.1578	46.6636	12	0
MZ2	Mata do Zagaia 2	20.1560	46.6721	8	18

Average straight-line distance among localities: 21.3 ± 14.7 km; range = 0.9 - 53.7 km

Supplemental Table 2. Hybrids between *Bokermannohyla ibitiguara* and *B. sazimai* found in this study, with respective private alleles from *B. ibitiguara*. A single allele was private for *B. sazimai* (*). CFBH-T = tissue number at Coleção de Anfíbios Célio F. B. Haddad, Universidade Estadual Paulista, Rio Claro, São Paulo, Brazil.

CFBH-T	Stream	Private alleles by loci								
		Bi179	Bi609	Bi1122	Bi1397	Bi1521	Bi2312	Bi2686	Bi3629	Bi4144
18117	MP3	-	339	-	-	278	202	-	-	-
18121	MP3	-	333	-	-	-	-	-	-	221
18124	MP3	-	-	-	-	-	-	-	-	-
18128	MP3	-	336	-	196	-	202	-	-	-
18192	MZ2	-	-	-	-	-	-	-	-	-
18164	MZ1	231*	-	-	-	-	-	-	-	-
18169	MZ1	-	-	-	-	-	-	-	-	-
18170	MZ1	-	-	-	-	-	-	-	-	-
18172	MZ1	-	-	-	-	-	-	-	-	-
17325	R25	-	-	-	-	-	-	-	-	225
17326	R25	-	-	218[h]	196	-	-	-	-	225
17261	NSF	-	-	-	-	-	202[h]	-	-	225
17290	RC	-	-	-	-	-	202	195	247	-
17242	RJ	-	336	-	-	-	-	-	-	-

[h] homozygous for the indicated allele.

Supplemental Table 3. 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*. CFBH-T = tissue number at Coleção de Anfíbios Célio F. B. Haddad, Universidade Estadual Paulista, Rio Claro, SP, Brazil.

CFBH-T	Stream	Private alleles by loci									
		Bi179	Bi383	Bi639	Bi1050	Bi1122	Bi1397	Bi1521	Bi3202	Bi3629	Bi4144
18117	MP3	-	-	-	-	-	355	-	278	203[h]	-
18121	MP3	-	-	-	-	-	-	-	-	203	-
18124	MP3	-	-	-	-	237[h]	-	-	-	-	-
18128	MP3	-	216	-	-	-	-	-	-	-	-
18192	MZ2	241	-	-	-	-	-	-	-	-	-
18164	MZ1	-	-	-	-	-	-	-	-	-	-
18169	MZ1	-	-	-	-	-	-	-	-	-	-
18170	MZ1	-	-	-	-	-	-	-	-	-	-
18172	MZ1	-	-	-	-	-	-	307[h]	-	-	-
17325	R25	-	238	-	-	-	-	-	-	-	-
17326	R25	-	-	-	-	-	-	-	-	-	-
17261	NSF	-	-	-	-	-	-	-	-	-	-
17290	RC	-	-	-	-	-	-	-	-	-	208
17242	RJ	-	-	313	184[h]	-	-	-	-	-	-

[h] homozygous for the indicated allele.

Supplemental Table 4. Body and acoustic measures of the two hybrids between *Bokermannohyla ibitiguara* and *B. sazimai* that emitted short notes different from either parental species. CFBH = voucher number at Coleção de Anfíbios Célio F. B. Haddad, Universidade Estadual Paulista, Rio Claro, SP, Brazil.

	CFBH 40568	CFBH 40569
Body measures		
Snout-vent length (mm)	36.75	38.15
Body mass (g)	4.2	4.9
Acoustic measures (means)		
Minimum frequency of the short note (Hz)	777.50	724.98
Dominant frequency of the short note (Hz)	1571.9	1894.9
Duration of the short note (s)	0.1185	0.1237
Minimum frequency of the short notes' sequence (Hz)	794.4	751.1
Dominant frequency of the short notes' sequence (Hz)	1636.5	1894.9
Duration of the short notes' sequence (s)	2.944	2.991
Number of short notes per sequence	12	12

Supplemental Table 5. Tissue (CFBH-T) and voucher specimen (CFBH) numbers of 44 additional *Bokermannohyla* individuals, deposited in the Coleção de Anfíbios Célio F. B. Haddad, Universidade Estadual Paulista, Rio Claro, São Paulo, Brazil. Most samples are from southeastern Brazil, except those from Paraná, Santa Catarina and Rio Grande do Sul, which are from south Brazil.

