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Diversificação das espécies de *Rhinella* do grupo *crucifer* (Anura, Bufonidae) na Mata Atlântica

MARIA TEREZA CHIARIONI THOMÉ

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

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Orientador: João Miguel de Barros Alexandrino

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COMISSÃO EXAMINADORA

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Rio Claro, ___de_____de_____

"No obstante, uma mirada más detenida nos permite descubrir que los seres vivos llevan su historia escrita em si mismos. La mariposa, la orquídea, la fuchsia, se mueven en el tiempo seguidas por su sombra. Ningún ser camina por la tierra sin imprimir en ella y en sí mismo la huella de su paso."

Crisci, Katinas, Posadas

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RESUMO

Nessa tese, utilizamos uma série de métodos filogeográficos para investigar a diversificação em *Rhinella* gr. *crucifer*, um grupo de espécies próximas de sapos endêmicos da Mata Atlântica. No primeiro capítulo fizemos uma primeira abordagem da estrutura genética no grupo utilizando amostragem grosseira. Foram analisadas seqüências de DNA mitocondrial e nuclear de DNA de 65 indivíduos representando as cinco espécies atualmente válidas. Encontramos que a diversidade genética é geograficamente estruturada. A divergência mais antiga, datada do Plioceno, separa as populações mais ao sul do bioma enquanto que o restante das populações estão distribuídas em clados que divergiram desde o Pleistoceno. Modelos de distribuição paleoecológica suportam fragmentação de habitat associada a ciclos glaciais, mas a congruência de padrões filogeográficos com os refúgios inferidos é limitada. Algumas quebras genéticas coincidem geograficamente com barreiras associadas à atividade neotectônica. Os dados refutam a hipótese recentemente proposta de colonização Holocênica do sul da Mata Atlântica, sugerindo persistência de habitat nessa região. No segundo capítulo, utilizamos amostragem em nível populacional para delimitar melhor as unidades genéticas no grupo. Utilizamos seqüências de DNA mitocondrial e nuclear de 404 indivíduos, métodos baseados em árvores e freqüência alélica, considerando um cenário de divergências recentes e hibridação. Ambos marcadores apoiaram a existência de cinco unidades genéticas, três distribuídas na área núclear de distribuição do grupo, e duas com distribuições isoladas. Encontramos evidência clara da existência de zonas de contacto para dois pares de unidades genéticas. A correspondência entre unidades genéticas e morfoespécies é limitada: existe clara associação entre a unidade mais ao sul e *R. henseli*, mas não entre outras unidades e espécies. *R. pombali* coincide com uma das duas zonas híbridas encontradas, e sugerimos que esta espécie deve ser invalidada. No terceiro capítulo aproveitamos a amostragem dos capítulos anteriores e a estrutura genética descrita para investigar os processos demográficos possivelmente associados com a diversificação no grupo. Nesse capítulo testamos e rejeitamos que refúgios pleistocênicos influenciaram a diversificação no grupo, segundo três hipóteses disponíveis na literatura. Em vez disso, a diversidade foi algumas vezes maior para as áreas de não-refúgio. Análises demográficas revelaram histórias diferentes para 3 regiões do bioma, com expansão populacional moderada na região norte, crescimento um pouco mais acentuado na região central e estabilidade demográfica nas populações do sul. Tempos de divergência para as quebras principais foram novamente datadas de um período Plio-

Pleistocenico. Simulações coalescentes desenhadas para testar cenários demográficos alternativos permitiram rejeitar hipóteses de diversificação envolvendo vicariância associada a refúgios e colonizações recentes, favorecendo a influência de barreiras geográficas na diversificação genética observada. Os nossos dados suportam uma história complexa para o grupo e para o bioma. No capítulo 4 fizemos uma breve releitura da variação de forma no grupo utilizando morfometria geométrica e fotos obtidas padronizadamente para 272 machos adultos. Nossas análises preliminares indicam que existe variação de forma no grupo como um todo, mas a variação é sutil. A história evolutiva do grupo prevê, em certo grau, a variação na forma desses animais, mas a influência de variáveis ambientais ainda precisa ser testada.

filogeografia, paleomodelagem de nicho, delimitação de espécies, demografia, refúgios, barreiras, testes de hipóteses

ABSTRACT

In this dissertation, we used phylogeographic methods to investigate diversification in the *Rhinella* gr. *crucifer*, a group of closely related toads endemic to the Brazilian Atlantic Forest. In the first chapter we described the genetic structure in the group using coarse sampling. We analyzed mitochondrial and nuclear DNA sequences of 65 individuals representing the five currently valid species. We found that genetic diversity is geographically structured; the oldest divergence, dating from the Pliocene, separates the southernmost populations. The remaining population are distributed in clades that diverged throughout the Pleistocene. Palaeoecological distribution models support habitat fragmentation associated with glacial cycles, but the congruence of phylogeographic patterns and inferred refugia is limited. Some genetic breaks coincide geographically with barriers related to neotectonic activity. The data refute the recently proposed hypothesis of Holocene southern colonization of the biome, suggesting instead habitat persistence in this region. In the second chapter, we used sampling at the population level to delimit genetic units within the group. We used DNA sequences of mitochondrial and nuclear markers from 404 individuals. We combined tree and frequency–based methods, assuming a scenario of recent divergence and hybridization. Both marker types supported the existence of five genetic units, three being distributed within the core

distribution of the group, and two with more isolated distributions. We found evidence of contact zones between two pairs of units. The correspondence between morphospecies and genetic units is limited: clear association is possible between the southern unit and *R. henseli*, but not between other units and species. *R. pombali* coincides with the distribution of one of the putative hybrid zones, and we suggest this species should be invalidated. In the third chapter we take advantage of sampling and genetic structure described in the previous chapters to investigate the demographic processes associated with diversification in the group. We tested and rejected that Pleistocene refugia influenced diversification in the group, according to three previously proposed hypotheses. Instead, diversity was sometimes higher at non-refuge areas. Demographic analyses revealed different histories for three regions of the biome, with moderate population expansion in the northern region, more intense growth in the central region, and demographic stability for southern populations. Divergence times were again dated to a Plio-Pleistocene period. Coalescent simulations designed to test alternative demographic scenarios allowed us to reject hypotheses of refugial vicariance and recent colonizations, supporting the influence of geographic barriers in genetic diversification. Overall, our data supported a complex history for the group and for the biome. In chapter 4, we briefly addressed shape variation in the group using geometric morphometrics based on pictures obtained for 272 adult males. Our preliminary analyses indicate that shape variation in the group as a whole is subtle. The evolutionary history of the group predicts shape variation in these animals to some degree, but the influence of environmental variables remains to be tested.

phylogeography, niche palaeomodeling, species delimitation, demography, refugia, barriers, hypothesis testing

INTRODUÇÃO GERAL

A riqueza de espécies da região Neotropical teve grande influência na visão de Darwin e Wallace sobre evolução. A diversidade nesta região é tão grande que o número exato de espécies está longe de ser conhecido. Muitos anos de intenso esforço humano e financeiro serão necessários para que esse todas estas espécies sejam catalogadas (Carbayo & Marques 2011). Tão importante quando obter boas estimativas acerca da diversidade existente é melhorar nosso entendimento de como ela se formou, que continua muito limitado (Rull 2008).

Os critérios para o reconhecimento do que é uma espécie para a comunidade científica costumam depender dos atributos biológicos do organismo em questão e do conceito de espécie empregado (Starkey et al. 2003). Desde que seqüências de DNA se tornaram disponíveis, o estudo da variação genética entre populações se tornou uma ferramenta–chave nessas decisões taxonômicas, mas também revolucionou a investigação da história evolutiva das populações. Como é nessa escala 'populacional' em que se dá a evolução propriamente dita, o uso destes marcadores moleculares contribuiu imensamente para a nossa compreensão sobre o surgimento das espécies. A filogeografia é a disciplina que aborda essa questão (Avise et al. 1987), investigando os processos demográficos que modelam as distribuições geográficas das linhagens, sejam estas pertencentes a uma espécie ou a várias espécies próximas (Avise 2000, Hewitt 2006). A natureza dos dados utilizados (i.e. marcadores moleculares) faz com que a filogeografia esteja em constante revolução, ao mesmo tempo contribuindo e se beneficiando do desenvolvimento de novas ferramentas computacionais, moleculares e teóricas (Hickerson et al. 2010).

Estudos filogeográficos nos permitem compreender como ocorreu a diversificação em grupos de espécies aparentadas e quais os mecanismos responsáveis pelo surgimento de tantas espécies em ambientes megadiversos, além de ter o desejável efeito colateral de testar a eficiência da classificação taxonômica vigente no grupo estudado. Consequentemente, estes estudos são especialmente relevantes para os Neotrópicos, que carecem desse tipo de informação para o melhor planejamento dos esforços de conservação. Nessa tese utilizamos uma série de métodos filogeográficos para investigar a diversificação em um grupo de espécies próximas de sapos, descrevendo como a diversidade genética de suas populações e

espécies está estruturada, os processos evolutivos envolvidos e sua consequência para o conhecimento da história de um dos biomas megadiversos mais ameaçado do planeta.

O grupo de *Rhinella crucifer* foi originalmente descrito por M.A.P. Wied–Neuwied em 1821 como uma única espécie cuja distribuição coincidia com os domínios morfoclimáticos da Mata Atlântica. Atualmente o grupo conta com cinco espécies taxonomicamente válidas (*R. abei, R. henseli, R. ornatus, R. crucifer* e *R. pombali,* Frost 2011), delimitadas morfologicamente em uma revisão recente (Baldissera et al. 2004). As distribuições estimadas para cada espécie são concordantes com a divisão da Mata Atlântica em sete unidades biogeográficas, delimitadas com base em distribuições de outros anfíbios endêmicos a este bioma (Lynch 1979, fig. 1).

Figura 1: Subdivisão da Mata Atlântica em sete unidades proposta por Lynch (1979) segundo o endemismo de anfíbios no bioma.

A suspeita de que "*Rhinella crucifer*" se tratava de um complexo de espécies já havia sido mencionada antes de sua revisão taxonômica por outros pesquisadores (Haddad & Sazima 1992, Izecksohn & Carvalho-e-Silva 2001). De fato, variações morfológicas no grupo vêm confundindo autores ao longo de muitos anos, deixando como testemunho diversas descrições antigas destas variações (fig. 2), que resultaram em um histórico vasto de nomes propostos e sinonímias (ver em Baldissera 2001). Muitas dessas variações parecem ser baseadas em caracteres aparentemente sem sinal evolutivo, como o padrão de coloração dorsal em “cruz” que deu nome ao grupo (Baldissera et al. 2004).

Figura 2: Variações morfológicas atribuídas ao grupo de *Rhinella crucifer* e sinonímia atual. *Bufo scaber* Spix 1824 (*R. ornata*), *B. ornatus* Spix 1824 (*R. ornata*), *B. crucifer* var. *stellatus* Spix 1824 (*R. crucifer*), *B. dorsalis* Spix 1824 (*R. ornata*), *B. crucifer* Wied-Neuwied 1821 (*R. crucifer*), *B. crucifer* var. *stellatus* (*R. crucifer*), *B. crucifer* var. *henseli* Lutz 1934 (*R. henseli*), *B. cinctus* Schinz 1822 (*R. crucifer*), *B. cinctus, B. crucifer* var. *roseanus* Miranda-Ribeiro1926 (*R. ornata*), *B. crucifer* var. *pfrimeri* Miranda-Ribeiro1926 (*Raebo guttatus*).

Mesmo após a revisão, a estrutura genética no grupo permanecia desconhecida e os relacionamentos de parentesco entre as espécies não estavam confirmados, impossibilitando a proposição de uma hipótese biogeográfica que explicasse sua diversificação. As propostas de relacionamentos disponíveis até então eram insatisfatórias, sendo uma baseada em dados de DNA de poucos indivíduos (Baldissera, 2001), e outra baseada na composição de secreções da pele dos animais (Maciel et al. 2006, fig. 3). Apenas a segunda hipótese de parentesco incluía todas as espécies do grupo, porém com resultados pouco confiáveis (Maciel et al. 2006).

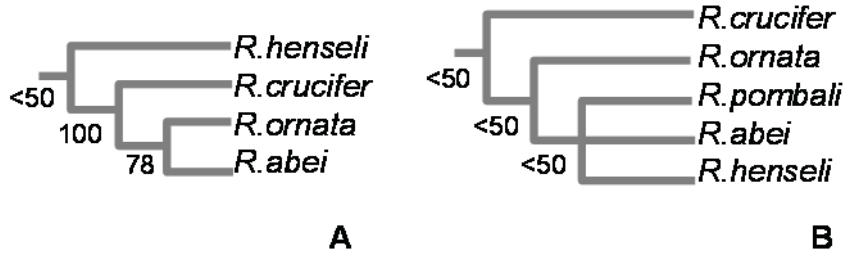

Figura 3: Árvores filogenéticas obtidas por Baldissera (2001) (A) e Maciel et al. (2006) (B) mostrando duas propostas diferentes para o relacionamento entre as espécies do grupo. Números após os nós indicam valores de suporte percentuais (*bootstrap*).

De acordo com o projeto inicial os objetivos deste estudo eram i) investigar o número de linhagens genéticas existentes no grupo, e ii) determinar seus relacionamentos e padrões de distribuição geográfica, com enfoque no conhecimento taxonômico pré-existente. Com o desenvolvimento do projeto, outros objetivos foram incorporados, como a investigação da história do bioma através da história das populações e uma releitura da variação morfológica no grupo utilizando um método alternativo. Assim, esta tese está organizada em quatro capítulos que se encontram em diferentes graus de desenvolvimento. No primeiro capítulo utilizamos uma amostragem grosseira para fazer uma primeira descrição da estruturada e diversidade genética no grupo, e um levantamento dos possíveis mecanismos envolvidos na sua diversificação. No segundo capítulo utilizamos uma amostragem mais refinada para delimitar em maior detalhe as unidades genéticas no grupo e avaliar a taxonomia atual. No terceiro capítulo utilizamos os dados produzidos nos capítulos anteriores em uma investigação preliminar dos mecanismos de diversificação que efetivamente afetaram a história do grupo,

utilizando uma abordagem de teste de hipóteses. Finalmente, no quarto capítulo fizemos uma tentativa de avaliar se a variação na forma desses animais reflete a variação genética encontrada nos capítulos anteriores.

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Capítulo 1

Phylogeography of endemic toads and post-Pliocene persistence of the Brazilian Atlantic Forest

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Phylogeography of endemic toads and post-Pliocene persistence of the Brazilian Atlantic Forest

Maria Tereza C. Thomé, Kelly R. Zamudio, João G. R. Giovanelli, Célio F. B. Haddad, Flávio A. Baldissera Jr., João Alexandrino

ABSTRACT

The Plio–Pleistocene refugia hypothesis recently gained support in explaining Brazilian Atlantic Forest megadiversity from combined analyses of species paleodistributions and genetic diversity. Here we examine genetic differentiation and historical distributions in the *Rhinella crucifer* group of toads, endemic to and widely distributed within this biome. We analyzed sequences of mitochondrial (control region, ND1, and ND2) and nuclear (β-crystallin and rhodopsin) DNA markers from 65 individuals representing five species. We found deep structure across the range at mitochondrial markers; genetic diversity is geographically structured in four main haplotype clades with the oldest divergence, dated to the Pliocene, between the southernmost populations and other regions of the species' range. Remaining populations are distributed in haplotype clades that may have diverged throughout the Pleistocene. Our paleoecological distribution models support a scenario of habitat fragmentation associated with glacial cycling, but we found limited congruence of phylogeographic patterns with the refugia. We found that some genetic breaks geographically coincide with putative barriers associated to neotectonic activity, but finer-scale sampling will be necessary to test the relative importance of distinct isolation mechanisms. Overall, the data refute the recently proposed hypothesis of a southern Holocene colonization of the Atlantic Forest from northern refugia, suggesting instead persistence of forested habitats in the south. Our unexpected results underscore the need to consider distinct organismal histories in planning biome-level conservation. We discuss species correspondence to clades recovered in our phylogenetic analyses.

1. Introduction

The Brazilian Atlantic Forest (AF) harbors a large diversity of animals, plants, and habitat types (Mittermeier et al., 2005; Myers et al., 2000). This biome occupies coastal regions of eastern Brazil and reaches its western maximum extent in eastern Paraguay and northeastern Argentina. Given this large geographic extent, the AF is floristically diverse with several regional forms of rainforest (ombrophilous) and semi-deciduous forest, depending on rainfall regimes (Oliveira-Filho and Fontes, 2000) (Fig. 1). The loss of habitat is a main threat for the AF biota (Myers et al., 2000); according to the most recent account the Brazilian share is already reduced to 11.4–16% of its original extent, and the landscape configuration of remaining reserves makes long-term conservation challenging (Ribeiro et al., 2009).

Studies of AF species diversification are still scarce and show limited agreement on general mechanisms to explain the origin of its diversity. Estimates of the timing of Neotropical diversification events indicate that lineages have originated continuously since the late Eocene/early Oligocene until the Pleistocene (Rull, 2008). A realistic view of AF diversity should consider multiple mechanisms operating at different spatial and temporal scales. Yet, patterns of endemicity in birds, small mammals, and other vertebrates are broadly concordant in geographic distribution (Costa and Leite, 2000; Sigrist and Carvalho, 2008; Silva et al., 2004), suggesting that a common mechanism may have played a role in shaping distributions of multiple taxa in this megadiverse biome.