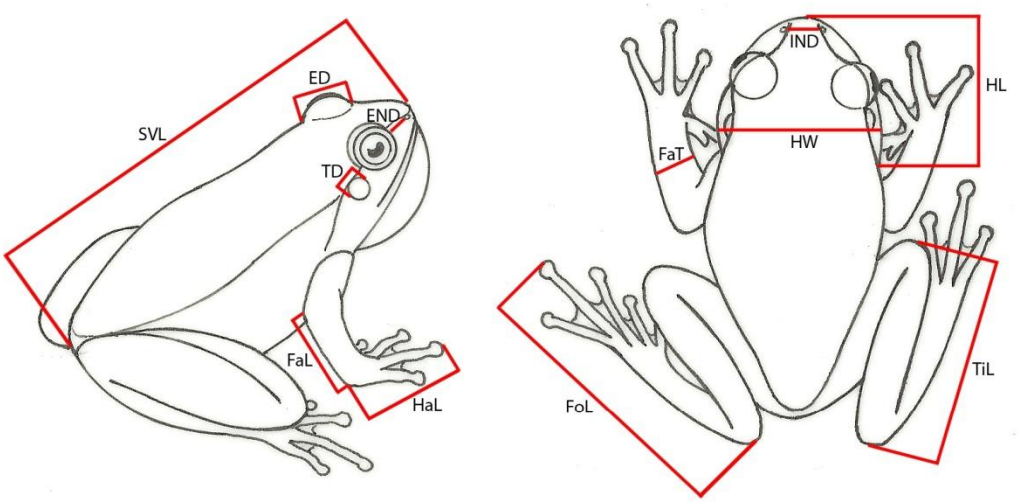
Species	CFBH-T	CFBH	Municipality	State
B. alvarengai	17426	*	Santana do Riacho	Minas Gerais
B. alvarengai	17427	*	Santana do Riacho	Minas Gerais
B. alvarengai	17428	*	Santana do Riacho	Minas Gerais
B. alvarengai	17429	*	Santana do Riacho	Minas Gerais
B. alvarengai	17430	*	Santana do Riacho	Minas Gerais
B. alvarengai	17431	*	Santana do Riacho	Minas Gerais
B. alvarengai	17432	*	Santana do Riacho	Minas Gerais
B. alvarengai	17433	*	Santana do Riacho	Minas Gerais
B. alvarengai	17434	*	Santana do Riacho	Minas Gerais
B. alvarengai	17435	*	Santana do Riacho	Minas Gerais
B. alvarengai	17436	*	Santana do Riacho	Minas Gerais
B. alvarengai	17437	*	Santana do Riacho	Minas Gerais
B. alvarengai	17438	*	Santana do Riacho	Minas Gerais
B. alvarengai	17439	*	Santana do Riacho	Minas Gerais
B. alvarengai	17440	*	Santana do Riacho	Minas Gerais
B. hylax	5523	11545	Cubatão	São Paulo
B. hylax	5526	11548	Cubatão	São Paulo
B. hylax	5872	13795	Peruíbe	São Paulo
B. hylax	6622	14795	São Luís do Paraitinga	São Paulo
B. hylax	15914	31093	São Paulo	São Paulo
B. hylax	15990	25675	Apiaí/Iporanga	São Paulo
B. hylax	16044	26775	Apiaí/Iporanga	São Paulo
B. hylax	16047	26778	Apiaí/Iporanga	São Paulo
B. hylax	16048	26779	Apiaí/Iporanga	São Paulo
B. hylax	16330	32310	São Paulo	São Paulo
B. hylax	8173	18191	Anitápolis	Santa Catarina
B. hylax	8175	18193	Anitápolis	Santa Catarina
B. hylax	10324	21924	Barracão	Rio Grande do Sul
B. hylax	15240	31018	Morretes	Paraná
B. circumdata	6514	14652	São Luís do Paraitinga	São Paulo
B. circumdata	6515	14653	São Luís do Paraitinga	São Paulo
B. circumdata	15623	30799	São José do Barreiro	São Paulo
B. circumdata	15640	30802	São José do Barreiro	São Paulo
B. circumdata	16042	26773	Apiaí and Iporanga	São Paulo
B. circumdata	16043	26774	Apiaí and Iporanga	São Paulo
B. circumdata	16523	33994	Silveiras/Cunha/Areias	São Paulo
B. circumdata	16545	33904	Silveiras/Cunha/Areias	São Paulo
B. circumdata	16546	33992	Silveiras/Cunha/Areias	São Paulo
B. circumdata	16548	33995	Silveiras/Cunha/Areias	São Paulo
B. circumdata	16549	33991	Silveiras/Cunha/Areias	São Paulo
B. circumdata	13691	28095	Santa Maria Madalena	Rio de Janeiro
B. circumdata	13692	28096	Santa Maria Madalena	Rio de Janeiro
B. circumdata	15235	31013	Campina Grande do Sul	Paraná
B. circumdata	15236	31014	Campina Grande do Sul	Paraná

* No voucher, only tissues collected

Supplemental Table 6. Tissue accession numbers (CFBH-T) of *Bokermannohyla ibitiguara*, *B. sazimai* and hybrid individuals (in bold) from the Serra da Canastra National Park, state of Minas Gerais, Brazil. Localities were ordered from west to east. When available, the collected specimen (CFBH) is stated in parentheses next to each tissue number, shown in italics when not recorded. Tissues were deposited at Coleção de Anfíbios Célio F. B. Haddad, Universidade Estadual Paulista, Rio Claro, São Paulo, Brazil.

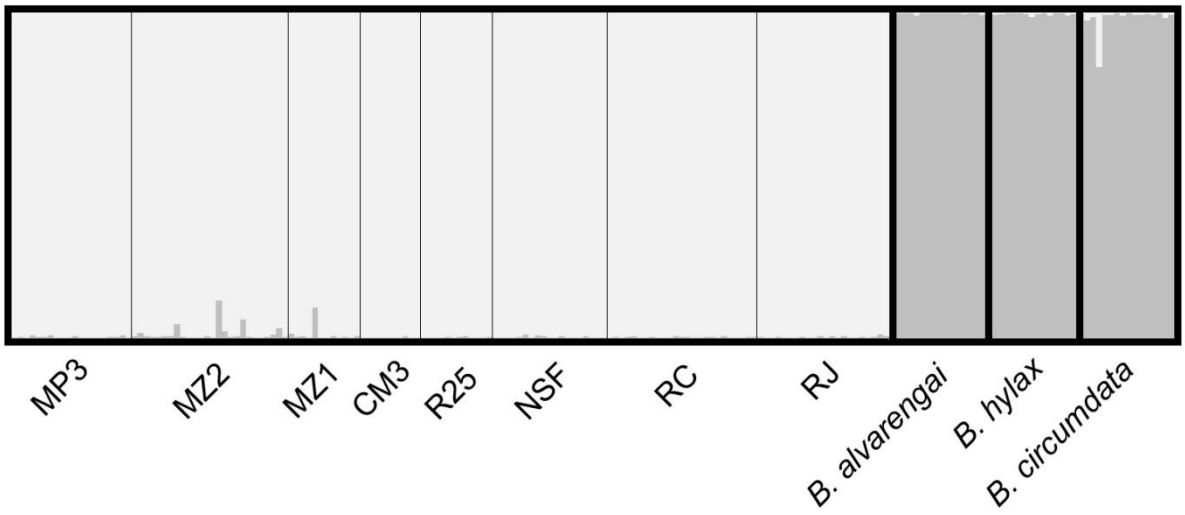
Locality	Vouchers
MP3	18112 *(40577)*, 18113-18116, **18117**, 18118, 18119 (40578), 18120 (40579), **18121 (40580)**, 18122 (40581), 18123, **18124**, 18125-18127, **18128**, 18129-18131
MZ2	18174-18181 (40609-40616), 18182-18191, **18192**, 18193-18199
MZ1	18162 (40597), 18163 (*40598*), **18164 (*40599*)**, 18165-18168 (*40600-40603*), **18169 (*40604*), 18170 (*40605***), 18171 (*40606*), **18172 (*40607*)**, 18173 (*40608*)
CM3	17300 (40566), 17301 (35867), 17305, 17308 (35868), 17309 (35869), 17310 (35870), 17321 (35871), 17322 (35872), 17323 (35873), 17324
R25	17306 (40562), 17307 (40563), 17316 (40567), **17325 (40568)**, **17326 (40569)**, 17327 (40570), 18200 *(40617)*, 18202 *(40618)*, 18203-18206
NSF	17238 (35859), 17239 (35860), 17240 (35861), 17245 (35862), 17246 (35863), 17253 (35864), 17254 (35865), 17255 (35866), 17256 (40564), 17257-17260, **17261**, 17275, 17276, 17277 *(40565)*, 17278, 17279
RC	17241 (40549), 17247-17249, 17262 (40556), 17263 (40557), 17264 *(40558)*, 17265, 17280 *(40559)*, 17281-17289, **17290**, 17298 *(40560)*, 17299 *(40561)*, 17317 (40571), 17318-17320
RJ	**17242 (40550)**, 17243 (40551), 17244 (40552), 17250 (40553), 17251 *(40554)*, 17252, 17266 (40555), 17267-17274, 17291-17297