The most commonly invoked mechanism for South American diversification is isolation of taxa in areas of stable habitat during Quaternary climatic fluctuations [the Pleistocene refugia hypothesis (Haffer, 1969)]. Habitat refugia would enable population persistence in allopatry during climatically unfavorable periods and should show higher genetic diversity and endemism than nonrefugial areas (Bennett and Provan, 2008; Hewitt, 1996, 2000). Additional predictions may include the presence of sister taxa in adjacent refugia, secondary contact zones between refugia, and range expansions out of refugial areas (Moritz et al., 2000). Although this model was initially proposed to explain the remarkable level of Neotropical speciation during the Pleistocene (Rull, 2008), climatic oscillations that occurred throughout the Tertiary may extend the timeframe for diversification due to refugial isolation (Haffer, 1997).

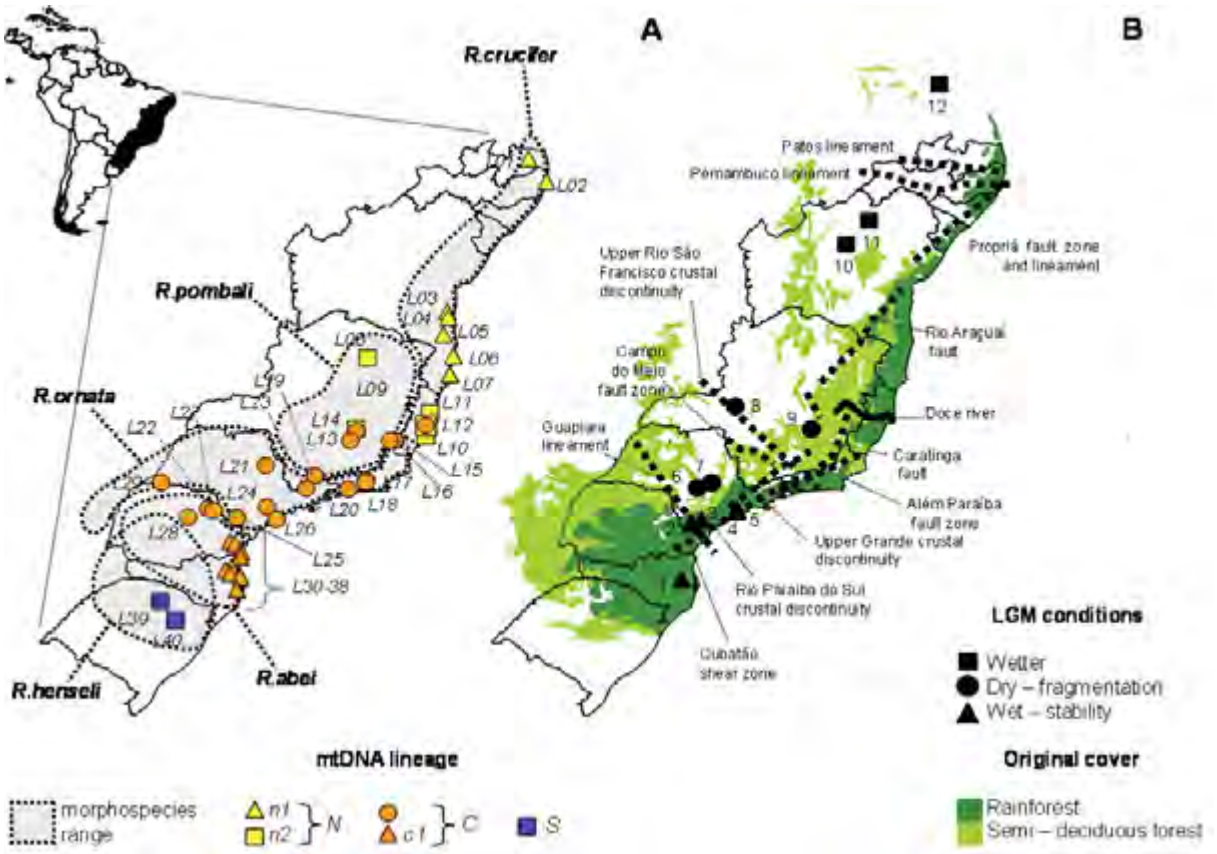

Fig. 1. (A) Sampling localities for the phylogeographic study and approximate geographic distributions of morphospecies in the Rhinella crucifer group. Locality codes follow Table 1. Symbols indicate mitochondrial clades. (B) Original area of occurrence of the Brazilian Atlantic Forests (ombrophylous and semi-deciduous), location of putative geographic barriers, and climatic/forest conditions for the LGM (last glacial maximum) according to other studies: 1, Botuverá cave; 2, Alto do Ribeira state park and Santana cave region; 3, Intervales state park region; 4, Curucutú; 5, Colônia crater; 6, Botucatú and Anhembi region; 7, Jaguariúna; 8, Salitre de Minas; 9, Catas Altas; 10, Toca da Boa Vista region; 11, Irecê; 12, Ceará shore (see text for details).

Paleomodeling of species' distributions complements phylogeographic inferences of Pleistocene refugia from genetic data (Carnaval and Moritz, 2008; Hugall et al., 2002; Waltari et al., 2007). Paleomodeling of the AF biome predicts severe forest contraction south of São Paulo state and large stable forested areas in northern regions during the last glacial maximum (LGM), followed by Holocene expansion (Carnaval and Moritz, 2008). This scenario is compatible with genetic lineage divergences observed within several northern AF taxa (Cabanne et al., 2008; Carnaval et al., 2009; Costa, 2003; Moraes-Barros et al., 2006; Fitzpatrick et al., 2009; Pellegrino et al., 2005), but divergent lineages have also been

observed in southern AF (Cabanne et al., 2007; Fitzpatrick et al., 2009; Grazziotin et al., 2006), where refugia are not predicted by paleomodels.

The AF lies in a geographically complex landscape with several features that could potentially foster diversification due to vicariance, or at least add complexity to a scenario of historical habitat fragmentation. River basins and mountain chains often delimit AF species' distributions, but few studies have established geomorphologic events as promoters of allopatric diversification in this biome. Major coastal rivers correlate both spatially and temporally with differentiation among lineages of geckos and small mammals (Costa, 2003; Pellegrino et al., 2005) and delimit some areas of endemism in the AF (Costa and Leite, 2000; Sigrist and Carvalho, 2008; Silva et al., 2004). River systems and mountain chains also coincide with phylogeographic breaks for pitvipers and birds, although the timing of genetic differentiation and barrier formation cannot be readily reconciled (Cabanne et al., 2008, 2007; Grazziotin et al., 2006). Refugia and geographic barriers have been proposed as mutually exclusive models for speciation in the Amazonian region, where some biogeographic disjunctions seem associated to either predicted climatic refuges or to geomorphology (Bush, 1994). In the geographically complex AF region it is unlikely that forest refugia or barrier hypotheses alone will account for general patterns of lineage diversification. Neotectonic activity has significantly remodeled the landscape of eastern Brazil during the Quaternary (Ricommini and Assumpção, 1999), confounding the signatures of isolating mechanisms along this Tertiary–Quaternary time scale.

The five toad species that compose the *Rhinella crucifer* group (Baldissera et al., 2004) are endemic to the AF, and their combined range is concordant with the distribution of that biome (Oliveira-Filho and Fontes, 2000). These five species, until recently considered conspecific, were described based on analyses of regional morphological differences (Baldissera et al., 2004). *Rhinella crucifer* occurs from the State of Ceará to the southern State of Espírito Santo and the northeastern State of Minas Gerais; *R. pombali* occurs in the State of Minas Gerais; *R. ornata* occurs from southern State of Espírito Santo to the northern State of Paraná; *R. abei* is distributed from the State of Paraná to the southern State of Santa Catarina and areas of the northern State of Rio Grande do Sul; and *R. henseli* from southern State of Santa Catarina to the coast of the State of Rio Grande do Sul, with isolated records in the state of Paraná (Lima et al., 2005). The range limits of these morphospecies are not well known,

but their distributions are thought to primarily be nonoverlapping (Baldissera et al., 2004), with the exception of *R. henseli* and *R. abei* (Fig. 1). However, genetic diversity within and among morphospecies is unknown, and their phylogenetic relationships have not been established. Their widespread distribution, endemic status, and regional morphological differentiation make this species group ideal for understanding diversification mechanisms and biogeographical patterns across the AF.

Here we quantified mitochondrial and nuclear sequence variation throughout the range of the *R. crucifer* species group to characterize the geographic distribution of genetic diversity and to infer processes of historical diversification. We compared the observed patterns to (i) contemporary and past species' range predictions from bioclimatic modeling and (ii) the location of major potential geographic barriers, to infer if climatic habitat changes, landscape features, or both, have promoted diversification in this AF taxon. Specifically, we examined whether the predictions under the southern AF Holocene colonization hypothesis (sensu Carnaval et al., 2009) apply to the observed phylogeographic pattern of clade divergence, i.e., older genetic divergence between toads of northern AF regions than between toads south of the state of São Paulo. Finally, we verified the concordance between genetic diversity and current taxonomic status of the species in this group.

2. Materials and methods

2.1. Population sampling and DNA extraction

In this study we obtained tissue samples from 65 field collected individuals in the *Rhinella crucifer* group and four outgroup taxa (Table 1). Vouchers are deposited in the following institutions: Célio F.B. Haddad amphibian collection at Departamento de Zoologia, Universidade Estadual Paulista ‘‘Júlio de Mesquita Filho” (CFBH), Coleção Herpetológica da Universidade Federal de Minas Gerais, Museu Nacional, Rio de Janeiro (MNRJ), Museu de Zoologia da Universidade de São Paulo (MZUSP), Coleção de Tecidos do Departamento de Zoologia do Instituto de Biociências da Universidade de São Paulo (UF), Museu de Ciências Naturais da PUCMG (MCN), and Museu de Ciências e Tecnologia da PUCRS (MCP). The initials MTR (Miguel T. Rodrigues) represent field numbers from ongoing studies. Our sampling covered most of the range of the species group and included all five currently

recognized species (Baldissera et al., 2004). We chose outgroups based on published molecular and morphological data (Duellman and Schulte, 1992; Maxson, 1984; Pramuk, 2006). According to those studies, the *Rhinella marina* group is the sister taxon to the *R. crucifer* group; and the *R. granulosa* group is the sister taxon to the *R. marina* + *R. crucifer* groups. Therefore, we included three species of the R. marina group (*R. icterica*, *R. marina* and *R. rubescens*) and one of the *R. granulosa* group (*R. merianae*). Samples consisted of liver, toe clips, or muscle preserved in 100% ethanol. We digested tissues in lysis buffer and Proteinase K and purified whole genomic DNA using QIA Quick DNEasy columns (Qiagen Inc.) according to the manufacturer's protocol. One microliter of the eluted extract, containing approximately 1–10 ng/ml of DNA, was used as template for polymerase chain reactions (PCR).

Table 1: Haplotype code, voucher number (see text for institution information), collection locality, state, and code of the Rhinella species analyzed in this study. State abbreviations: PB, Paraíba; PE, Pernambuco; BA, Bahia; MG, Minas Gerais; ES, Espírito Santo; RJ, Rio de Janeiro; SP, São Paulo; PR, Paraná; SC, Santa Catarina; RS, Rio Grande do Sul; AM, Amazonas; AC, Acre; GO, Goiás (numbers marked with '*' represent tissue samples without vouchers).

haplotype	**voucher number**	**Locality/ state**	**code**
h1	CFBH2911	Areia/ PB	*L1*
h2	CFBH2914, 3650	Recife/ PE	*L2*
h3	CFBH18717	Aurelino Leal/ BA	*L3*
h4	MTR13599*	Camacan/ BA	*L5*
h5	CFBH13401	Uruçucá/ BA	*L4*
h6	VD191*	Camacan/ BA	*L5*
h7	VD195*	Camacan/ BA	*L5*
h8	MNRJ38921	Porto Seguro/ BA	*L6*
h9	MNRJ38920	Porto Seguro/ BA	*L6*
h10	FSFL1480*	Prado/ BA	*L7*
h11	CFBH10220	Grão Mogol/ MG	*L8*
h12	AF402*	Santa Bárbara/ MG	*L9*
h13	CFBH14966	Sooretama/ ES	*L11*
h14	CFBH2866	Aracruz/ ES	*L12*
h15	CFBH14948	Sooretama/ ES	*L11*
h16	CFBH2877	Vila Velha/ ES	*L10*
h17	CFBH2867	Aracruz/ ES	*L12*
h18	MNRJ38327	Catas Altas/ MG	*L14*
h19	FSFL 1046*	Ouro Branco/ MG	*L13*
h20	FSFL 1044*	Ouro Branco/ MG	*L13*
h21	FSFL1045*	Ouro Branco/ MG	*L13*
h22	MTR1252*	Ibitirama/ ES	*L15*

h23	MTR11548*	Santa Marta/ MG	*L16*
h24	MTR11543*	Santa Marta/ MG	*L16*
h25	CFBH18808, 18815	Teresópolis/ RJ	*L17*
h26	CFBH18822	Guapimirim/ RJ	*L18*
h27	CFBH2865	Aracruz/ ES	*L12*
h28	CFBH14647	Cristina/ MG	*L19*
h29	CFBH2869	Itaguaí/ RJ	*L20*
h30	CFBH2870, 2871	Itaguaí/ RJ	*L20*
h31	CFBH11497	S. Rita do Passa Quatro/ SP	*L21*
h32	II-H046*	Pinhalão/ PR	*L22*
h33	CFBH7193, 7194	S. Antônio do Pinhal/ SP	*L23*
h34	M60*	Piedade/ SP	*L24*
h35	CFBH9937	Cristina/ MG	*L19*
h36	CFBH6803	Ribeirão Branco/ SP	*L25*
h37	CFBH13792, 3793	Peruíbe/ SP	*L26*
h38	M61*	Piedade/ SP	*L24*
h39	II-H114*	Ortigueira/ PR	*L28*
h40	CFBH6802	Ribeirão Branco/ SP	*L25*
h41	IIH-172*	Wenceslau Brás/ PR	*L27*
h42	IIH-*	Wenceslau Brás/ PR	*L27*
h43	IIH-149*	Wenceslau Brás/ PR	*L27*
h44	CFBH18361	Teodoro Sampaio/ SP	*L29*
h45	CFBH18310	Teodoro Sampaio/ SP	*L29*
h46	CFBH8458	Itapema/ SC	*L37*
h47	CFBH2837	Morretes/ PR	*L31*
h48	CFBH18141	Quatro Barras/ PR	*L30*
h49	PUCRS*	S. Amaro da Imperatriz/ SC	*L38*
h50	CFBH2841	Itapoá/ SC	*L33*
h51	CFBH2916	Guaratuba/ PR	*L32*
h52	CFBH2917	Guaratuba/ PR	*L32*
h53	CFBH18175	Massaranduba/ SC	*L34*
h54	CFBH2842	Rio dos Cedros/ SC	*L35*
h55	CFBH2844	Rio dos Cedros/ SC	*L35*
h56	CHBH18164	Blumenau/ SC	*L36*
h57	CFBH2857	Blumenau/ SC	*L36*
h58	CFBH18238	Bento Gonçalves/ RS	*L40*
h59	MNRJ33006	Mato Castelhano/ RS	*L39*
h60	MNRJ33012	Mato Castelhano/ RS	*L39*
R. granulosa	CFBH16651	Manaus/ AM	
R. icterica	CFBH11027	Bom Jardim da Serra/ SC	
R. marina	CFBH15711	Tarauacá/ AC	
R. rubescens	CFBH7696	Cocalzinho de Goiás/ GO	

2.2. DNA amplification and sequencing

We sequenced three mitochondrial and two nuclear gene fragments. We obtained mtDNA sequences for all 65 samples, and nuclear sequences for a representative subsample of those. The mitochondrial fragments were the control region and a short segment of the adjacent cytochrome b gene (hereafter called the control region, 930 bp), a fragment including the 50 end of the 16S gene, the complete tRNAleu, the complete NADH dehydrogenase subunit 1, the tRNAIle and part of the tRNAGln genes (hereafter called ND1, 1414 bp), and a partial sequence of the NADH dehydrogenase subunit 2 (hereafter called ND2, 895 bp). The nuclear fragments were segments of the exons 1 and 4 of the rhodopsin gene (hereafter referred as rhodopsin, 280 bp) and the b-crystallin gene (hereafter called crystallin, 358 bp). Amplicons of nuclear fragments were cloned using the pGEM-T Vector System (Promega Corporation) and transformed into One Shot TOP10 competent *Escherichia coli* (Invitrogen Corporation) following manufacturer's protocols.

Table 2: Target gene fragment, origin, and annealing temperature of the primers used in this study.

Fragment	**Primers**	**Origin**	**Anealling**
control region	cytbA-L/ controlP-H.b	Goebel et al., 1999	48.3-51.3° C
ND1	tmet-frog/ 16S-frog	Wiens et al., 2005	55.3° C
	rhiND1int3R (internal)	this study (TGA GAT CAA ATG GGG CTC GGT TTG)	
ND2	L4437/	Macey et al., 1997	53.3-59° C
	intHalbo2/	Prado et al., in prep	
	rhiND2R	this study (TTA AAT CAG AGT GAG GAA GAG GAG)	
rhodopsin	RHOD1A/ RHOD1D	Bossuyt and Milinkovitch, 2000	59.7° C
β-crystallin	CRYB1LS/ CRYB2LS	Dolman and Phillips, 2004	55.3° C

We PCR amplified each target gene using specific primers (Table 2). Amplification conditions included an initial denaturation step at 94° C (5 min), 35 cycles consisting of 94° C (1 min) denaturation, 48.3–60.2° C (1 min) annealing (Table 2), 72° C (1 min) extension, and a final extension step at 75° C (5 min). We purified successful amplicons or clones with 10 units of Exonuclease I and one unit of shrimp alkaline phosphatase. We used the products as template in sequencing reactions with the same primers used for amplification, with Big Dye

termination sequencing chemistry (Applied Biosystems). We purified sequencing products using Sephadex G-50 columns and electrophoresed on ABI PRISM 3100 or 3730 Genetic Analyzers (Applied Biosystems). We checked electropherograms for errors and assembled contiguous sequences. We aligned all contigs with ClustalW (Larkin et al., 2007) and checked alignments by eye. GenBank Accession Numbers are provided in Appendix 1.

2.3. Phylogenetic and population genetic analyses

We used Bayesian Inference (BI) and Maximum Likelihood (ML) methods to infer gene genealogies, and to evaluate nodal support. We selected the model of nucleotide evolution that best fit our data using the AIC criterion (Akaike, 1973) in MODELTEST 3.7 (Posada and Crandall, 1998). We inferred a BI topology and estimated posterior probabilities using MRBAYES 3b4 (Huelsenbeck and Ronquist, 2001) and inferred ML topology and bootstrap support values using PAUP* 4b10 (Swofford, 2001). For BI we used the Markov chain Monte Carlo (MCMC) analysis with two independent runs, each with four chains sampling every 100 generations for 6 million generations. We changed parameters (substitution rates) according to nucleotide models of evolution; we kept all priors under default settings. We verified convergence among runs by confirming that the average standard deviation of split frequencies was <0.01, and checking trends of log likelihood values of the cold chains. We eliminated the first 15,000 trees as burn-in, and estimated a 50% majority rule consensus from the remaining 45,000 trees. ML support values were obtained though 1000 bootstrap replicates with starting trees obtained by stepwise addition, using a nearest-neighbor interchange heuristic search for ML. We performed independent analyses for each fragment and a final analysis with all three mtDNA genes concatenated. We used a single model of nucleotide evolution in the ML concatenated analysis, while in the BI concatenated analysis we allowed partitions to evolve under different models with unlinked rates. To test for the monophyly of the *Rhinella crucifer* group, we set R. merianae as outgroup in all trees, and then checked the position of other outgroups relative to members of the *R. crucifer* group. For nuclear alleles we produced unrooted median joining networks (Bandelt et al., 1999) in the software Network 4.5.1.0 (available at fluxus-engineering. com) eliminating sites containing gaps. We avoided ambiguous phase assignments by using only sequences with no heterozygote positions, a single heterozygote position, or cloned sequences. We included alleles from the same individual as independent sequences in the analysis. We estimated the

minimum number of recombination events RM using the four-gamete test (Hudson and Kaplan, 1985) in DNAsp 4.20 (Rozas et al., 2003). For both mtDNA and nuclear fragments we calculated summary statistics (number of haplotypes, haplotype and nucleotide diversity, and respective standard variations) in DNAsp 4.20 (Rozas et al., 2003).

2.4. Estimates of divergence times

The only gene fragment for which we have an independently calibrated diversification rate for another Neotropical anuran is the ND2 gene (0.957% per lineage per million years; Crawford, 2003); thus, we inferred divergence-time estimates based only on this fragment. Times of divergence between clades were estimated with net sequence divergence between groups (Da, Nei, 1987) using the Tamura–Nei distance (Tamura and Nei, 1993) with the gamma model estimated by the composite likelihood method (Tamura et al., 2004) as implemented in MEGA 4.0 (Tamura et al., 2007). This distance measure corrects for multiple hits, taking into account the different nucleotide frequencies and rates of substitution, and models evolutionary rates among sites using the gamma distribution (Tamura and Nei, 1993). Standard deviation values were obtained with 1000 replicates.

We applied the coalescent approach implemented in BEAST 1.4.7 (Drummond and Rambaut, 2007) to calculate the time to the most recent common ancestor (tMRCA) for divergent haplotypes within clades. We checked codon positions and used the SRD06 model suggested by Shapiro et al. (2006) that allows 3rd positions to have a different relative rate of substitution, transition–transversion ratio, and gamma distribution. The selected tree prior was ‘birth and death process’; scale operators were set at default values with the ‘auto-optimize’ option on, and the length of chains set to 15 million generations. We first ran the analysis using an uncorrelated relaxed lognormal clock (Drummond et al., 2006) and used the standard deviation and coefficient of variation to ascertain that our data conformed to a strict clock. We then performed three independent runs using the strict clock and checked stationarity of the posterior distributions for all model parameters using Tracer v.1.4 (Rambaut and Drummond, 2007).

2.5. Mapping areas of historical stability

To identify areas within the distribution of our focal taxa that were most likely to have remained stable during the Pleistocene period, we produced past and current geographic distribution maps using ecological niche modeling, and interpreting areas of intersection among maps as putative stable areas (e.g., Carnaval et al., 2008; Waltari et al., 2007). We used the maximum entropy algorithm implemented in MAXENT (Phillips et al., 2006); species presence points are listed in Appendix 2. We first modeled the current distribution using the following current climate variables at a spatial resolution of 1 km: mean diurnal range, temperature seasonality, maximum temperature of warmest month, minimum temperature of coldest month, temperature annual range, annual precipitation, precipitation seasonality, precipitation of wettest quarter, precipitation of warmest quarter (Hijmans et al., 2005). To avoid spurious projections, the model was calibrated using the AF domain and results were considered valid only in areas where environmental conditions fall within this range ('dontextrapolate' function in MAXENT, e.g., Giovanelli et al., 2008). For threshold definition we used the minimum presence criterion that equals the minimum model prediction value for any occurrence data, allowing for no omission. Probabilities below the threshold were transformed to zero, producing a binary map. We checked model accuracy by dividing occurrences into training data (75% of occurrence point data), used for model prediction, and test data (remaining 25%) used for model validation. The resulting model was evaluated with the underlying area (AUC) of Receiver Operating Characteristics Curve (ROC) for the modeling algorithm (Fielding and Bell, 1997; Manel et al., 2001). The AUC ranges from 0.5 (random accuracy) to a maximum value of 1.0 (perfect discrimination). We projected the obtained current time model to three paleoclimate scenarios, one simulating the last interglacial period (LIG) 130,000 years ago (Otto-Bliesner, 2006, available at: http://pmip2.lsce.ipsl.fr/), and two simulating the last glacial maximum period (LGM) 21,000 years ago: CCSM3 (Community Climate System Model, available at: http://www.ccsm.ucar.edu), and MIROC (Model of Interdisciplinary Research on Climate, availableat: http://www.ccsr.utokyo.ac.jp/kyosei/hasumi/MIROC/tech-repo.pdf). Settings, threshold, and background for the past projections were the same used for current time modeling.

3. Results

3.1. Phylogenetic and population genetic analyses: mtDNA variation

We found 198 polymorphic sites in the control region fragment (926 bp), of which 177 were parsimony informative and 21 were autapomorphic. In the ND1 fragment (1413 bp) 181 sites were polymorphic, 138 parsimony informative, and 43 autapomorphic; in the ND2 fragment (895 bp), among 142 polymorphic sites 115 were parsimony informative and 27 autapomorphic. The control region sequence alignment required 21 sites with gaps, while the other mitochondrial fragments required none.

Table 3: Diversity indices for each gene fragment and clade. H, number of haplotypes; hd, percentage of haplotype diversity and respective standard deviations (SD); P, percentage of nucleotide diversity and respective standard deviations (SD); N, number of sequences.

Fragment	**mtDNA**	**H**	**%hd (SD)**	**%Π (SD)**	**N**
control region	All	52	99 (0.5)	3.93 (0.48)	65
	N	17	99.3 (2.1)	2.44 (0.1)	18
	N+C	49	99 (0.6)	3.09 (0.15)	62
	C	32	98.1 (1.1)	2.0 (0.08)	44
	S	3	100 (27.2)	0.66 (0.2)	3
ND1	all	57	99.6 (0.4)	1.68 (0.26)	65
	N	17	99.3 (2.1)	0.83 (0.06)	18
	N+C	54	99.5 (0.4)	1.21 (0.06)	62
	C	37	99.2 (0.7)	0.81 (0.06)	44
	S	3	100 (27.2)	0.8 (0.28)	3
ND2	all	45	97.9 (0.9)	2.24 (0.3)	65
	N	15	97.4 (2.9)	1.24 (0.08)	18
	N+C	42	97.7 (0.9)	1.7 (0.11)	62
	C	27	95.9 (1.7)	0.94 (0.05)	44
	S	3	100 (27.2)	0.89 (0.32)	3
rhodopsin	-	17	78.8 (2.7)	0.72 (0.05)	98
β-crystallin	-	33	97.8 (0.7)	1.63 (0.11)	54

The three fragments showed different numbers of unique haplotypes (Table 3). We found 52 haplotypes for the control region fragment, of which 12 were shared (nine were among individualswithin collected localities and three among individuals of different

localities). For the ND1 fragment we found 57 haplotypes, of which seven were shared (five within and two among localities). Finally, the ND2 fragment showed 45 haplotypes, of which ten were shared (four within and six among localities). Considering the concatenated sequences of all three mtDNA gene regions we found 60 unique haplotypes, with haplotypes shared only among individuals collected at a same locality (Table 1). Genetic diversity indices varied among fragments (Table 3); overall nucleotide diversity was statistically higher for the control region than for the ND1 and ND2 fragments. Haplotype diversity was higher for the ND1 than ND2 fragment. The best fit models for each fragment independently (control region and ND2 fragments: TIM + I + G, Rodriguez et al., 1990; ND1 fragment: TVM + I + G, Zharkikh, 1994) were similar in that all include six nucleotide substitution categories, a proportion of invariant sites, and a gamma distribution. The nucleotide substitution model GTR + I + G (Lanave et al., 1984) was the best fit for the concatenated mtDNA region (used in the ML concatenated analysis).

In all phylogenetic analyses, whether based on individual genes or concatenated sequences, the *Rhinella crucifer* group remained monophyletic, confirming R. marina as the closest taxon. Individual gene tree topologies were broadly in agreement for the three mitochondrial fragments (Appendix 3). The control region topology showed higher resolution than did ND1 and ND2 trees at shallower divergence levels, a pattern expected given the higher mutation rate of that gene. Differences among mtDNA topologies were restricted to poorly supported clades, justifying the use of total mtDNA sequence evidence for phylogenetic inference. The Bayesian topology based on all mitochondrial data revealed four well supported clades corresponding to geographical regions of the AF (Fig. 2). The first divergence separates a clade including samples from the extreme southern part of the distribution, in the state of Rio Grande do Sul (*S* clade), and its sister clade containing samples from the rest of the distribution (*C + N* clade). The *C + N* clade is further structured into two units; a clade containing populations from the central part of the range (*C* clade) and a clade of northern populations (*N* clade). The *N* clade, ranging from northern Espírito Santo and Minas Gerais states to Paraíba state, is further divided in two subclades, one representing samples from states of Bahia to Paraíba (*n1* subclade) and the other with samples from Minas Gerais and Espírito Santo (*n2* subclade). The C clade is the mostgeographically widespread and includes samples from southern Espírito Santo, Minas Gerais, São Paulo, Paraná and Santa Catarina states. It is also the least geographically structured clade; however, it contains

one subclade (*c1*) that is highly supported, with samples from Santa Catarina and eastern Paraná states. The topologies based on individual mtDNA genes show the same four regional clades and any differences among fragment topologies are restricted to poorly supported subclades (Appendix 2).

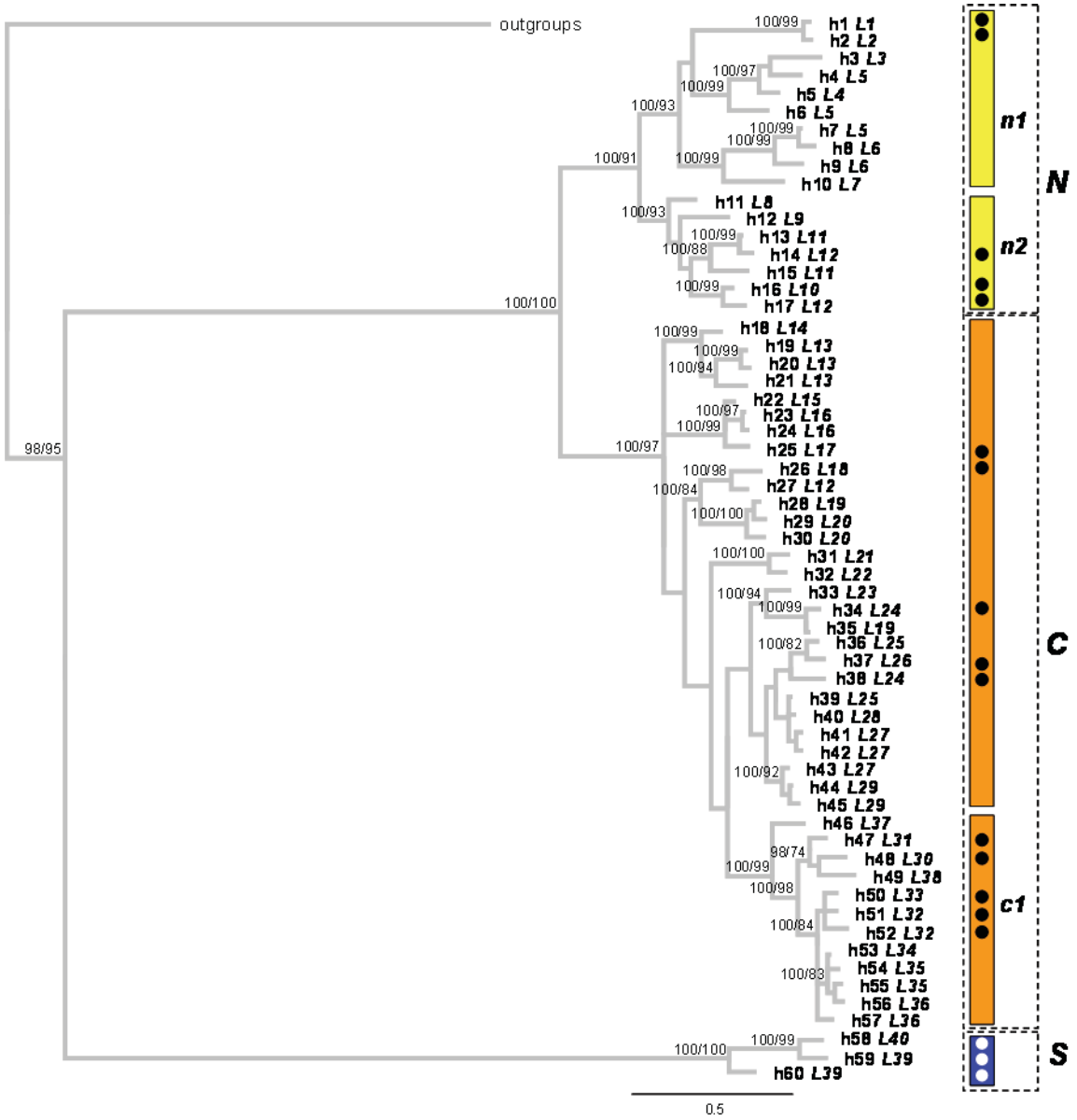

Fig. 2. Bayesian tree based on control region, ND1 and ND2 mitochondrial fragments combined. The scale measures the number of expected changes per site. Node numbers indicate support values for Bayesian Inference and Maximum Likelihood, respectively. Values under 80 are not represented. Haplotype (h) and locality (L) numbers at the tips of the tree refer to those in Table 1 and Fig. 1, respectively. Bars represent main clades and subclades, dots represent samples located in refugia predicted by paleomodeling.

Haplotype diversity values were similar among the four regional clades. Nucleotide diversity was significantly higher for the *C* + *N* clade than for the *S* clade (for all fragments). The *N* clade showed higher values of nucleotide diversity than the *C* clade for the control region and ND2 fragments, and similar values for the ND1 fragment (Table 3).

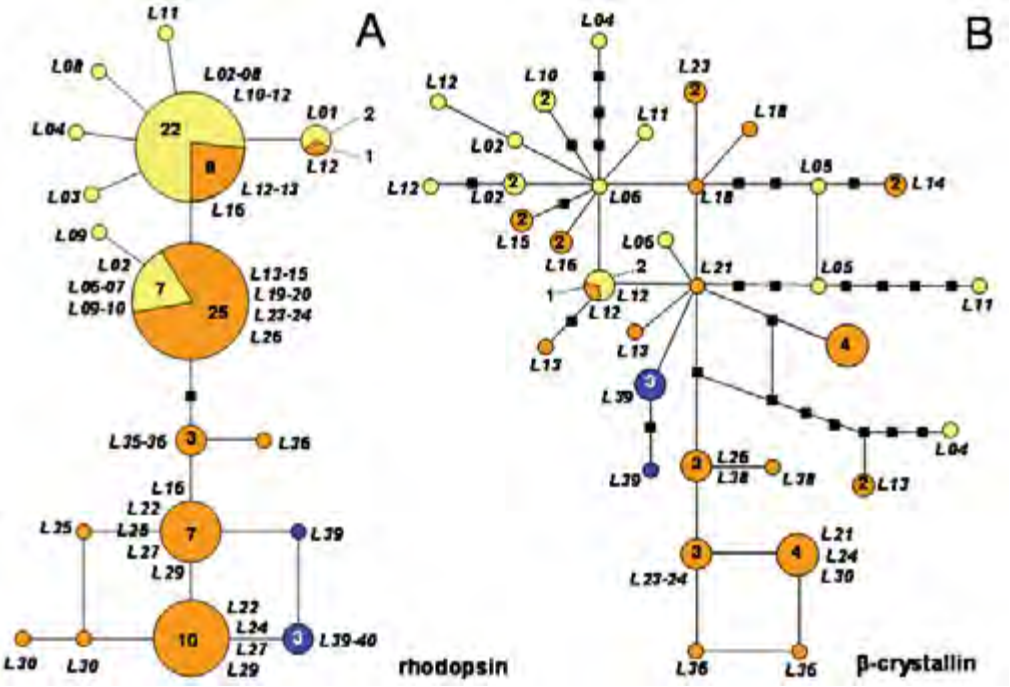

Fig. 3. Median joining networks of the nuclear gene fragments. A, rhodopsin; B, b-crystallin. Black squares represent unsampled alleles. Circle size is proportional to number of sequences. Locality codes outside circles follow Table 1. Shading corresponds to associated mitochondrial clades yellow for the *N* clade, orange for the *C* clade, and dark blue for the *S* clade). Number of contained sequences are inside the circles, except for singleton alleles.