Localities MZ1 and MZ2 contained parental *B. sazimai* and *B. sazimai*-like hybrids; the others contained parental *B. ibitiguara* and *B. ibitiguara*-like hybrids.

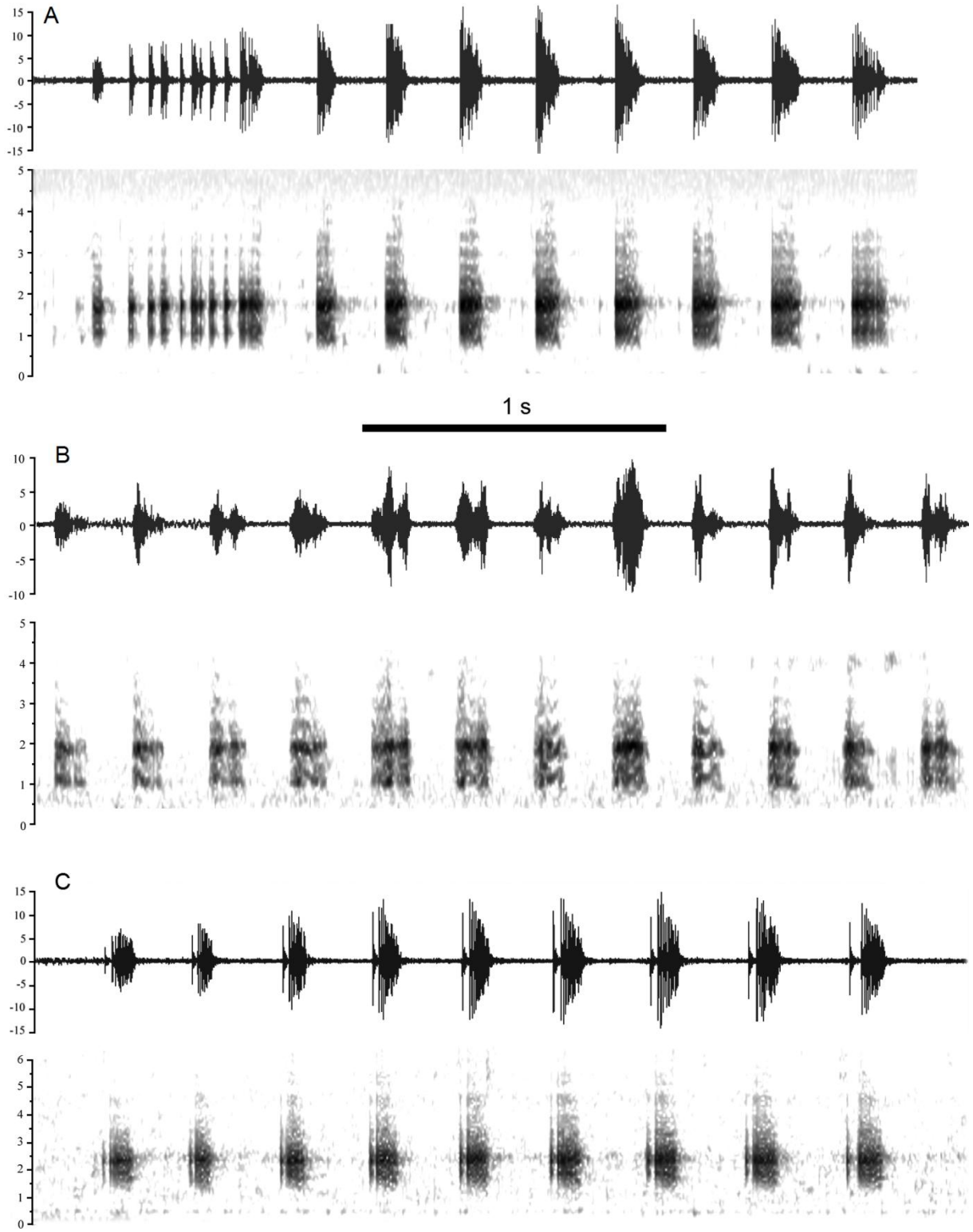


Supplemental Figure 1. Twelve morphometric variables measured in this study (see text for full names).

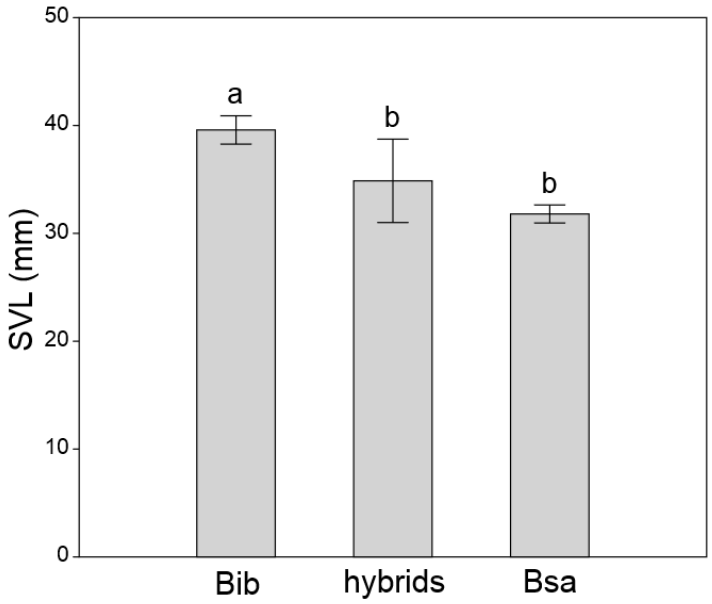
Supplemental Figure 2. Examples of adult *Bokermannohyla ibitiguara* (top left, voucher CFBH 40563), *B. sazimai* (top right, voucher CFBH 40614) and hybrids (bottom) found in this study. Hybrids were most similar to *B. ibitiguara* (bottom left, voucher CFBH 40550) or *B. sazimai* (bottom right, voucher CFBH 40605) depending on the population of origin. Scale bar = 2cm.