3.2. Phylogenetic and population genetic analyses: nuclear DNA variation

We obtained 98 rhodopsin sequences from 49 samples and 54 crystallin sequences from 27 samples. In the rhodopsin alignment (280 bp) we found 13 polymorphic sites, of which seven were parsimony informative and six autapomorphic. The crystallin alignment (358 bp) had 38 polymorphic sites, 20 parsimony informative, and 18 autapomorphic. The crystallin fragment alignment also included 46 sites containing gaps, while the rhodopsin showed none. In the networks (Fig. 3) all nuclear alleles were connected with 95% certainty. Both networks showed poor geographic structure compared to mtDNA trees; however, the rhodopsin gene was more structured and showed a north–south trend in haplotypedistribution,

three alleles corresponding to different mtDNA main clades, and only one unsampled allele. Less geographic structure was evident for the crystallin network and the number of unsampled alleles was higher. We detected recombination at both nuclear fragments; for the rhodopsin we found six pairs of sites with four gametic types and a minimum of two recombination events, and for the crystallin we found 46 pairs of sites with four gametic types and a minimum of four recombination events. Haplotype diversity was higher for the crystallin gene, but nucleotide diversity was statistically similar for both nuclear fragments (Table 3).

3.3. Estimates of divergence time

Average net distances (*Da*) obtained between clades were as follows: 0.076 (±0.013) between *S* and *C + N* clades; 0.016 (±0.004) between *C* and *N* clades; and 0.006 (±0.002) between *n1* and *n2* subclades. Calibrating these values with the available rate of lineage diversification (Crawford, 2003), we obtained the following times of divergence: 3.97 MYr between the *S* and *C + N* clades; 0.836 MYr between the *C* and *N* clades; and 0.313 MYr between *n1* and *n2* subclades. Clade age inferred by the tMRCA ranges from 6.26 MYr for the whole species group, to 0.24 MYr for the shallowest subclade *c1* (Table 4).

Table 4: Mean times to the most recent common ancestor (tMRCA) for ND2 mitochondrial clades and 95% confidence intervals.

mtDNA	**t_{MRCA} (MYr)**
all	6.26 (4.13-7.49)
N	1.03 (0.64-1.29)
n1	0.82 (0.51-1.03)
n2	0.57 (0.27-0.81)
C	0.96 (0.60-1.18)
c1	0.24 (0.09-0.37)
N+C	1.87 (1.19-2.25)
S	0.73 (0.30-1.09)

3.4. Mapping areas of historical stability

The current time distribution model performed better than a random model, as evidenced by AUC values (0.95). Predictive maps (Fig. 4) were generated after a minimum presence threshold of 0.043. The current time model predicted an area compatible with the

current distribution of the group but overpredicted areas in northern Amazon, central Brazil (Mato Grosso, Mato Grosso do Sul, Goiás, and Tocantins states), and eastern Bolivia. Similar overpredictions are observed in the LIG, MIROC, and CCSM past projections. The current time model, the LIG and MIROC-LGM projections yielded similar distribution maps while the CCSM-LGM projection predicted pronounced fragmentation with several isolated areas. The intersection map of the current time model plus the three past projections shows relatively large isolates predicted as areas of continuous occupancy (stable areas) (i) the coastal region of northeastern Brazil, ranging from Alagoas to Rio Grande do Norte (hereafter called the Pernambuco region), (ii) southeastern Brazil, ranging from Rio de Janeiro to Espírito Santo and eastern Minas Gerais (hereafter called the Southeastern region), (iii) coastal south-southeastern Brazil, ranging from north Santa Catarina to São Paulo (hereafter called the Serra do Mar region), (iv) the interior of Paraná state (hereafter called the Paraná inland region), and (v) central-north Rio Grande do Sul state and western Santa Catarina state (hereafter called Rio Grande do Sul inland region) (Fig. 4).

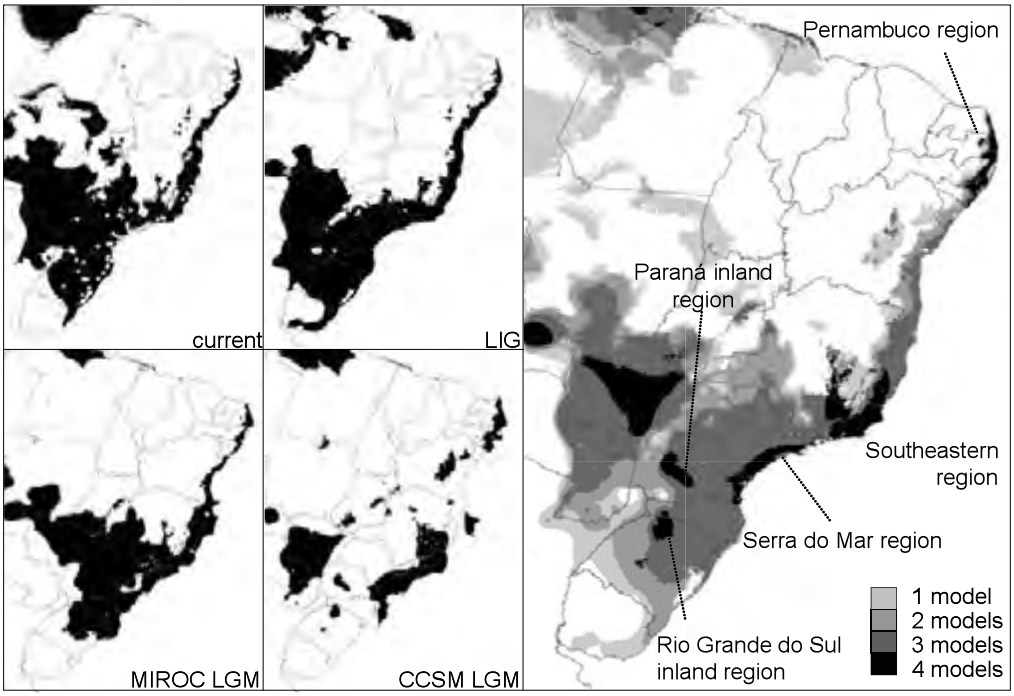

Fig. 4. Models of habitat distribution for current time, last interglacial period (LIG), last glacial maximum period (LGM), and intersection of the four models. White dots (in the current time model) indicate occurrence points used to generate the models. On the intersection map (right) dark shading indicates putative stable areas.

4. Discussion

We report genetic diversity within a widespread toad complex endemic to the Brazilian Atlantic Forest to examine the hypothesisthat southern populations are derived from a recent Holocene colonization from northern Pleistocene refugia. Contrary to expectations based on earlier studies of frogs in this same biome (Carnaval et al., 2009; Fitzpatrick et al., 2009), we found (i) deep lineage divergence both within northern and southern AF, (ii) the oldest divergence (Pliocene) to be between the southernmost populations and the rest of the range, (iii) that habitat fragmentation during glacial cycles is supported by paleoecological modeling, but congruence of genetic divergence patterns with putative refugia is limited, (iv) geographical barriers coincide with some genetic breaks between clades. Overall, our data refute the hypothesis of recent southern AF colonization, although they do not completely reject the role of refugial dynamics in determining lineage diversification in the *Rhinella crucifer* group. We also found that the current taxonomy of morphospecies within this group (Baldissera et al., 2004) is problematic, and we propose revisions based on the inferred historical diversification in this group.

4.1. Divergence at mitochondrial and nuclear markers

Mitochondrial diversity revealed that the earliest phylogenetic split in the *Rhinella crucifer* group separated the geographically disjunct southernmost populations from all others (Figs. 1 and 2, Table 2). Populations in the remaining clades range from Santa Catarina to the northernmost sampled sites in Paraíba. Among these populations we found strong support for a northern haplotype clade (subdivided into subclades *n1* and *n2*), and a central one (*C*), with a single contact zone between populations containing subclade *n2* and clade *C*. Haplotypes from both clades were collected at a single locality (Aracruz, *L12* in Fig. 1) in the state of Espírito Santo.

The deep phylogeographic structure evident in northern populations was not present among populations in central AF (the *C* clade). Closely related haplotypes were found in geographic proximity, and this clade is characterized by shallow divergences and few strongly supported subclades (Figs. 1 and 2). One exception occurs in haplotypes of the subclade *c1*, which is well supported in the total mtDNA topology (Fig. 2). Haplotypes in the *c1* subclade were sampled from the southern coastal regions of Paraná and Santa Catarina. Despite the

shallow divergences among some *Rhinella gR. crucifer* morphospecies, we found no haplotypes shared among the sampled localities (Figs. 1 and 2); however, more detailed population sampling may reveal higher levels of haplotype sharing across this continuous distribution, especially in central Atlantic Coastal Forest.

The signal of differentiation is less conspicuous in the nuclear gene genealogies (Fig. 3) as expected due to differential retention of ancestral polymorphisms, lower nucleotide substitution rates, and large effective population sizes of nuclear DNA (Avise, 2000; Edwards and Beerli, 2000; Moore, 1995). Several studies have reported incomplete nuclear lineage sorting despite reciprocal monophyly among mtDNA lineages (Broughton and Harrison, 2003; Dolman and Moritz, 2006; Machado and Hey, 2003; Sequeira et al., 2008). The recombination events we detected may also contribute noise to the nuclear allele network. Nonetheless, our nuclear genes show two patterns; the first is that nuclear alleles are shared only between individuals of northern and central mtDNA clades. Secondly, alleles found in southernmost populations (*S* clade) are always connected by few mutational steps to each other or to other alleles found in *C* clade individuals. Overall, the nuclear data show higher allele sharing between individuals of the *N* and *C* mitochondrial clades and support an independent evolutionary history of the *S* clade.

Relatively coarse population sampling across a continuous distribution can mimic a disjunct pattern of population structure (Bridle et al., 2004). Despite sampling gaps in our study, especially in the northern part of the range, we do not believe that sampling has biased our evolutionary inferences. The widest sampling gapin our study occurs between Pernambuco/Paraíba and southern Bahia (Fig. 1), but is not concordant with any major phylogeographic break; in fact, haplotypes collected on either margin of this gap are entirely contained within the subclade *n1*. A smaller gap occurs between the states of Santa Catarina and Rio Grande do Sul, a region corresponding to the major phylogeographic break between *S* and remaining populations in the *C* + *N* region. This deep divergences and high genetic diversity between S haplotypes and those of other regions suggests that differentiation is due to historic evolutionary processes rather than an artifact of sampling. Thus, our results suggest allopatric differentiation of populations among the three regions, and further sampling will allow us to clarify the spatial dynamics of putative contact zones and geographic limits of clades.

4.2. Temporal context for divergence

Accepting the allopatric model of diversification and given that the mtDNA data conformed to a molecular clock, we estimated divergence times between major haplotype clades using the ND2 gene geneology and a calibration inferred from another Neotropical anuran (Crawford, 2003). Divergence times between major clades based on *Da* were estimated at _4 MYr (Pliocene) between the *S* and *C* + *N* haplotype clades (~7.6%), ~0.8 MYr between the *N* and *C* haplotype clades (~1.6%), and ~0.3 MYr between n1 and n2 subclades (~0.6%, Table 3). With the exception of the split between the *S* and *C* + *N* haplotype clades, all diversification events within the *R. crucifer* group occurred in the Pleistocene. These estimates are compatible with the range of estimated tMRCA across haplotype clades (6.26–0.24 MYr; Table 3). Recent phylogeographical studies of AF endemics have generally shown a Pliocene–Pleistocene range of haplotype divergence times (Carnaval et al., 2009; Grazziotin et al., 2006; Pellegrino et al., 2005; present study). Some studies also present some earlier divergences (Fitzpatrick et al., 2009; Mata et al., 2009), supporting a continuous rate of diversification for taxa in this particular biome as described in a recent meta-analysis of divergences across Neotropical taxa (Rull, 2008). Among the AF amphibians examined thus far, the treefrog Hypsiboas faber and the toads *Rhinella crucifer* (sensu group) are the only ones spanning all regions of the AF (Lynch, 1979). Despite the tight overlap in tempo of diversification and geographic distribution, only limited agreement is observed in their geographic pattern of lineage divergence. Codistributed species may indeed evolve different patterns of genetic diversity due to stochasticity in the rates of extinction and speciation (Moritz et al., 2000; Rabosky and Lovette, 2008).

4.3. Putative refugia and the phylogeographic pattern

Estimated times of clade divergence in *R. crucifer* are compatible with the hypothesis that allopatric diversification was determined by Pliocene–Pleistocene climate-driven range fragmentation. Paleoclimatic modeling scenarios of range stability over the last 130 kYr showed areas of stable habitat that could have acted as isolated refugia (Fig. 4). Assuming that the last 130 kYr in our modeling are representative of earlier Pliocene–Pleistocene range fragmentation, we can evaluate lineage divergences in the *R. crucifer* species group relative to the geographic positions of putative refugia. The only predicted refuge that approximately coincides with a divergent clade in our mtDNA tree topology is the Rio Grande do Sul inland

region, which may have harbored the ancestors of the extant S populations. The remaining putative stable areas show loose correlation with haplotype clades; the lack of genetic divergence observed between samples in the two central refugia (Southeastern and Serra do Mar stable regions, Fig. 4) is not compatible with the predicted Pleistocene isolation of these areas. Additionally, the phylogeographic structure observed across the whole range of n1 subclade does not support a recent Holocene expansion and colonization from the Pernambuco stable region (Figs. 2 and 4). Caveats related to paleomodeling may explain this poor agreement between predicted refugia and phylogeographic pattern. The two distinct LGM scenarios (CCSM and MIROC) produced highly discordant predictions, a pattern explained by distinct initial climatic conditions of the simulations from which they were derived (Weber et al., 2007). The fact that fragmentation in the intersection map is recovered only by the CCSM-LGM scenario raises questions about the predictive performance of our paleodistribution models. In addition, it has been argued that general circulation models perform poorly in South America because they typically lack the resolution needed to capture the effects of the complex topography in the region (Cook and Vizy, 2006; Rojas et al., 2009).

Independent evidence of the effects of Quaternary climate changes on habitat dynamics show limited agreement among different regions of the AF (Fig. 1). Paleoclimatic studies are most common for southern and southeastern Brazil and show differences in the degree of fragmentation among rainforest and semideciduous forest sites, and also between inland and coastal sites. Four sampled regions that support stable wet conditions and/or forest persistence during the LGM are all in areas of rainforest relatively close to the ocean (Fig. 1). At Botuverá and Santana caves, composition of stalagmites indicates that longer and more intense summertime rainfall occurred during glacial times causing persistence of wet conditions from 70 to 17 kYr (Cruz et al., 2007, 2006). At Alto do Ribeira and Intervales state park regions, isotopes and charcoal present in the soil indicate a drier LGM, but forest elements were always present (Saia et al., 2008). At Colônia crater, palynological data indicate three episodes of diminished arboreal pollen during the last glacial period (including the LGM), followed by rapid forest expansions (Ledru et al., 2009). However, palynological and soil data obtained for Curucutú, an area neighboring Colônia crater, indicate that forest fragmentation was only a localized effect of coastline retraction and lower ocean humidity (Pessenda et al., 2009). In contrast, paleoclimatic studies at semideciduous regions with more inland distributions west of the Serra do Mar formation (Botucatú, Anhembi, Jaguariúna, Fig.

1) show clear cases of forest fragmentation (Behling, 2002; Behling and Lichte, 1997; Gouveia et al., 2002). Thus, paleoclimatic conditions may have been highly variable even within this one region of the AF.

Paleoclimatic studies at intermediate latitudes (Salitre de Minas and Catas Altas, state of Minas Gerais) show a very different pattern; semi-deciduous forests were historically less stable, with partial reduction in range and formation of savanna-forest mosaic landscapes during the LGM (Behling and Lichte, 1997; Ledru, 1993; Ledru et al., 1996; Pessenda et al., 1996). Paleoclimatic records from northeastern Brazil sites are sparser but highlight a contrasting pattern of higher rainfall during the LGM than at the present time. In the Caatinga biome (a xerophytic formation in northeastern Brazil) fossil travertine deposits and speleothems in caves at Toca da Boa Vista region suggest three wetter past periods, including the last two glacial maxima (Auler and Smart, 2001; Wang et al., 2004). A wetter LGM is also supported by isotope data at Irecê (Dever et al., 1987) and by an increase in flux of material to the ocean near Ceará state shoreline (Arz et al., 1998). Thus, the data available do not support the generalization of past climatic conditions across the AF. Rather, regional paleoclimates varied significantly in the timing and intensity of dry and wet periods, underscoring the inappropriateness of treating the AF as a single climatic unit.