Supplemental Figure 3. Bayesian analysis with genotypes from five microsatellites that amplified for *B. ibitiguara* and *B. sazimai* across our localities (acronyms), as well as for additional non-sympatric congeners (*B. alvarengai*, *B. hylax* and *B. circumdata;* see supplemental table 5). We found two genetic clusters, one composed by *B. ibitiguara* + *B. sazimai* (light gray, separated by localities), and the other composed by the other three congeners combined (dark gray).



Supplemental Figure 4. Comparison between a sequence of short notes of (A) *B. ibitiguara*, also with an introductory long note (voucher CFBH 35867; SVL = 38.2 mm), (B) an identified hybrid (CFBH 40569; 38.2 mm) and (C) *B. sazimai* (CFBH 40616; 33.3 mm). For each individual, we show the oscillogram above (ku) and spectrogram below (kHz). Air temperatures were 20°C (A and B) and 18°C (C).



Supplemental Figure 5. Means and standard errors of body size values (SVL) among parental *B. ibitiguara* (Bib), parental *B. sazimai* (Bsa) and hybrids found in this study. The three groups were statistically different by a Kruskal-Wallis test. Post-hoc significant differences (Student-Newman-Keuls) are represented by different letters.

CONCLUSÃO

- *Bokermannohyla ibitiguara* é um anuro habitante de riachos que apresenta variação genética, morfológica e acústica na sua área de distribuição (Serra da Canastra, no Cerrado de Minas Gerais), e hibridação com a espécie *Bokermannohyla sazimai* dentro do Parque Nacional da Serra da Canastra original (PNSC).

- Para *B. ibitiguara*, análises genéticas com indivíduos de 12 localidades, utilizando 21 microssatélites, revelaram quatro grupos genéticos distintos, separando, grosseiramente, populações que ocorrem em riachos dentro do PNSC de populações que ocorrem em áreas alteradas nas propriedades rurais ou não desapropriadas do parque.

- Indivíduos de diferentes riachos apresentaram diferenciação genética, exceto em riachos dentro do PNSC, onde os indivíduos apresentaram também maior heterozigosidade e riqueza alélica. Consequentemente, observamos um maior grau de diferenciação entre indivíduos localizados fora do PNSC comparados aos de dentro do PNSC.

- A diferenciação genética foi explicada, além da distância geográfica, pela complexidade topográfica, enquanto a cobertura vegetal apresentou uma fraca correlação. De forma semelhante, a variação de alguns parâmetros genéticos das populações foi explicada apenas por aspectos topográficos.

- A importância do PNSC para a conectividade espacial entre populações de *B. ibitiguara* pode ser explicada por sua baixa complexidade topográfica, facilitando a dispersão de indivíduos. Características de relevo devem ser levadas em consideração para o estabelecimento de medidas de conservação, não apenas o uso de hábitat e/ou cobertura vegetal. Tal caracterização é particularmente importante em áreas prioritárias de preservação do Cerrado como a Serra do Espinhaço (também em MG) e a Chapada dos Guimarães (MT), as quais também apresentam particularidades quanto ao seu relevo.

- Na análise fenotípica de *B. ibitiguara*, a variação acústica foi muito maior do que morfológica através da área de distribuição, sugerindo uma seleção direcional de fêmeas sobre parâmetros acústicos dos machos.

- Variáveis acústicas significativamente diferentes entre populações podem ser associadas à preferência de fêmeas e/ou reconhecimento individual nesta espécie, demonstrando um papel da seleção sexual na variabilidade acústica.

- A diferenciação genética não explicou a diferenciação acústica entre populações, excluindo a evolução neutra por deriva e isolamento genético como um mecanismo responsável pela variação geográfica dos cantos da espécie.

- Não encontramos suporte para a hipótese de adaptação acústica (AAH) como mecanismo de variação acústica populacional. No entanto, dois parâmetros das notas associados à agressividade (notas curtas) correlacionaram-se com a vegetação ripária de forma contrária ao previsto pela AAH. Tal fato poderia ser explicado por uma maior densidade psossível de machos reproduzindo-se em áreas com menos vegetação ripária, aumentando a seleção intrasexual (competição entre machos), o que por sua vez impõe pressões seletivas sobre tais parâmetros do canto agressivo.

- Nossos resultados sugerem que a seleção sexual promove diferenciação acústica nesta espécie em detrimento dos outros mecanismos analisados, confirmado por observações comportamentais anteriores. Demonstramos que caracteres fenotípicos e genéticos, bem como diferentes mecanismos devem ser analisados na evolução de sinais de comunicação, especialmente em animais com comportamento reprodutivo complexo.

- Em uma análise interespecífica na zona de contato entre *B. ibitiguara* e *B. sazimai* (PNSC), identificamos híbridos genéticos em sete dos oito riachos amostrados, os quais apresentam estrutura genética sobreposta, porém intermediárias aos parentais.

- Análises com três espécies de *Bokermannohyla* não simpátricas reforçaram a grande similaridade genética entre *B. ibitiguara* e *B. sazimai*, apesar de estarem atualmente incluídas em grupos taxonômicos distintos.

- Análises multivariadas com caracteres morfométricos e acústicos demonstraram que os híbridos identificados não são intermediários fenotipicamente entre as espécies parentais (*B. ibitiguara* e *B. sazimai*), mas sim apresentam um mosaico de fenótipos. Especificamente com relação à vocalização, dois machos híbridos apresentaram algumas notas diferentes dos parentais, e outros dois apresentaram cantos semelhantes.

- Sugerimos que a hibridação nestas espécies pode ocorrer por falhas no principal sistema de reconhecimento (comunicação acústica), devido à similaridade das vocalizações entre machos de ambas as espécies. Além disso, táticas alternativas de acasalamento, como machos satélites (observados em *B. ibitiguara*), e diferenças morfológicas entre os sexos poderiam explicar a ocorrência de reprodução interespecífica.

- Apesar de estudos com hibridação em anuros serem aparentemente enviesados para espécies com reprodução explosiva e pouca oportunidade de escolha de machos por fêmeas, demonstramos que eventos de hibridação podem ocorrer também em espécies de reprodução prolongada (*B. ibitiguara* e *B. sazimai*), com mecanismos de seleção sexual e comportamento de corte elaborados, bem como territorialidade/agressividade (*B. ibitiguara*). Reiteramos também a necessidade de incorporar análises filogenéticas na caracterização dos grupos de *Bokermannohyla*, levando em conta a possível hibridação entre espécies simpátricas do gênero.

- A diversificação multifacetada de *Bokermannohyla ibitiguara* traz contribuições para as áreas de conservação, biologia evolutiva e ecologia comportamental. Acreditamos que os enfoques e resultados desta tese possam ser aplicados a diferentes organismos e biomas, já que a história evolutiva das espécies é sempre mediada pela interação dos indivíduos com a paisagem, com indivíduos coespecíficos (do mesmo sexo ou do sexo oposto) e com outras espécies simpátricas.

APÊNDICE A

Microsatellite markers for *Bokermannohyla* species (Anura, Hylidae) from the Brazilian Cerrado and Atlantic Forest domains

Renato C. Nali[1,*], Kelly R. Zamudio[2], Cynthia P.A. Prado[3]

Abstract. We characterized 22 polymorphic microsatellite markers for the Brazilian treefrog *Bokermannohyla ibitiguara* and tested their cross-amplification in *B. alvarengai*, *B. circumdata* and *B. hylax*. Our focal species occurs in protected and disturbed Brazilian Cerrado landscapes, a highly threatened savanna in central Brazil. Fourteen markers successfully cross-amplified for at least one congener. These microsatellites will be useful for studies of mating systems, relatedness and landscape genetics of Cerrado populations under various deforestation levels. Moreover, variable markers for *B. circumdata* and *B. hylax* will also be useful for landscape genetic studies of taxa typical of the threatened Atlantic Forest domain.

Keywords: conservation, cross-amplification, Illumina MiSeq, marker identification, Neotropical frog, next-generation sequencing.

Landscape genetics explores the interaction between landscape variables and the evolutionary history of populations, thus quantifying the processes of migration and gene flow, genetic drift, and selection within and among populations (Manel et al., 2003; Holderegger and Wagner, 2008). Due to their high degree of polymorphism, microsatellite markers have been extensively used in landscape genetics for inference of recent demographic events, such as bottlenecks, reduced migration, and other genetic impacts of human activities (Pearse and Crandall, 2004). However, microsatellite marker specificity is typically high (Galbusera, Dongen and Matthysen, 2000; Duryea, Brasileiro and Zamudio, 2009) and for most non-model organisms, such as the frog genus *Bokermannohyla*, few microsatellite markers have been developed (Eterovick et al., 2012).

The hylid genus *Bokermannohyla* includes 33 species, distributed in both open and forested formations in central, south, southeastern, and northeastern Brazil (Faivovich et al., 2005; Frost, 2014). *Bokermannohyla ibitiguara* (Cardoso, 1983) is a stream-dwelling frog endemic to the Brazilian Cerrado, typical of the Serra da Canastra, State of Minas Gerais (MG), and surrounding mountainous areas, inhabiting gallery forests in both protected and anthropogenically altered landscapes (Haddad, Andrade and Cardoso, 1988; Nali and Prado, 2012, 2014). This species is classified as Data Deficient by the IUCN (Caramaschi and Eterovick, 2004), thus its true conservation status is unknown, precluding inference of its actual extinction risk (Morais et al., 2013). Very few studies have investigated the processes leading to genetic differentiation in frogs from open formations in the Neotropics (e.g., Maciel et al., 2010; Prado, Haddad and Zamudio, 2012). The Brazilian Cerrado is the most diverse and threatened savanna in the world (Silva and Bates, 2002); thus,

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this species is an excellent model for conservation genetic studies investigating the historical processes leading to diversification in the Cerrado and the genetic consequences of landscape fragmentation.

Here we characterize 22 microsatellite markers for *Bokermannohyla ibitiguara* and their cross-amplification in three closely related congeners: *B. alvarengai*, *B. circumdata*, and *B. hylax*. *Bokermannohyla alvarengai* also occurs in the Brazilian Cerrado, and the last two species occur in the Atlantic Forest, a highly diverse hotspot extremely threatened due to habitat loss (Ribeiro et al., 2009). For initial marker discovery, we created an enriched genomic library and used Illumina MiSeq next-generation sequencing to identify di-, tri- and tetra-repeats throughout the genome (Andres and Bogdanowicz, 2011). First we extracted whole genomic DNA from tissue of a single tadpole of *B. ibitiguara* with Qiagen DNeasy extraction columns. This individual was collected in the municipality of Sacramento, Minas Gerais state, Brazil, preserved in absolute ethanol, and deposited at Coleção de Anfíbios Célio F.B. Haddad, Departamento de Zoologia, I.B., Universidade Estadual Paulista, Rio Claro, São Paulo state, Brazil (tissue accession number: CFBH-T 11888). Genomic DNA (50-100 ng) was endonuclease-digested with AluI, RsaI, and Hpy166II, and pooled for subsequent adenylation with Klenow (exo-) and dATP. Restricted/adenylated DNA was then ligated to an Illumina Y-adaptor sequence using T4 DNA ligase in the presence of 1 mM ATP. Genomic fragments with repeats were captured by hybridization to biotinylated repeats and streptavidin-coated magnetic beads, and amplified/indexed with Platinum *Taq* polymerase and a pair of Illumina primers (one universal, one index primer). PCR products were quantified with a Qubit 2.0 fluorometer (Life Technologies, Carlsbad, CA), verified by electrophoresis on a 1.0% agarose gel, and size selected (300-600 bp) with AMPure beads (Beckman Coulter, Indianapolis, IN). We enabled the "design primers" function of msatcommander 1.0.3 software (Rozen and Skaletsky, 2000; Faircloth, 2008), also enabling the "repeats" and "primers" output files. Dimeric to tetrameric microsatellites with a product size range of 150-450 bp were selected.