Although the observed phylogeographic pattern in the *R. crucifer* group refutes large scale AF range contraction (sensu Carnaval and Moritz, 2008), our data do not rule out smaller scale habitat fragmentation. Populations could have persisted throughout the Pleistocene with more local/regional climate-driven isolation events, contributing to genetic diversification. Our genetic results are, however, in strong disagreement with the recently proposed hypothesis that most endemic taxa diverged primarily due to isolation in northern AF refugia, and that the southern regions were only recently colonized via southward dispersal of migrants from these refugia (Carnaval et al., 2009; Carnaval and Moritz, 2008). Phylogeographic studies of other AF taxa have already provided evidence consistent with southern or central refugia (Cabanne et al., 2007; Grazziotin et al., 2006; Fitzpatrick et al., 2009), but here we report the first case of deep lineage divergence consistent with the persistence of AF habitats in southern Brazil during the climatic cycles of the Plio–Pleistocene period.

4.4. Neotectonics as an alternative explanation?

Because the Atlantic margin of the South American Plate is tectonically passive, the South American continent east of the Andes was considered stable for a long period (Lima, 2000). Faults and fractures affecting dated sedimentary deposits, regional uplifts, and seismically induced structures indicate that Neotectonism has been remodeling this landscape (Ricommini and Assumpção, 1999). Recent revisions on active faults, many of which present evidence for Quaternary surface rupture, may provide alternative explanations for diversification of the AF biota (Ribeiro, 2006; Ricommini and Assumpção, 1999; Saadi, 1993; Saadi et al., 2005, 2002). The split between N and C clades is spatially concordant with the Doce River system, a complex and dynamic geologic formation that comprises many faults along its basin (Mello et al., 1999; Ricommini and Assumpção, 1999; Saadi et al., 2005). This is a known barrier for other taxa endemic to the AF (Sigrist and Carvalho, 2008 and references therein). Other neotectonic barriers that coincide with genetic breaks recovered in our phylogeny are the Guapiara lineament and the Cubatão shear zone, both including recent surface ruptures (Ricommini and Assumpção, 1999; Saadi et al., 2002) that separate the c1 subclade from the remaining populations of the C clade. Other potential neotectonic barriers occur within the group's distribution, and their associations with minor genetic breaks deserve future testing with finer sampling. Among others, the Upper Grande crustal discontinuity may isolate populations within the C region, and the Rio Araçuaí fault may have played a role in isolating the n1 and n2 subclades (Fig. 1). We found no geographic barrier compatible with the deep divergence between the *S* and *C + N* clades. This split is the only one associated with habitat fragmentation suggested by paleomodeling. Because postulated refugia and neotectonic barriers intercalate geographically, finer scale population sampling will be required to detect the complex patterns of population divergence and expansions caused by multiple mechanisms.

4.5. Taxonomic implications

We found taxonomic correspondence of mtDNA clades to three of the five morphospecies currently recognized (Baldissera et al., 2004; Frost, 2009). Only the southernmost *R. henseli* corresponds to an exclusive mtDNA clade (*S*). This is the most distinguishable species in the *R. crucifer* group due to its distinct color pattern, body size, shape of the head and parotoid glands (Baldissera et al., 2004). The *N* clade corresponds to the

R. crucifer morphospecies and to some individuals of *R. pombali* (*L8* and *9* in Fig. 1). The *C* clade include all the *R. ornata* and *R. abei* along with the rest of the *R. pombali*. Thus, *Rhinella pombali* is polyphyletic, and its recognition as an independent taxon may be invalid from a phylogenetic point of view. *Rhinella abei* appears to be a monophyletic taxon (subclade *c1*) nested within the *C* clade, but recognizing that taxon renders *R. ornata* paraphyletic relative to *R. abei*. The shallowness of most mtDNA clades, and incomplete lineage sorting in the nuclear markers suggest that the current morphological taxonomy might over-split species boundaries in the *Rhinella crucifer* group. Further sampling is needed to clarify species boundaries, and combining morphological and molecular data will be a necessary approach to infer species status in this group.

The morphological diagnosability of *R. pombali* and, to a lesser extent, *R. abei* (Baldissera et al., 2004) raises the challenging hypothesis that these morphotypes originated from differentiation along ecological gradients. *Rhinella pombali* is in fact the only one amongst the morphospecies in the *R. crucifer* group that occurs exclusively in inland semi-deciduous forests. This hypothesis should be examined in the context of uncovering the mechanisms of biological speciation in this species group.

4.6. Conservation implications

According to Carnaval et al. (2009), the Brazilian system of conservation units is not effective in preserving the main historical refugial areas. By focusing conservation priorities toward southern and southeastern Brazil, conservation measures favor regions of lower diversity occupied mostly by post-LGM expansion taxa, while potentially richer regions in the northeastern remain exposed to high rates of deforestation. There is no question that preserved areas and conservation units are scarcer and more poorly studied in Northeastern Brazil (Ribeiro et al., 2009), making this region a priority. However, we caution against using evidence from a single model of diversification as a guideline for conservation. Our results corroborate other recent studies showing continuous diversification of taxa across the AF since the Tertiary, a timeframe coincident with isolation due to refugial and/or geographical barrier dynamics (Carnaval et al., 2009; Fitzpatrick et al., 2009; Grazziotin et al., 2006; Mata et al., 2009; Pellegrino et al., 2005; present study). In many cases diversification does not fit a refugial model and even in cases where it does, we should not assume that any single model will prevail over others. Historical refugia and geographical barriers may have interacted

regionally or locally to isolate populations that later became genetically and phenotypically divergent. We predict these interactions may have contributed to very different patterns of diversification, albeit with some geographic commonalities, which must be uncovered to provide the best guidelines for biodiversity conservation in the Brazilian Atlantic Forest.

4.7. Conclusions

Genetic diversity in the *Rhinella crucifer* species group is structured in geographically disjunct clades across the AF. Diversification started in the Pliocene with most of the genetic structure originating in the Pleistocene. Isolated stable refugial areas may have existed, but we found limited evidence that refugia predicted from paleomodeling played a role in the diversification of this species group. Our findings contradict the model of AF diversification

driven by refugial isolation in northern regions with subsequent colonization of the south. We found that geographic barriers coincide with some of the phylogeographic breaks, suggesting that refugia and barrier models of diversification are not mutually exclusive. Our phylogeographic results indicate that conservation measures based on a single refugial model for diversification of AF taxa may not encompass some highly distinct lineages, especially in the southern regions of AF where habitat stability has been predicted to be lowest.

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Appendix 1– GenBank Accession numbers: GU907122–GU907480.

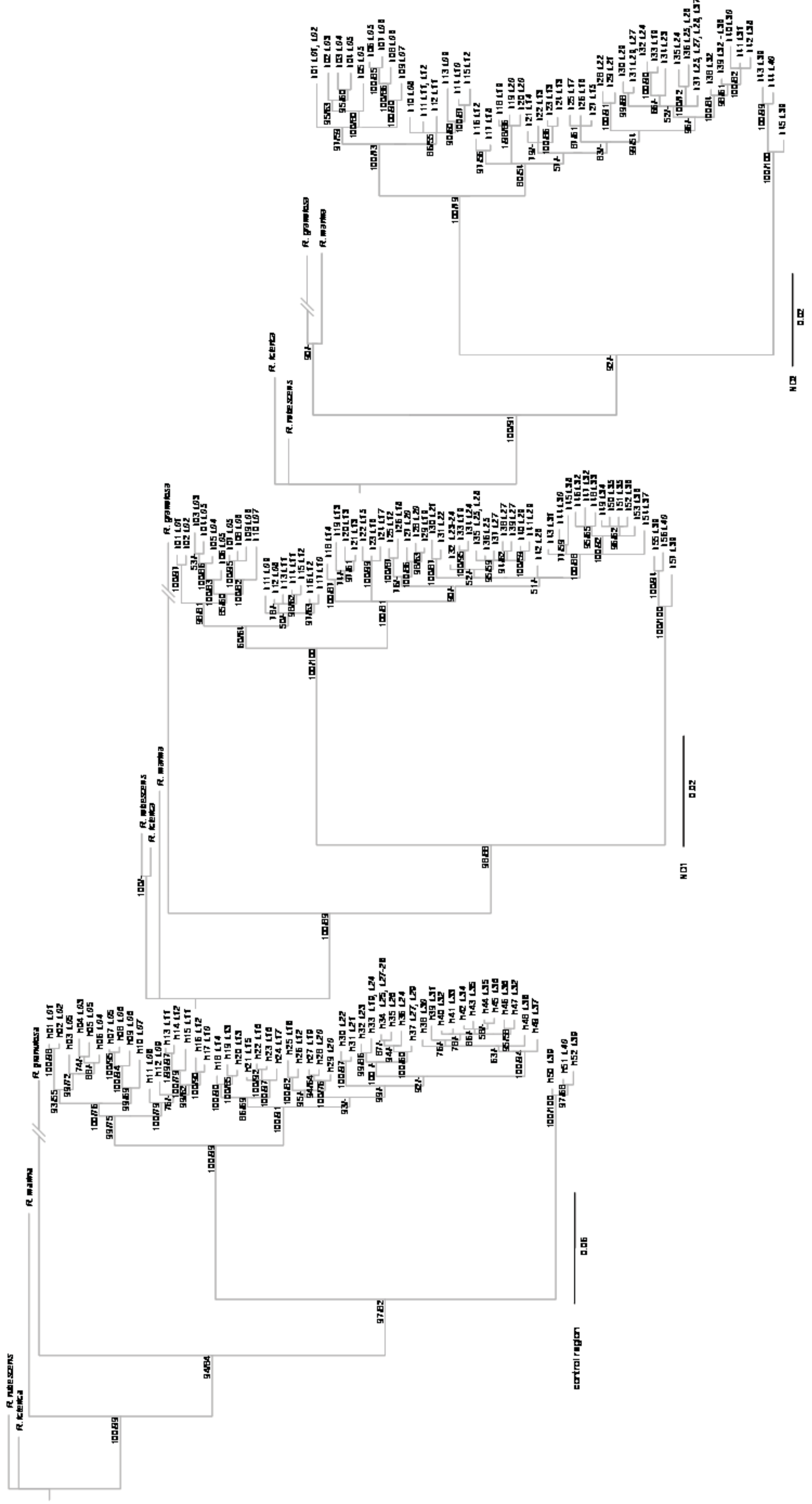
Appendix 2– Species presence points used for paleomodeling.

Municipality	State	longitude	latitude
Igarassu	PE	-34.94780	-7.82420
Aurelino Leal	BA	-39.32223	-14.33844
Uruçucá	BA	-39.28972	-14.58667
Ilhéus	BA	-39.23060	-14.76112
Caraíva	BA	-39.14698	-16.80024
Sooretama	ES	-39.88722	-19.13583
Linhares	ES	-40.07056	-19.15139
Linhares	ES	-40.07064	-19.15139
Sooretama	ES	-40.07056	-19.15139

Municipality	State	longitude	latitude
Sooretama	ES	-40.04611	-19.15306
Povoação	ES	-39.81700	-19.55070
Santa Tereza	ES	-40.54000	-19.95889
Vila Velha	ES	-40.32767	-20.41859
Santa Leopoldina	ES	-41.01000	-20.47333
S. Rita do Passa Quatro	SP	-47.62900	-21.62950
Divinolândia	ES	-46.60355	-21.67737
Baurú	SP	-49.09164	-22.23547
Itirapina	SP	-47.83136	-22.23667
Cristina	MG	-45.23333	-22.25000
Rio Claro	SP	-47.52292	-22.41193
Teresópolis	RJ	-42.98660	-22.44908
Petrópolis	RJ	-43.25377	-22.47206
Guapimirim	RJ	-43.00138	-22.49451
Teodoro Sampaio	SP	-52.30128	-22.60567
Itaguaí	RJ	-43.71233	-22.72560
Parati	RJ	-44.59447	-23.04395
Parati	RJ	-44.70677	-23.13247
Jundiaí	SP	-46.96686	-23.21694
S. Luis do Paraitinga	SP	-45.12161	-23.34491
Ubatuba	SP	-44.84563	-23.34505
S. Luis do Paraitinga	SP	-45.13459	-23.35072
S. Luis do Paraitinga	SP	-45.13377	-23.35193
S. Luis do Paraitinga	SP	-45.13377	-23.35193
Ubatuba	SP	-44.82526	-23.36461
Ubatuba	SP	-45.01472	-23.39513
Ubatuba	SP	-45.11658	-23.40948
Natividade da Serra	SP	-45.24366	-23.43743
Ubatuba	SP	-45.13370	-23.50066
Cotia	SP	-46.93573	-23.59546
Caraguatatuba	SP	-45.40970	-23.60750
São Sebastião	SP	-45.72736	-23.70207
São Sebastião	SP	-45.75317	-23.72562
São Sebastião	SP	-45.62000	-23.78000
Santos	SP	-46.29981	-23.81620
Cubatão	SP	-46.27567	-23.90256
Cubatão	SP	-46.42064	-23.92458
Itanhaém	SP	-46.74562	-23.99620
Guapiara	SP	-48.46468	-24.22012
Eldorado	SP	-48.11856	-24.49481
Iguape	SP	-47.22631	-24.56605
Iguape	SP	-47.50187	-24.64360
Pariquera-Açú	SP	-47.81108	-24.64578
Cananéia	SP	-47.92322	-25.06952
Cananéia	SP	-47.92489	-25.07689
Cananéia	SP	-47.92362	-25.15191
Quatro Barras	PR	-49.01014	-25.31066
Antonina	PR	-48.68228	-25.31067
Quatro Barras	PR	-49.00096	-25.38742
São João do Triunfo	PR	-50.09889	-25.57167
Matinhos	PR	-48.54080	-25.78560

Municipality	State	longitude	latitude
Massaranduba	SC	-49.02728	-26.74434
Blumenau	SC	-49.08191	-26.98802
Blumenau	SC	-49.09558	-27.02968
Nova Teutonia	SC	-52.41020	-27.03860
Itapema	SC	-48.62178	-27.09021
Capinzal	SC	-51.61190	-27.34360
Rancho Queimado	SC	-49.02170	-27.67250
Anitápolis	SC	-49.09484	-27.81603
Mato Castelhano	RS	-52.19170	-28.27830
Treviso	SC	-49.51749	-28.47038
Cambará do Sul	RS	-50.08090	-29.03320
Cotiporã	RS	-51.63080	-29.07150
Caxias do Sul	RS	-51.26250	-29.14160
Bento Gonçalves	RS	-51.46310	-29.17790
Santa maria	RS	-53.84310	-29.79390
Viamão	RS	-51.05650	-30.07090
Camaquã	RS	-51.81220	-30.85110
Missiones/ Argentina	-	-54.95200	-27.08880

Appendix 3–Bayesian trees of the ND1, ND2, and control region mitochondrial fragments. The scale measures the number of expected changes per site. Node numbers indicate support values for Bayesian Inference and Maximum Likelihood. Values under 50 are not represented.


control region
0.05
ND1
0.02
ND2
0.02

Capítulo 2

Delimiting genetic units in the *Rhinella crucifer* group of toads under incomplete lineage sorting and hybridization

Delimiting genetic units in the Rhinella crucifer group of toads under incomplete lineage sorting and hybridization

Maria Tereza Chiarioni Thomé, Kelly Raquel Zamudio, Célio Fernando Baptista Haddad, João Alexandrino

ABSTRACT

Delimiting genetic units is the first step toward understanding evolutionary mechanisms generating diversification of closely related organisms. Here we analyze mitochondrial and nuclear sequence data resulting from extensive geographic sampling to delimit genetic units in the *Rhinella crucifer* species complex of toads, a group endemic to the Atlantic Forest biome. We used tree–based and frequency–based approaches of species delimitation in a scenario of recent divergences and hybridization. Analyses of both mitochondrial and nuclear markers supported a genetic structure of five units within this group of toads. Three genetic units are distributed in the geographic core area of the group, while two other units show more isolated distributions. Genetic evidence for hybridization exists for two pairs of units and unclear evidence of admixture for another pair. The correspondence between genetic units and the five presently currently recognized morphospecies is limited: clear association exists only for the southern most diverged unit and *R. henseli* but not between other units and species *R. abei*, *R. ornata* and *R. crucifer*. Finally, *R. pombali*, as currently described, coincides with an area of genetic intermediacy (hybrid zone), suggesting that this species should be invalidated. These and other taxonomic issues will need to be considered, so the taxonomy of the *R. crucifer* species group properly represents evolutionary history of its lineages. The species limits inferred here will allow further studies on aspects of diversification and evolution of this group.

1. Introduction

The use of molecular data in taxonomical studies has deeply impacted the field by bringing new perspectives on species concepts (De Queiroz 1998, Agapow et al. 2004), making possible the use of barcoding (Hebert et al. 2003, DeSalle et al. 2005), and leading to a new discipline of integrative taxonomy (Dayrat 2005, Will et al. 2005, Padial et al. 2010). Organismal categorization based on molecular markers was advocated for conservation purposes through the delimitation of Evolutionary Significant Units (Ryder 1986) based on a set of controversial criteria (Moritz et al. 1994; but see Crandall et al. 2000). At the core of these discussions is the recognition of species as segments of population lineages against a view of species as operational units of a taxonomic rank (De Queiroz 2011). Independent of differing opinions, delimitation of genetic units can be a useful proxy for improving taxonomy and is the first step towards the understanding of evolutionary mechanisms acting on the diversification of closely related organisms.