From the above enriched microsatellite library, we optimized a total of 22 loci for *B. ibitiguara* (table 1) that met the follow-

Table 1. Primer sequences (Forward – F and Reverse – R), annealing temperatures (T_a), marker sizes (bp), allelic diversity (N_a), observed (H_o) and expected (H_e) heterozygosity of 22 microsatellite loci genotyped across three *Bokermannohyla ibitiguara* populations. Forward primers had the following sequence added to the 5′ end: CGAGTTTTCCCAGTCACGAC (see text for details).

Locus (Genbank #)	Primer (5′ – 3′)	Repeat	T_a (°C)	Size (bp)	N_a	H_o/H_e
Bi1 (KF977107)	F: AGGTGCGCTTGTAAGTATGAAAG R: GTTTAATACAGGGCGGTTCAGG	$(AGAT)_{10}$	64.5	219-274	9	NSF: 0.375/0.775 CM3: 0.571/0.813 R1: 0/0.7*
Bi94 (KF977108)	F: GATGATGAAACCTGTCACTGACC R: AATTCCCAAACCATCATTTCCAG	$(AGAT)_{14}$	61.5	179-220	11	NSF: 1/0.917 CM3: 0.857/0.879 R1: 0.429/0.703
Bi179 (KF977109)	F: ACAACCTGATGATGTTACCAACC R: ATAGTTTGGACATGAGGACCCTG	$(AG)_9$	59.5	216-235	8	NSF: 0.75/0.692 CM3: 0.714/0.714 R1: 0.625/0.758
Bi383 (KF977110)	F: GATATTACATCCAACAGAGGGCG R: GTTGGTATAAAGTCCTGCCTCAC	$(AC)_{13}$	59	208-234	8	NSF: 0.143/0.736* CM3: 0.5/0.682 R1: 0.143/0.143
Bi609 (KF977111)	F: CTACTGCAGAGCCATACAAACTG R: CAGATGTCCATGAACCAACTCAG	$(ACT)_7$	62	321-357	10	NSF: 0.75/0.875 CM3: 0.427/0.846 R1: 0.5/0.864

(Continued.)

Table 1. (Continued.)

Locus (Genbank #)	Primer (5′ – 3′)	Repeat	T_a (°C)	Size (bp)	N_a	H_o/H_e
Bi639 (KF977112)	F: AGTGGCCGTATATTTGATATGCAG R: AACAAAGTCCAGATCTTCACCTG	$(AAT)_{13}$	62	281-307	10	NSF: 1/0.868 CM3: 0.833/0.864 R1: 0.625/0.75
Bi1032 (KF977113)	F: ATGTGGTAAGATCTCCCTAGTGG R: TTCCAGTCTTGAGAAATTCTTTGG	$(AAAC)_7$	59	327-353	7	NSF: 0.5/0.893 CM3: 0.333/0.758 R1: 0.4/0.6
Bi1050 (KF977114)	F: GGCAGGCATCAGAGAAACTATG R: AATGCGTAGGTGTCTTTCTGAAC	$(AAT)_{11}$	64.5	203-222	7	NSF: 0.429/0.494 CM3: 0.286/0.560 R1: 0.333/0.561
Bi1122 (KF977115)	F: CTCATATGATTCAGAGGGTGGAC R: CCCTTTGGTCTTTCTTGTTCTCC	$(AC)_{13}$	59.5	213-235	5	NSF: 0.25/0.8* CM3: 0.143/0.363 R1: 0.143/0.143
Bi1397 (KF977116)	F: CTGCTTGAATCCAGGTCTGAATG R: CCGTCCCTTTATTGTCTCATTGG	$(AGAT)_{16}$	61	182-244	15	NSF: 0.429/0.857 CM3: 0.857/0.934 R1: 0.6/0.8
Bi1521 (KF977117)	F: TCAAACCACTCTCCATACGAATG R: GTTGCTGTAGACACTCTCTCTTG	$(AAT)_{10}$	59	278-313	7	NSF: 0.286/0.791* CM3: 0.667/0.712 R1: 0.375/0.342
Bi2312 (KF977118)	F: TCCCAACCTAGAATGCAGAGATC R: TCTCCCTGTAAATCTTGACTTTCC	$(AG)_9$	61	184-222	15	NSF: 0.625/0.858 CM3: 0.571/0.802 R1: 0.286/0.835*
Bi2686 (KF977119)	F: CCCTTCAGTACTGTGACATCATG R: ATTGCGGAGAATTATTGACTCCC	$(ACAG)_{13}$	64.5	187-273	9	NSF: 0.625/0.75 CM3: 0.5/0.818 R1: 0.571/0.758
Bi2761 (KF977120)	F: GCCTGAAAGTGGAAGATGAGATC R: GGGCATCAACATTAAATCTCAAGC	$(AGG)_8$	59	240-278	10	NSF: 0.667/0.712 CM3: 0.5/0.848 R1: 0.875/0.867
Bi3003 (KF977121)	F: CGGCATCTTGGACCATTTATAGG R: TATCTCACAAACTGTCTGTGTCC	$(AGAT)_9$	64.5	322-460	11	NSF: 0.571/0.824 CM3: 0.571/0.824 R1: 0.75/0.808
Bi3029 (KF977122)	F: TTTGTTGAAATTTGGCACCCTGG R: ACTGCATCTCTCCTCACTAACC	$(AG)_{15}$	64	244-267	9	NSF: 0.75/0.783 CM3: 0.714/0.857 R1: 0.571/0.802
Bi3202 (KF977123)	F: AGGTATCCTCTTAGTTCTTGCCC R: GTCCAGTAAATATCAACCTGCCC	$(AAAG)_{13}$	57.5	204-281	8	NSF: 0.75/0.742 CM3: 0.714/0.714 R1: 0.875/0.733
Bi3370 (KF977124)	F: GAGACCACTGCCATAGACCATG R: GGAAACGTTCTCCAGTCAACTAC	$(AAC)_{12}$	56.5	266-288	7	NSF: 0.625/0.683 CM3: 0.285/0.439 R1: 0.5/0.733
Bi3438 (KF977125)	F: GTTGTCTTAGTAGTGCACGTGTG R: GTGACTTAACCCTTCACGTTCTC	$(AC)_7$	59	207-231	4	NSF: 0.375/0.458 CM3: 0.429/0.385 R1: 0.4/0.711
Bi3629 (KF977126)	F: CTTCCTCAGGGTCCTCAATCATC R: ATGATGGTGTGTACAGAACGAAC	$(AT)_{10}$	59	217-353	13	NSF: 0.625/0.925 CM3: 0.429/0.846 R1: 0.4/0.711
Bi3836 (KF977127)	F: GAAATTGCAGAGGGTCCTACTTG R: TGGAAGCGCATGTATATCAGTTC	$(AC)_{10}$	59	299-320	11	NSF: 0.5/0.917 CM3: 0.286/0.648 R1: 0.5/0.817
Bi4144 (KF977128)	F: CATACATGTGCCAACTTTGCTTC R: TAGTTATGCTGCACTTGTTGAGG	$(ACAG)_{12}$	59.5	191-289	17	NSF: 0.625/0.942 CM3: 1/0.956 R1: 1/0.875