The *Rhinella crucifer* species group is a widespread group of toads distributed along 30 latitudinal degrees of the Atlantic Forest biome, following the eastern coastline of South America and occupying the interior of the continent in the central–southern region of its distribution, where it reaches eastern Paraguay and Argentina. Along with this widespread distribution there is morphological variation, both within and among populations (Baldissera et al. 2004), which has led to a messy taxonomic history for this group (Frost 2011). In a recent review, Baldissera et al. (2004) recognized five species based on of morphological and morphometric variation. This work is the basis for the current taxonomy for this group (Frost 2011), but a recent broad scale phylogeographical survey revealed that mitochondrial clades do not fully correspond to these morphological species (Thomé et al. 2010). The distribution of mitochondrial clades compared to that of the five species suggests that hybridization may occur among these lineages (Thomé et al. 2010), a hypothesis that is supported by the fact that species are mostly diagnosable at the core of their ranges while identification is confusing or impossible at boundaries. Nuclear sequence data for the species complex as a whole showed evidence of incomplete lineage sorting (Thomé et al. 2010). The lack of fine-scale sampling in the previous study prevented both detailed mapping of mtDNA lineages and description of geographic patterns of nuclear allele frequency, resulting in a incomplete description of the genetic structure in the group.

The interest in using genetic criteria for unit delimitation has increased along with a recent change of paradigm in systematics (Edwards 2009). Newly available computational tools based on multilocus methods were developed for 'species' delimitation (Yang & Rannala 2010), inference of the relationships among previously defined species (Liu & Pearl 2007, Knowles & Carstens 2007, Heled & Drummond 2010), or both (O'Meara 2010, Carstens & Dewey 2010). However, the delimitation of genetic units is not always straightforward because the absence of horizontal gene flow is a requisite of these tree–based methods. Analyses of allele frequencies that jointly explore genetic structure and infer levels of migration (Pritchard et al. 2000) have been used in delimiting species where closely related species potentially hybridize (e.g. Dépraz et al. 2009, Meud et al. 2009, Larson et al. 2010, Reeves & Richards 2011, Ross et al. 2010). These methods have disadvantages over the species-tree methods such as restricted phylogenetic signal, a potential sensitivity to very recent genetic isolation (Yang & Rannala 2010), and a tradeoff between the costs of sampling at population–level and the number of markers required to detect genetic structure, especially when the efficiency of the markers (sensu Yang et al. 2005) is not known *a priori*.

In this study we greatly increase both the geographic and population sampling of previous work (Thomé et al. 2010) and use sequence data from a set of three mitochondrial and three nuclear genes to delimit genetically distinguishable units in the *Rhinella crucifer* group. Given the widespread distribution of the group, and under a scenario of recent divergences and likely hybridization, we employ a combination of tree and allele frequency–based methods. We discuss the results in the context of available information on the taxonomy and history of the group, and provide a perspective on the potential for other evolutionary studies.

2. Methods

Study System

The *Rhinella crucifer* group includes five morphospecies (Baldissera et al. 2004, Frost 2011): *Rhinella crucifer* occurs from the State of Ceará to northern State of Espírito Santo; *R. pombali* is restricted to the State of Minas Gerais with isolated records in the state of Rio de Janeiro; *R. ornata* occurs from southern State of Espírito Santo to northern state of Paraná; *R. abei* is distributed from the State of Paraná to the State of Santa Catarina; and *R. henseli*

occurs from southern State of Santa Catarina to the State of Rio Grande do Sul, with isolated records in the state of Paraná (Baldissera et al. 2004, Lima et al. 2005, Silveira et al. 2009) (fig. 1). Previous findings (Thomé et al. 2010) indicate that *R. henseli* corresponds to a highly divergent mitochondrial clade at the southern limit of the species' range. A northern clade includes *R. crucifer* and part of *R. pombali*, and a central clade includes remaining populations of *R. pombali*, along with *R. ornata* and *R. abei*. *Rhinella pombali* is found along an east-west axis where the northern and central clades meet, ranging from the coast throughout the inland plateau. Its morphology is intermediate between *R. crucifer* and *R. ornata* (Baldissera et al. 2004), which taken together with the mitochondrial DNA is suggestive of a hybrid origin. Hybridization between the morphospecies *R. abei* and *R. henseli* is also possible; we are aware of one locality where individuals reproduce in syntopy.

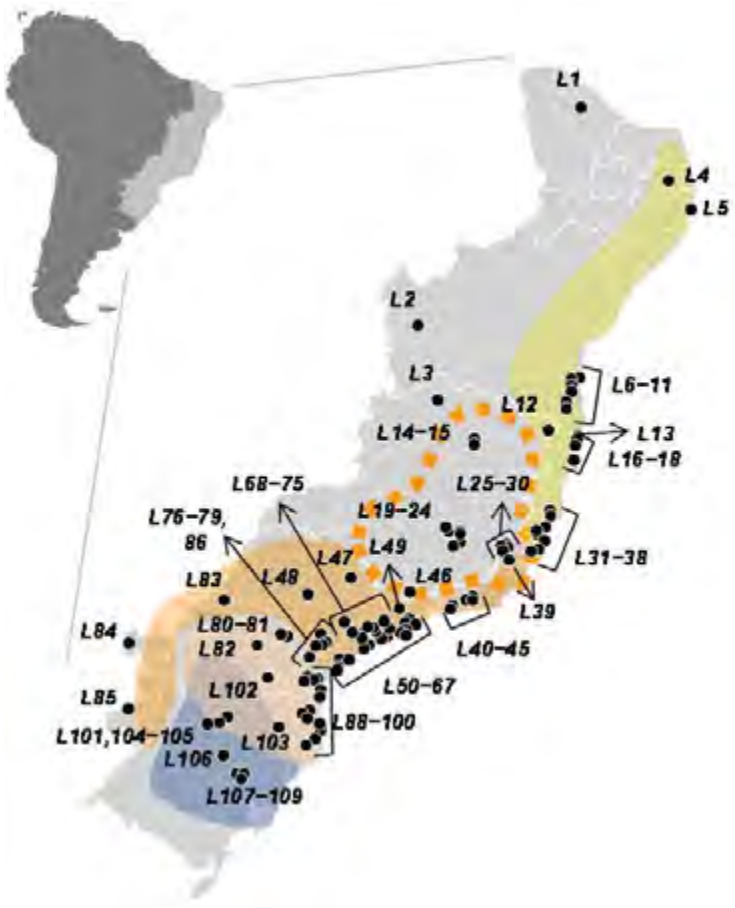


Figure 1: Localities sampled for this study and distribution of morphospecies in the *Rhinella crucifer* group. *Rhinella crucifer* in yellow, *R. pombali* in dashed orange, *R. ornata* in solid orange, *R. abei* in pale orange and *R. henseli* in blue.

Sampling and molecular protocols

We pooled sequences from newly collected individuals with previously published sequences (Thomé et al. 2010) reaching a total of 404 individuals of the *Rhinella crucifer* group (appendix 1). The 109 sampled localities were distributed across the Atlantic Forest biome and covered most of the species groups' distribution (fig. 1). We included fragments from 3 mitochondrial and 2 nuclear genes previously characterized for this species group (Thomé et al. 2010) and one additional nuclear fragment. The sequenced mitochondrial fragments were the control region and a short segment of the adjacent cytochrome b gene (hereafter referred to as the control region, 935 bp), a fragment including the 50 end of the 16S gene, the complete tRNAleu, the complete NADH dehydrogenase subunit 1, the tRNAIle and part of the tRNAGln genes (hereafter ND1, 1350 bp), and a partial sequence of the NADH dehydrogenase subunit 2 (hereafter ND2, 906 bp). The nuclear fragments were segments of the exons 1 and 4 of the rhodopsin gene (hereafter rhodopsin, 279 bp), the β-crystallin gene (hereafter crystallin, 357 bp), and the intron 1 of the A alpha polypeptide (hereafter alpha polypeptide, 707 bp, Bell et al. in prep).

We digested the samples and extracted whole genomic DNA using QIA Quick DNEasy kits following the manufacturer's protocol (Qiagen Inc.). We amplified target fragments with polymerase chain reactions (PCR) using one microliter of the eluted extract (~ 1–10 ng DNA) as template. Amplification conditions included an initial denaturation step at 94 ºC (5 min) followed by 35 cycles consisting of denaturation at 94 ºC (1 min), annealing at 48.3–60.2 ºC (1 min) (Table 2), extension at 72 ºC (1 min), and a final extension step at 75 ºC (5 min). Amplicons were purified with 10 units of Exonuclease I (Exo I) and one unit of shrimp alkaline phosphatase (SAP) as template for sequencing reactions. We used the same primers as in amplifications for sequencing using Big Dye termination sequencing chemistry (Applied Biosystems). We purified sequencing products using Sephadex G-50 columns and electrophoresed on ABI PRISM 3100 or 3730 Genetic Analyzers (Applied Biosystems). We checked electropherograms for errors, heterozygotic positions, and indels in the assembled contiguous sequences. A subset of the nuclear fragments containing either multiple heterozygotic positions or insertions/deletions were cloned using the pGEM-T Vector System (Promega Corporation) and transformed into One Shot TOPO10 competent *Escherichia coli* (Invitrogen Corporation) following manufacturer's protocols. After cloning we amplified the

desired fragments directly from the transformed colonies and sequenced as described above. Potential cloning errors were eliminated by comparing the heterozygotic sites of cloned sequences with those of the originals sequences. We aligned all contigs with ClustalW (Larkin et al. 2007) and checked alignments by eye.

Delimitation of genetic units

We used three methods to assess genetic structure and identify clusters of individuals corresponding to evolutionary units: i) we constructed phylogenetic trees, ii) performed population assignment analyses based on allele frequencies, and iii) checked for correspondence between genotypes. Final evaluation of species limits was achieved by comparing the clusters detected by the different methods.

For the mitochondrial data we constructed a haplotype tree by concatenating the three fragments. We used *Anaxyrus americanus, Rhinella icterica, R. rubescens, R. granulosa, R. schneideri* and *R. marina* as outgroups. We used maximum likelihood in the program RAxML (Stamatakis 2006a) with the GTR model and CAT approximation of rate heterogeneity (Stamatakis 2006b). We ran 10 replicates producing support values with 1,000 thorough bootstrap repetitions. We divided the alignment in 8 partitions as follows: control region, first, second and third codon positions of the coding region of the ND1 fragment, remaining regions of the ND1 fragment, and the first, second and third codon positions of the ND2.

For the nuclear dataset we first resolved heterozygotic positions using the coalescent-based Bayesian method PHASE (Stephens & Donelly 2003) implemented in DNAsp software (Librado & Rozas 2009), setting the number of interactions and burnin to 1,000 and maintaining one for thinning interval. We excluded from further analyses the sequences phased with a posterior probability inferior to 0.9. We then tested the three markers for intragenic recombination by checking the correlation between linkage disequilibrium (LD) and physical distance using two permutation tests: D−prime (D', Lewontin 1964) and the four−gamete test (G4, Hudson & Kaplan 1985). Both tests were performed in the omegaMap program (Wilson & McVean 2006) using 100,000 permutations. We also used the Difference of Sums of Squares method (DSS) implemented in TOPALi v. 2.5 (Milne et al. 2008). The program slides two windows along the alignment (a left−hand and right−hand window) and

calculates the sums−of−squares (SS) between observed genetic distances and distances based on a estimated tree. By comparing the SS of the left window to the right window the program infers putative recombination breakpoints. We included all sequences using a window size of 93 bp, steps ten bp long, and 100 bootstrap repetitions.

Genetic structure was accessed from nuclear data using allele trees and population assignment analyses. We constructed trees for each nuclear marker using maximum likelihood as described above. For population assignment analysis we used the algorithm implemented in the program STRUCTURE 2.3 (Pritchard et al. 2000) coding phased sequences as alleles. In this analysis we only included individuals from which we obtained a complete nuclear dataset (3 nuclear fragments). We used the admixture model with independent allele frequencies keeping lambda fixed at 0.7 after preliminary tests. We used 1,000,000 iterations as burnin and counted the next 3,000,000 iterations as our run. We considered 1 to 15 as a plausible range of putative populations (K) and performed 20 replicates for each K value. Because of the reduced number of nuclear loci, we avoided making inferences on optimal clustering. Instead, we observed the genetic breaks that appeared repeatedly over suitable values of K. To identify the most suitable values of K we plotted the average log likelihood values [Ln Pr (*X*/*K*)] for each K, calculated delta K values (ΔK, Evanno et al. 2005) with Structure Harvester v0.6. (Earl 2011) and checked for biologically meaningful population clusters.

We combined nuclear and mitochondrial data in a three-dimensional factorial correspondence analyses (FCA, Benzécri 1973) using GENETIX 4.05 (Belkir et al. 2004). FCA uses correspondences between genotypes to graphically plot individuals in a three-dimensional hyperspace based on their allele frequencies. FCA was used here because it enables combined analysis of all markers to help identify genetic structure in populations with no *a priori* information. We coded phased nuclear sequences as alleles as in the STRUCTURE analysis. Because of low levels of haplotype sharing in the mitochondrial data, we coded well-supported major clades as alleles (see results). We performed hierarchal FCA runs, starting with the complete dataset and removing individuals forming divergent clouds systematically until clouds were no longer separable.

Relationships among units

After identifying the clusters that were common to all methods we inferred the relationships among units with the species tree ancestral reconstruction method *BEAST (Heled & Drumond, 2010) in the software package BEAST v.1.6 (Rambaut & Drummond 2007). We excluded individuals with genotypes that could result from hybridization between units based on the previous analyses. To avoid over–parameterization we included a single mitochondrial fragment (ND2) with the three nuclear markers. We used the SRD06 model for the ND2, HKY +G for the rhodopsin and anonymous fragment, and GTR+I+G for the crystallin, estimating base frequencies in all cases. We used the Yule species tree prior linking all gene trees randomly generating starting trees for all partitions. We performed 3 independent runs of 100 million generations sampling log parameters each 10,000 steps. After comparing independent run results we used a ten percent burnin and combined the runs to extract average parameter values and intervals. We checked convergence of runs based on combined ESS values in TRACER v. 1.4 (Rambaut & Drummond 2007).

3. Results

Delimitation of genetic units

We obtained 386 sequences for the control region fragment, 401 for the ND1 and 398 for the ND2. After concatenation we found 305 distinct haplotypes among the 404 sampled individuals. The topology of the mitochondrial tree (fig. 2) recovers the monophyly of the *Rhinella crucifer* group with high support. The two most divergent clades within the group are located at the latitudinal extremes of the groups' distribution (fig. 3); the clade '*S*' is restricted to the southern region of the AF, states of Rio Grande do Sul, Santa Catarina and Paraná (L100−109), and the northernmost clade '*G*' is only found at the sampled locality of Guaramiranga, state of Ceará (L1). Haplotypes from the geographic region in between these two extremes form a main clade that contains most of the sampled haplotypes, ranging from the state of Santa Catarina to Paraíba (L2–101), which is itself structured with clades '*N*', '*P*', and '*C*'. Clade *N* has a northern distribution (fig. 3) ranging from the state of Paraíba to the state of Rio de Janeiro, including the eastern and northeastern regions of the inland state of Minas Gerais (L4–20, 26, 29, 31–38, 45). This clade showed deep substructure with several well supported sub–clades distributed sequentially from north to south, overlapping only at

geographic transitions. Clade *P* is geographically restricted to two western localities in the states of Minas Gerais and Bahia (L2 and L3) (fig. 3). Clade *C* covers the central AF ranging from the state of Espírito Santo to the state of Santa Catarina, including most of the interior regions of the AF (western limits of the states of Minas Gerais and Bahia to Paraguay) (fig. 3). Substructure in this clade is evident, with several well-supported sub–clades including a derived sub–clade restricted to eastern localities of the states of Paraná and Santa Catarina (subclade *c1*). Other sub–clades showed partially or completely overlapping distributions mainly across the state of São Paulo and surroundings.

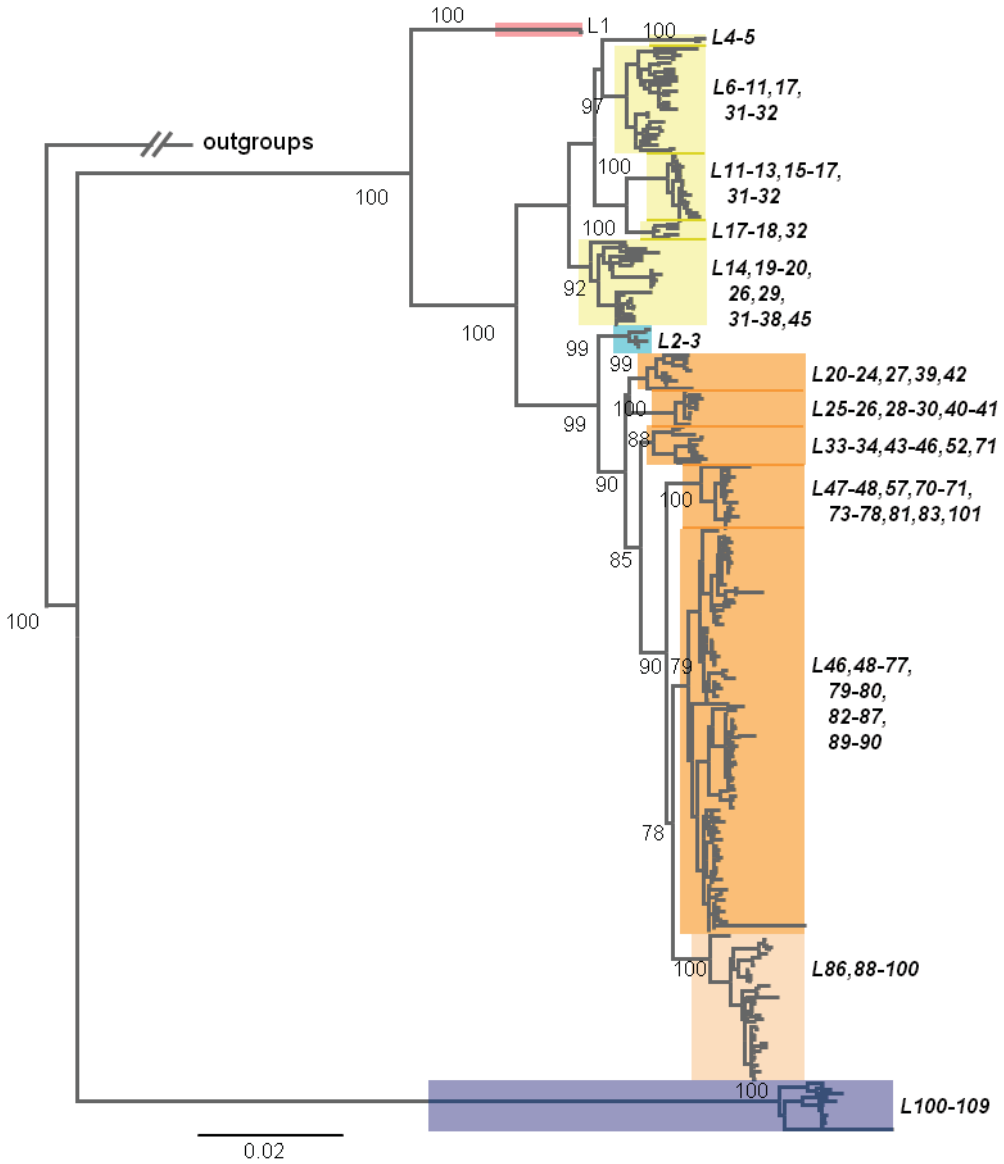


Figure 2: mitochondrial haplotype and nuclear allele trees inferred by maximum likelihood for the *Rhinella crucifer* group. Numbers before clades indicate support values, number after clades code for localities.