* Showed deviation from HWE, considering a P value corrected for multiple comparisons (Bonferroni $P < 0.00227$).

ing criteria: (1) polymorphic, (2) with reliable amplification, and (3) clean genotyping peaks in preliminary scoring. Loci were optimized across 23 individuals of *B. ibitiguara* from three independent populations from Minas Gerais state, Brazil (population NSF: 20.2434°S, 46.4466°W; population CM3: 20.2273°S, 46.6164°W; population R1: 20.2727°S, 47.0735°W; see Supplementary table S1 online). The abbreviations represent population names (in Portuguese): NSF = Nascente do São Francisco, CM3 = Capão de Mata 3, and R1 = Riacho 1. All individuals were euthanatized by spraying a solution of lidocaine 10% in the gular region (McDiarmid, 1994), and liver tissue samples were collected. To test cross-amplification (table 2) we additionally genotyped 15 samples of *B. alvarengai* and *B. hylax*, and 16 samples of *B. circumdata*, collected in southeastern and southern Brazil (table S1). We extracted DNA from all individuals with DNeasy extraction columns (Qiagen, Valencia, CA, USA), following 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 (table 1), 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), 1× buffer, 1.5 μM $MgCl_2$, 0.1 μg/μl bovine serum albumin, 0.4 mM dNTP, 0.1 μM of the forward and reverse primers, 0.3 μM of universal dye-labelled primer, and 0.25 U *Taq* polymerase. PCR products included a 20 bp tag on the 5′ end of the forward primer (table 1) and were co-amplified with a fluorescently tagged third 'universal' oligonucleotide that hybridized to that tag. After amplification, we combined 1 μl of individual PCR products from various 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 Genomics facility. We used GeneMarker v. 2.4.0 (SoftGenetics LLC, State College, PA) to analyze genotyping profiles, Microchecker v. 2.2.3 (Van Oosterhout et al., 2004) to check the final genotype assignments for null alleles, and Arlequin v. 3.5 (Excoffier and Lischer, 2010) to estimate observed/expected heterozygosities for each locus and population and test for linkage disequilibrium across all pairs of loci.

Table 2. Microsatellite amplification and number of alleles for *Bokermannohyla ibitiguara* and congeners (*B. alvarengai*, *B. circumdata* and *B. hylax*). Dashes represent non-genotyped loci due to poor or failed PCR amplifications. Sample sizes (n) are reported for each species, and numbers of successfully amplified congeners are shown in parentheses for loci with less successful genotyping.

Locus	*B. ibitiguara* $n = 23$	*B. alvarengai* $n = 15$	*B. circumdata* $n = 16$	*B. hylax* $n = 15$
Bi1	9	–	10	7
Bi94	11	7	–	–
Bi179	8	10	15	7
Bi383	8	–	1	1
Bi609	10	5	6	2
Bi639	10	6	9	9
Bi1050	7	–	–	1
Bi1397	15	16	13	8
Bi2312	15	–	8	6
Bi2761	10	–	4 (10)	3
Bi3029	9	3 (11)	4	6
Bi3202	8	2 (7)	4	6
Bi3370	7	1	6	4
Bi3438	4	1	–	4

For *B. ibitiguara*, the 22 polymorphic loci ranged in allele numbers from 4-17 across all individuals (table 1). We found evidence for linkage disequilibrium between 12 pairs of loci (Bi3370/Bi1, Bi3003/Bi2312, Bi 3370/Bi2312, Bi2312/Bi3836, Bi1/Bi1397, Bi1032/Bi1397, Bi3029/Bi609, Bi2312/Bi2761, Bi1397/Bi2761, Bi1397/Bi639, Bi3836/Bi383 and Bi1397/Bi383) across all populations. For the CM3 population, all loci were under Hardy-Weinberg Equilibrium (HWE), but two loci showed possible null alleles (Bi609 and Bi3629). For the R1 population, two loci showed deviation from HWE (Bi1 and Bi2312), both possibly with null alleles. The NSF population showed nine loci with possibility of null alleles (Bi1, Bi1032, Bi4144, Bi3836, Bi3629, Bi1397, Bi1122, Bi1521 and Bi383), the last three loci

showing deviation from HWE. Null alleles can cause excess homozygosity, which in turn can cause linkage disequilibrium between markers (Sabatti and Risch, 2002). However, excess homozygosity may also be caused by other factors such as inbreeding within populations, or recent bottlenecks (Falush, Stephens and Pritchard, 2007). We plan further studies with larger sample sizes to investigate population-specific factors mediating genetic structure.

For the three *Bokermannohyla* congeners, 14 of the 22 loci showed visible PCR amplicons of the appropriate size (table 2). For *B. alvarengai*, seven loci were polymorphic, although two of these genotyped poorly, and two were monomorphic. *Bokermannohyla circumdata* individuals were genotyped at one monomorphic and ten polymorphic loci, although one of the latter genotyped poorly. For the *B. hylax* samples, eleven loci were polymorphic and two were monomorphic. We found no relationship between cross-amplification success and phylogenetic distance among species; *B. alvarengai*, which is closely related to *B. ibitiguara* (Faivovich et al., 2005), showed less successful amplification than the other two more distantly-related species. Although loci varied from monomorphic to highly polymorphic for the three congeners (1-16 alleles), *B. alvarengai* and *B. circumdata* had loci with the highest numbers of alleles (16 and 15, respectively) when compared to *B. hylax* (9 alleles at most).

The microsatellite markers designed and optimized in this study will be useful for future landscape genetic and mating system studies in *B. ibitiguara*, as well as in the three additional *Bokermannohyla* species. Combined, these species have dominant ranges that include the threatened domains of the Brazilian Cerrado and Atlantic Forest. Successful cross-amplification of the optimized markers demonstrates the utility of this technique, and increases the availability of genetic resources for this poorly known Neotropical frog genus.

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Amphibia-Reptilia

Microsatellite markers for *Bokermannohyla* species (Anura, Hylidae) from the Brazilian Cerrado and Atlantic Forest domains

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Supplementary material

Table S1. Tissue (CFBH-T) and voucher specimen (CFBH) numbers of *Bokermannohyla* individuals used in this study and deposited in the Coleção de Anfíbios Celio F. B. Haddad, UNESP, Rio Claro, São Paulo, Brazil. Brazilian state names are: MG: Minas Gerais; SP: São Paulo; SC: Santa Catarina; RS: Rio Grande do Sul; PR: Paraná; RJ: Rio de Janeiro.

Species	CFBH-T	CFBH	Municipality, State
B. ibitiguara	17238[1]	35859	São Roque de Minas, MG
B. ibitiguara	17239[1]	35860	São Roque de Minas, MG
B. ibitiguara	17240[1]	35861	São Roque de Minas, MG
B. ibitiguara	17245[1]	35862	São Roque de Minas, MG
B. ibitiguara	17246[1]	35863	São Roque de Minas, MG
B. ibitiguara	17253[1]	35864	São Roque de Minas, MG
B. ibitiguara	17254[1]	35865	São Roque de Minas, MG
B. ibitiguara	17255[1]	35866	São Roque de Minas, MG
B. ibitiguara	17301[2]	35867	São Roque de Minas, MG
B. ibitiguara	17308[2]	35868	São Roque de Minas, MG
B. ibitiguara	17309[2]	35869	São Roque de Minas, MG
B. ibitiguara	17310[2]	35870	São Roque de Minas, MG
B. ibitiguara	17321[2]	35871	São Roque de Minas, MG
B. ibitiguara	17322[2]	35872	São Roque de Minas, MG
B. ibitiguara	17323[2]	35873	São Roque de Minas, MG
B. ibitiguara	17349[3]	31756	Sacramento, MG
B. ibitiguara	17350[3]	31757	Sacramento, MG
B. ibitiguara	17351[3]	31758	Sacramento, MG
B. ibitiguara	17352[3]	31759	Sacramento, MG
B. ibitiguara	17353[3]	31760	Sacramento, MG
B. ibitiguara	17354[3]	31761	Sacramento, MG
B. ibitiguara	17355[3]	31762	Sacramento, MG
B. ibitiguara	17356[3]	31763	Sacramento, MG

B. alvarengai	17426	*	Santana do Riacho, MG
B. alvarengai	17427	*	Santana do Riacho, MG
B. alvarengai	17428	*	Santana do Riacho, MG
B. alvarengai	17429	*	Santana do Riacho, MG
B. alvarengai	17430	*	Santana do Riacho, MG
B. alvarengai	17431	*	Santana do Riacho, MG
B. alvarengai	17432	*	Santana do Riacho, MG
B. alvarengai	17433	*	Santana do Riacho, MG
B. alvarengai	17434	*	Santana do Riacho, MG
B. alvarengai	17435	*	Santana do Riacho, MG
B. alvarengai	17436	*	Santana do Riacho, MG
B. alvarengai	17437	*	Santana do Riacho, MG
B. alvarengai	17438	*	Santana do Riacho, MG
B. alvarengai	17439	*	Santana do Riacho, MG
B. alvarengai	17440	*	Santana do Riacho, MG
B. hylax	5523	11545	Cubatão, SP
B. hylax	5526	11548	Cubatão, SP
B. hylax	5872	13795	Peruíbe, SP
B. hylax	6622	14795	São Luís do Paraitinga, SP
B. hylax	8173	18191	Anitápolis, SC
B. hylax	8175	18193	Anitápolis, SC
B. hylax	10324	21924	Barração, RS
B. hylax	10686	22316	Iguape, SP
B. hylax	15240	31018	Morretes, PR
B. hylax	15914	31093	São Paulo, SP
B. hylax	15990	25675	Apiaí e Iporanga, SP
B. hylax	16044	26775	Apiaí e Iporanga, SP
B. hylax	16047	26778	Apiaí e Iporanga, SP
B. hylax	16048	26779	Apiaí e Iporanga, SP
B. hylax	16330	32310	São Paulo, SP
B. circumdata	6514	14652	São Luís do Paraitinga, SP
B. circumdata	6515	14653	São Luís do Paraitinga, SP
B. circumdata	13691	28095	Santa Maria Madalena, RJ
B. circumdata	13692	28096	Santa Maria Madalena, RJ
B. circumdata	15235	31013	Campina Grande do Sul, PR
B. circumdata	15236	31014	Campina Grande do Sul, PR
B. circumdata	15623	30799	São José do Barreiro, SP
B. circumdata	15640	30802	São José do Barreiro, SP
B. circumdata	15918	31085	São Paulo, SP
B. circumdata	16042	26773	Apiaí e Iporanga, SP
B. circumdata	16043	26774	Apiaí e Iporanga, SP
B. circumdata	16523	33994	Silveiras, Cunha e Areias, SP
B. circumdata	16545	33904	Silveiras, Cunha e Areias, SP
B. circumdata	16546	33992	Silveiras, Cunha e Areias, SP
B. circumdata	16548	33995	Silveiras, Cunha e Areias, SP
B. circumdata	16549	33991	Silveiras, Cunha e Areias, SP

[1] NSF population; [2] CM3 population; [3] R1 population; * No voucher, only tissues collected