The number of nuclear sequences obtained after phasing was 562 for the crystallin, 664 for the rhodopsin, and 688 for the alpha polypeptide, among the 394 sampled individuals. The number of unique alleles for each gene was 25, 161, and 61, respectively. No recombination was detected by the DDS method or by permutation tests for the rhodopsin (D'=−0.15, p=0.08; G4=−0.12, p=0.11), crystallin (D='−0.02, p=0.17; G4=−0.02, p=0.19), and alpha polypeptide (D'=−0.03, p=0.14; G4=−0.05, p=0.08). The structure of allele trees shows poorly supported clades and polytomies across all genes (appendix 2). Sharing of alleles among localities was frequent for the rhodopsin and alpha polypeptide, and less common for the crystallin marker (appendix 1).

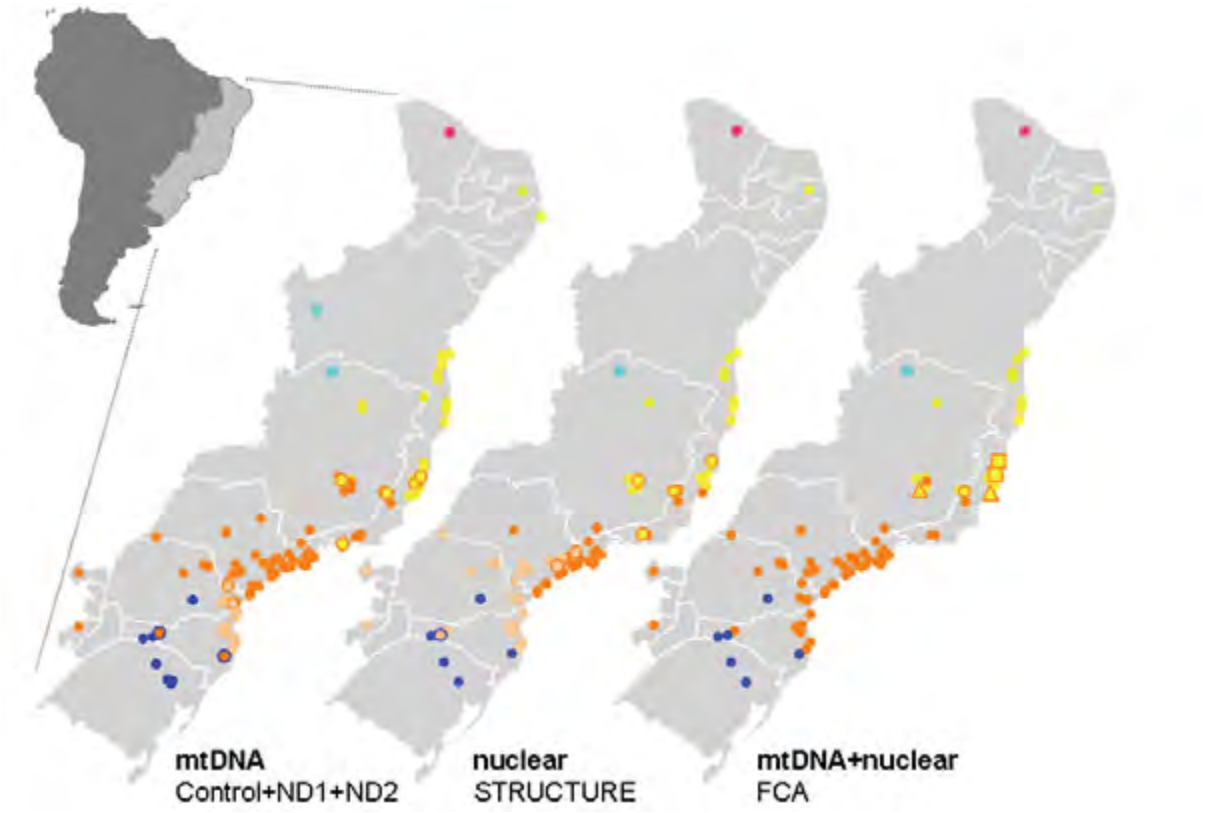


Figure 3: distribution of mitochondrial clades, nuclear demes and FCA clouds. Mitochondrial clade *G*, deme *G* and FCA cloud *G* in magenta; mitochondrial clade *N*, deme *N* and FCA cloud *N* in yellow; mitochondrial clade *P*, deme *P* and FCA cloud *P* in light blue; mitochondrial clade *C*, deme *C1* and FCA cloud *C* in orange; mitochondrial sub–clade *c1* and deme *C2* in pale orange; mitochondrial clade *S*, deme *S* and FCA cloud *S* in dark blue. Localities with more than one mitochondrial clade or more that one nuclear deme are coded with two colors. In FCA, squares code for localities where clouds C and N co–occur, triangles code for localities with individuals in the intersection of these two clouds, and the two–colored circle code for both situations.

A complete dataset for the three nuclear fragments was available for 196 individuals from 83 localities. STRUCTURE results were evaluated according to the distribution of Ln Pr (*X*/*K*), and ΔK. According to these criteria the values of K that best describe the structure of the nuclear data range from K=2 to K=6 (fig. 4 and fig. 5). Five genetic breaks were consistently recovered indicating the clustering of individuals in six geographically distributed demes: deme '*G*' with individuals from the locality of Guaramiranga, state of Ceará (L1), deme '*P*' containing individuals restricted to western Bahia (L3), deme '*N*' clustering individuals from the state of Paraíba to the state of Rio de Janeiro including northern and eastern Minas Gerais (L4, 6, 8, 10–11, 13–14, 16–23, 26, 28–29, 31–39, 41), deme '*C1*' with individuals from the states of Espírito Santo, Rio de Janeiro, southern Minas Gerais, and the eastern region of the state of São Paulo (L23, 26, 28, 31, 37, 39, 43, 46, 48–63, 65–68, 70–71, 73), deme '*C2*' with individuals from the inland part of the state of São Paulo, states of Paraná and Santa Catarina, and deme '*S*' with individuals from the states of Rio Grande do Sul, Santa Catarina and southern Paraná (fig. 2, fig. 5).

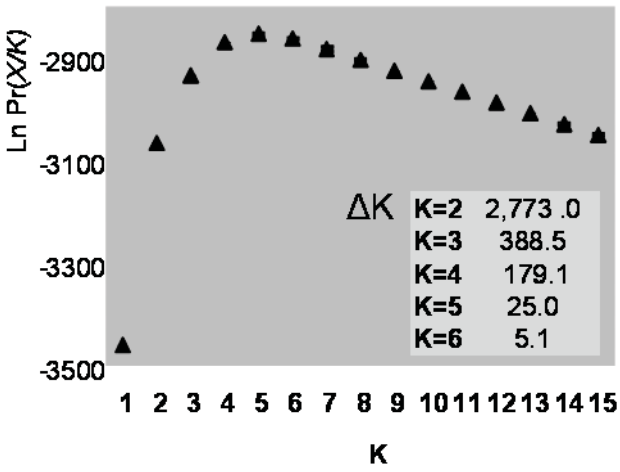


Figure 4: distribution of Ln Pr (*X*/*K*) for K=1–15 in the STRUCTURE analysis and values of ΔK for K=1–6.

The FCA performed with the complete dataset showed individuals clustering in three clouds corresponding to geographic groups: a cloud containing individuals from Rio Grande do Sul, Santa Catarina and Paraná states (cloud '*S*', L100, 102, 104–106, 109), a cloud with individuals from western Bahia (cloud '*P*', L3), and a cloud containing the rest of the individuals. After the removal of clouds *S* and *P*, the subsequent analysis shows the clustering of a third cloud with individuals from Guaramiranga (cloud '*G*', L1). In the analysis with the remaining individuals no more isolated clouds were found. Instead, we found two partially

overlapping clouds, the first with individuals from the state of Paraíba to the state of Bahia (cloud '*N*', L4, 6, 8, 10–11, 13–14, 16–21, 28–29, 31–36) at one geographic extreme, and the second with individuals from the state of São Paulo to the state of Santa Catarina (cloud '*C*', L23, 26, 28, 39, 41, 43, 46, 48–63, 65–68, 70–71, 73–86, 88–90, 93–97, 99, 101) at the opposite geographic extreme of the population distribution. Individuals distributed in the intersection of both clouds were from localities in the states of Espírito Santo and Minas Gerais (L22, 31, 33, 37) (fig. 6).

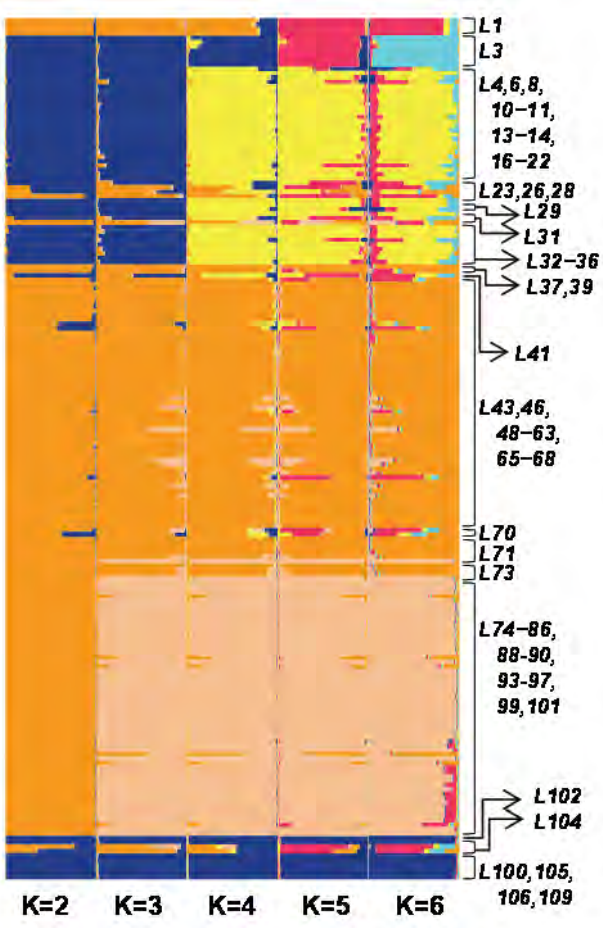


Figure 5: STRUCTURE results for K2–6. Individuals are represented as bars and colors state for the proportion of assignment to each nuclear deme. Colors are coded as in figure 3 and locality numbers as in figure 1.

Taking together the results of the three analyses we delineated five genetic units: G (corresponding to the main clade *G*, deme *G* and cloud *G*), P (containing the sublade *P*, deme *P* and cloud *P*), N (with the main clade *N*, deme *N* and cloud *N*), S (corresponding to the main clade *S*, deme *S* and cloud *S*) and C (including the main clade *C*, demes *C1* and *C2*, and cloud *C*). Considering the analyses individually, uncertainty of limits occurred at boundary zones: i)

we found overlap of the mitochondrial clades *N* and *C* at L20, 26, 29, 33–34, 45, and *C* and *S* at L100 and 101; ii) localities with individuals from more than one deme and individuals presenting low values of average q (<0.85 considering K=2 to K=6) were more frequent at the boundary between demes *N* and *C1* (L20, 22–23, 26, 28, 31, 33, 37, 41, 43); individuals distributed in the intersection of clouds in FCA also occurred at this region (L22, 31, 33, 37). Discordance between results of distinct analyses also occurred at boundary zones. We found individuals with relatively high values of average q (≥0.85 considering K=2 to K=6) in the STRUCTURE analyses that pertained to conflicting mtDNA clades at localities L21–22 and L28–29. Incongruence between mtDNA clades and clouds inferred by FCA also occurred at L21, 28–29.

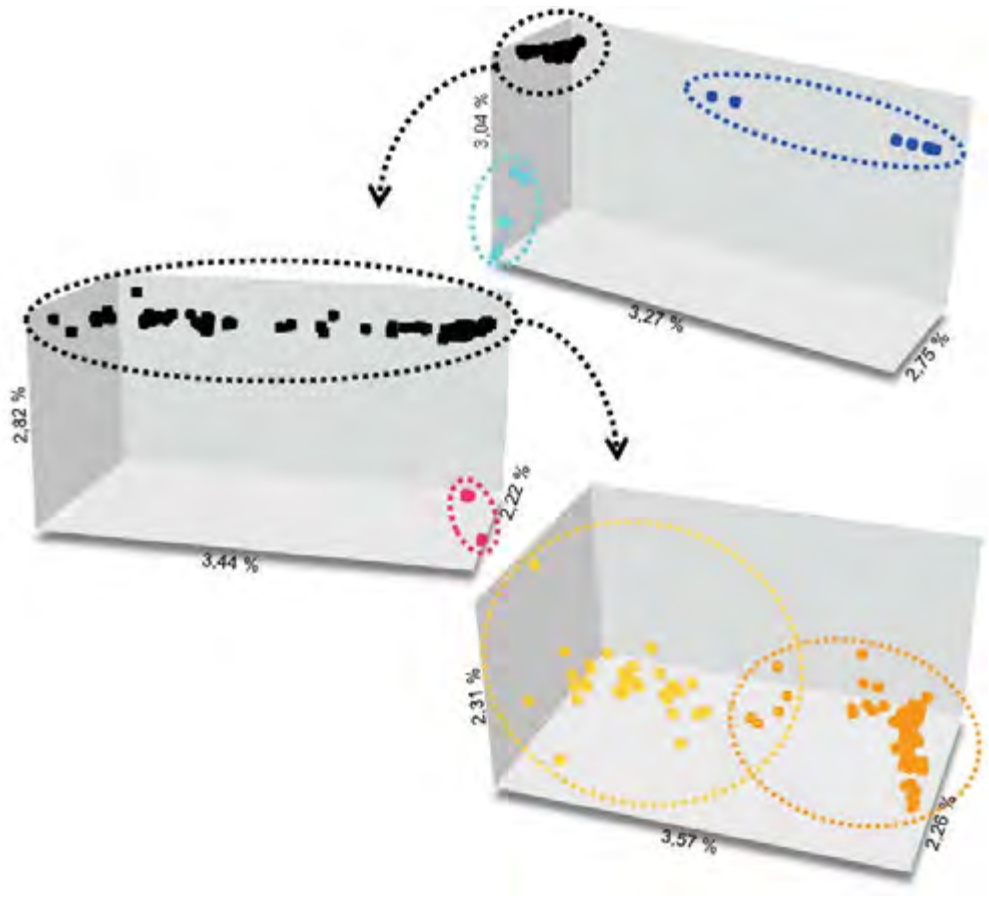


Figure 6: FCA combining nuclear alleles and mitochondrial haplogroups. Cloud *S* in dark blue, cloud *P* in light blue, cloud *G* in magenta, cloud *N* in yellow, and cloud *C* in orange. Individuals in the intersection of clouds *N* and *C* are represented by two colored squares.

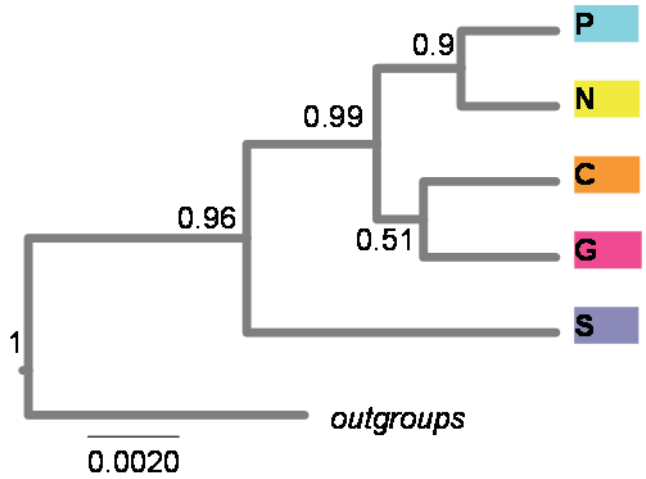


Figure 7: species tree inferred with *BEAST for the inferred genetic units. Values near nodes represent posterior probabilities.

Relationships among units

After excluding potential hybrids and individuals from localities at the boundaries between inferred genetic units, a total of 145 individuals were included in the final species tree analysis. The three runs separately showed relatively low numbers of independent samples for a few parameters, but combined ESS values were > 400. All independent runs produced the same topology for the species tree with similar values of posterior probabilities. The species tree with combined results from the three runs presented well-supported nodes, with the exception of the node uniting clades C and G (fig. 7).

4. Discussion

Analyses of the genetic structure of mitochondrial and nuclear marker within the *Rhinella crucifer* species group revealed five distinct genetic units corresponding to the core geographic area of the group (N, C, and S), and two units with more isolated distributions (G and P). Overlap occurs between units N and C and between C and S, with evidence of admixture between both pairs of units. The concordance of genetic units to the currently recognized species boundaries is very limited. Correspondence is clear between *R. henseli* and

unit S, and less obvious for *R. crucifer* and unit N, and *R. ornata* and unit C. The distribution of *R. pombali* within the putative hybrid zone between N and C explains its intermediate morphology and argues that it is not distinct from neighboring units. *Rhinella abei* corresponds to a mtDNA sub-clade, but its correspondence to a genetic group in combined marker analyses is not clear and might depend on the inclusion of more markers. Morphological distinctiveness of genetic units G and P remains to be tested.

Delimitation and relationships among genetic units

We found deep mitochondrial structure within the *R. crucifer* group; haplotypes cluster in sub–clades that belong to larger well-supported major clades (*N, C, S, G* and *P*). Geographic distributions of sub–clades overlap greatly while major clades have more exclusive areas of occurrence. The mtDNA tree presented here (fig. 2) differs from the previously published topology (Thomé et al. 2010). All main clades (*N, C* and *S*) identified earlier were recovered by our current analysis, but the addition of previously unsampled geographically isolated populations revealed two new clades with apparently narrow distributions (clades *G* and *P*). Also due to addition of samples from previously unsampled regions, the known geographic limit of clade *S* has been extended considerably to the north, and the southern limit of clade *N* was extended to the southern region of the state of Rio de Janeiro (fig. 3). Increased sampling also extended the distribution of the *C* clade towards the southwest, and improved the support for the relationships within this clade, especially for sub–clades in the northern part of its range. These sub–clades showed more restricted distributions than sub–clades in the state of São Paulo and western regions of the states of Paraná and Santa Catarina, which overlapped extensively, but this pattern might reflect unbalanced sampling. In eastern regions of the states of Paraná and Santa Catarina individuals clustered within sub–clade *c1*, a pattern that was recovered before (Thomé et al. 2010). The overlapping geographic distributions of all *N* sub–clades confirmed that the allopatric distribution shown in Thomé et al. (2010) was a sampling effect.

The nuclear gene genealogies (appendix 2) confirmed the incomplete lineage sorting previously described for the *Rhinella crucifer* group (Thomé et al. 2010). The nuclear genetic structure was better accessed through an unusual application of individual assignment analysis; we used the STRUCTURE method of Pritchard et al. (2000) with three nuclear loci and a model without prior information of the clustering of individuals. This scheme avoids

circularity in cross–validating results with other evidence (i.e. geography and mtDNA structure) but the limited number of loci makes inference on optimal number of clusters unreliable. We were able to estimate a relatively safe interval of possible values of K (K=2–6) by comparing scores produced by different criteria [ΔK, Ln Pr(*X/K*), and interpreting those results in terms of biological distribution of those demes]. Assignment analyses for that K interval consistently recovered five genetic breaks and six demes could be delimited based on the nuclear data alone (fig. 5). The presence of individuals with low average assignment coefficients was mostly restricted to the geographic boundaries of demes (figs. 1 and 5), which argues for admixture at contact zones between diverged lineages. Clusters defined after STRUCTURE analysis of nuclear markers correspond well to mtDNA structure; of the six inferred demes, demes *G*, *N*, *P*, and *S* correspond largely to mitochondrial main clades, and demes *C1* and *C2* together account for the mitochondrial clade *C* (fig. 3). Substructure in the mitochondrial clade *C* does not correspond to the genetic break between demes *C1* and *C2*. However, at values of K ≥7 a cluster geographically coincident with the distribution of mtDNA sub–clade *c1* was revealed (data not shown), which suggests that additional data (more nuclear markers) will likely uncover a nuclear genetic structure consistent with that of mtDNA for the eastern regions of the states of Paraná and Santa Catarina.

The limited number of loci used in the STRUCTURE analysis limits the definitive interpretations of our assignment results; clearly more conclusive inferences will require additional markers. Nonetheless, the performance of the method with only three loci was surprisingly convincing, suggesting that even these tree loci contain the signature of geographic history of the major lineages. Assignment analyses based on sequence data from five nuclear loci were also successful in revealing cryptic species in the West African forest gecko *Hemidactylus fasciatus* complex (Leaché & Fujita 2010). The success of these studies in detecting relevant genetic structure may rely on sufficient sampling of individuals and marker choice; optimal clustering in assignment analysis can be achieved with a reduced number of loci (up to two orders of magnitude less that in regular analysis) as long as chosen markers are highly efficient (Yang et al. 2005). For example, conservative markers might be more informative in exploring boundaries in widespread species complexes where noise from within species variation is likely to be high. This prediction was somewhat supported in Rosenberg et al. (2003) that found single nuclear polymorphisms (SNP) to be less sensitive for population structure than microsatellites, especially at large K values. It is thus plausible

that SNP might be a less efficient marker than sequence data due to intrinsic restriction to four possible alleles, a number that sequence data overcomes easily. A comparative study on the sensitivity of different types of markers to detect inter–specific genetic structure in assignment analysis should be of great utility in studies concerning the boundaries of closely related species.

The combination of mitochondrial and nuclear data in FCA repeated the results obtained for these markers individually (with the exception of nuclear genealogies) (fig. 6). Therefore, the existence of five units within the *Rhinella crucifer* group is strongly supported by the overall genetic data. The units C, N and S form the main range of the species group with parapatric distributions (S–C and C–N). Units G and P have very restricted distributions at the northern and western limits of the species groups' range, and appear to be geographically isolated. These allopatric distributions likely reflect the patchy nature of the habitat in the transition zones between the Atlantic Forest and other neighboring biomes, but sampling artifacts might also explain this isolation pattern to some degree.

The relationships among genetic units in the species tree analysis (fig. 7) are very different to those recovered in the mtDNA tree topology, and the nuclear gene trees. Topology changes for all units with the exception of unit S that remains the most divergent. The clustering of units G and C is poorly supported and should be interpreted as a polytomy. The species tree construction method we applied here is relatively new and the effects of using a few loci to infer relationships between closely related species, although not tested yet, probably affected our analysis. The discrepancy in population sizes of the 'species' (coarsely estimated by the geographic distributions of the genetic units) might cause drift to shape the allele composition of smaller populations in a way that phylogenetic signal is at least partially eliminated, so the species tree topology does not reflect true relationships. Additional analyses with a subset of the current data using BEST (Liu & Pearl 2007) revealed a different topology (data not shown), which attests against the reliability of our results. Therefore, the current tree of genetic units should be tested with further data (more loci) and analyses before conclusions can be made.

Two potential hybrid zones

Geographic overlap of units occurs along the borders of units N and C and more extensively between units C and S (fig. 3). Strong evidence of admixture was found for the N/C contact zone, with four individuals showing cyto–nuclear introgression. These individuals were distributed in localities along an east–west axis near the limits of the states of Espírito Santo, Rio de Janeiro, and Minas Gerais. Albeit spatially restricted relative to the ranges of units C and N, hybridization likely occurs across a wider region along this longitudinal axis. This is supported at the level of both mtDNA and nuclear data given the distribution of admixed individuals relative to the geographic distributions of clade overlap (figs. 3); and by the combined effect of both types of markers in showing a lack of clear delimitation in the FCA clouds (figs. 3 and 6). The hybrid zone between units N and C should therefore have a putative range across most of the states of Espírito Santo and southern Rio de Janeiro, also occupying the central region of the state of Minas Gerais.

Evidence for a second hybrid zone (between units C and S) is less conclusive. Two individuals from one locality (L104, fig. 1) have a nuclear genetic signature that indicates mixing according to the STRUCTURE analysis, but the lack of evidence for mitochondrial introgression makes incomplete sorting of lineages an equally likely explanation. Co–occurrence of the two mitochondrial clades was detected for two localities (L100 and 101, fig. 1), with individuals of both clades reproducing synchronously at the same pond at locality L100 (L.M. Giassom, personal communication). We sampled seven individuals at this pond but obtained a complete nuclear dataset for only one individual, for which we found no signal of introgression both with STRUCTURE and FCA. Other six individuals with incomplete nuclear datasets were not included in STRUCTURE and FCA analyses but their allelic compositions, as shown in the allele trees, provided some evidence of mixing for this locality. Sharing of nuclear alleles occurred exclusively within members of the same mitochondrial clade (also considering members from other localities) with the exception of one individual from clade *C* which contained alleles that are otherwise exclusive to members of the clade *S* (appendix 1). This occurred for the two nuclear markers available for this individual (rhodopsin and alpha polypeptide), showing that incomplete sorting of lineages is a much less parsimonious explanation than hybridization in this case.

Admixture between the genetic units in the *Rhinella crucifer* group is not a surprise; natural hybridization in bufonids is very frequent and cases of natural hybrids have been reported for almost every continent in which this cosmopolitan family occurs (e.g. Masta et al. 2002, Stöck et al. 2006, Cunningham & Cherry 2004, Yamazaki et al. 2008). In our case, we found stronger evidence for hybridization between units N and C than between units C and S, a result that might be explained by the amount of genetic divergence accumulated between each of these pairs. Malone & Fontenot (2008) revisited a large dataset of experiments crossing species from all major clades of bufonids (see Blair 1972) and re–interpreted results in the light of newly available phylogenies. The authors found intrinsic postzygotic reproductive isolation to be a gradual and likely outcome related to the degree of divergence, with high levels of divergence (~9% mtDNA) being required for hybrid offspring mortality during early developmental stages. It is possible that hybrid survival might be rarer for crosses between C and S than N and C, given that genetic divergences inferred from mtDNA data are greater between the S unit and all other units, reaching values up to 7% (ND2, Thomé et al. 2010). Colliard et al. (2010) found similar results for Plio–Pleistocene diverged populations of toads from the *Pseudepidalea viridis* group with a slight fitness reduction in F1 hybrids, a strong hybrid breakdown in backcrossed offspring, and complete mortality in F2 hybrids. However, hybridization was a weak indicator of phylogenetic relationship for the African 20-chromosome toads since it occurs erratically among species in that group (Cunningham & Cherry 2004).

A problematic taxonomy

Five species of the *Rhinella crucifer* group are recognized on the basis of morphological and morphometric data (Baldissera et al. 2004, Frost 2011). The diagnosis of these morphospecies can be difficult depending on the geographic origin of the specimen. An evaluation of the taxonomy based on the results of our present work reveals that the current classification does not reflect evolutionary relationships within the group and identifies several problems. Unequivocal correspondence between the genetic units and recognized species exists only between the unit S and *R. henseli*, which corresponds to a deeply diverged mitochondrial lineage (Thomé et al. 2010), and is the most clearly diagnosable species in the group (Baldissera et al. 2004). Strong but problematic associations are evident between unit N and *R. crucifer*, and between unit C and *R. ornata*. These species are easily diagnosable

morphologically if one compares individuals from across the core ranges of units C and N (i.e., not including individuals from the putative hybrid zone). The difficulty is that other named species appear to be nested within these 2 units. This is the case for *R. abei*, a species that occurs within the distribution of unit C, and with very subtle diagnostic characters (Baldissera et al. 2004). *Rhinella abei* cannot be associated to a genetic unit but its distribution roughly coincides to a haplogroup (subclade *c1*). Interestingly, Baldissera et al. (2004) found a similar pattern in morphometric analysis in that the distribution of *R. abei* is confined to an area within the polygon of the wider distribution of *R. ornata*. Despite these data, we cannot make any statement on the status of *R. abei* at this point because the failure of our analyses in detecting this entity may be caused by the limited number of nuclear markers (see above). A second problem arises when establishing correspondence between unit C and *R. ornata*: the type locality for this species ("probably Rio de Janeiro" according to Bokermann 1966) and type localities for all its synonyms (Frost 2011) lies within the putative hybrid zone between unit N and C, meaning that the type series may actually include hybrids. A careful examination of the type series is necessary to solve this issue.

Hybridization is probably the cause of the most problematic case in the taxonomy of the group. *Rhinella pombali* was originally described based on individuals from the central region of the state of Minas Gerais (Baldissera et al. 2004). This distribution has been expanded to the state of Rio de Janeiro near its limits with the state of Espírito Santo (Silveira et al. 2009), resulting in an overall area of occurrence that is largely concordant with the putative distribution for the hybrid zone between units N and C. The morphology of *R. pombali* shows characters that are intermediate between *R. crucifer* and *R. ornata* such as tarsal tubercles forming a row in *R. ornata* and in small individuals of *R. pombali*; tarsal tubercles forming a fringe in *R. crucifer* and in large individuals of *R. pombali* (Baldissera et al. 2004). This species has an intermediate size as well, a feature that can mislead the interpretations of traditional morphometric analysis (fig. 27 in Baldissera et al. 2004). Projected scores of *R. pombali* occupied a distinct area of the morphometric space but most of the variation contained in the canonical axis is explained by the size component, and not a significant change in shape. Our data argues in favor for the hypothesis that *R. pombali* was described based on hybrids formed from crosses between *R. crucifer* and *R. ornata* (Thomé et al. 2010). Difficulties in inferring species within closely related Bufonidae have been attributed to hybridization by taxonomists and phylogeneticists (Masta et al. 2002, Fontenot et

al. 2011), with true toads (genus *Bufo* as considered before Frost et al. 2006) being referred as a test–case in anuran systematics (Cunningham & Cherry 2004). The International Code for Zoological Nomenclature (ICZN 1999) states that names proposed for hybrid specimens are excluded from the provisions of the Code (article 1.3.3), and may enter into homonymy (article 23.8). On the basis of article 1.3.3, we suggest that *R. pombali* should not remain a valid species.

Further investigation of the status and distribution of the genetic units G and P will be another requirement to reach a taxonomical consensus for this group. A first inspection of specimens from these regions revealed considerable morphological divergence of unit P (P. H. Valdujo, personal communication), which may justify the description of a new species under morphological, phylogenetic and ecological criteria (the last being the type of habitat occupied). The same might be valid for unit G but morphological distinction remains to be examined.

Implication for evolutionary studies

Genetic diversification in the *Rhinella crucifer* group was associated with Plio-Pleistocene divergences and the earlier studies of the geographic distribution of mtDNA lineages suggested a major role of geographic barriers as promoters of divergence (Thomé et al. 2010). However, this first broadscale account of genetic diversity suffered from low and uneven sampling, and precluded further inferences on the demographic history of the group. Recent studies of Atlantic Forest taxa support a scenario of regional differences in evolutionary forces promoting diversification in this biome (Álvarez–Presas et al. 2011, D'Horta et al. 2011) resulting in patterns that are hard to interpret or generalize for the whole biome (but see Carnaval et al. 2009). Both fine scale sampling and the delimitation of units in this study will allow for the investigation of demographic patterns at regional scales, contributing to a better understanding and hypothesis testing of the microevolutionary processes affecting the *R. crucifer* group. The delimitation of the units also revealed two hybrid zones that may have originated through differentiation under gene flow or from secondary contact after a long period of isolation.

Other possibilities arise from the identification of the two contact zones. Hybridization is common in bufonids (Blair 1972), and has been useful for studying mechanisms of

reproductive isolation and speciation (Malone & Fontenot 2008, Colliard et al. 2010). Natural hybridization in Neotropical toads was shown by Haddad et al. (1990) and suggested by karyotype data (Azevedo et al. 2003), but as of yet, we still do not have a good description of dynamics occurring in a detailed Neotropical hybrid zone. The *Rhinella crucifer* group represents an excellent candidate system for studies of how hybridization works in Neotropical toads with the two putative hybrid zones enabling experiments and hypothesis testing of hybrid survival and reproductive success against genetic divergence.

Conclusions

The existence of five genetic units within the *Rhinella crucifer* species group is supported by mitochondrial and nuclear data, with three units composing the core area of the group and two units located at extremes of the species group's distribution. Evidence for hybridization was found between two pairs of units although cyto–nuclear introgression was restricted to a less diverged pair of genetic units. The concordance between inferred genetic units and previously recognized species was limited and sets more accurate distribution limits for *R. crucifer* and *R. ornata.* Hybridization supports that *R. pombali* is not a valid species, and underscores the need for examining hybridization in the type series of *R. ornata.* The recognition of *R. abei* as a independent unit might rely on more markers, while the morphological distinctiveness of genetic units G and P remains to be tested. Our results provide the basis for future evolutionary studies in the *R. crucifer* species group to contribute to a better understanding of diversification in the biome and of hybridization in Neotropical toads.

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Appendix 1– individuals samples in this study. Columns indicate voucher or tissue number, localities (with codes), sequenced mtDNA fragments, haplotypes, mitochondrial clade, nuclear alleles and individual codes for the STRUCTURE analysis.

				mtDNA			nuclear						
							a.polypep.		crystallin		rhodopsin		STR
number	municipality	state	L	fragments	Hap.	clade	al.1	al.2	al.1	al.2	al.1	al.2	
CFBH-T10831	Guaramiranga	CE	1	control,ND1,ND2	hap1	G	1	2	1	1	1	2	1
CFBH-T10832	Guaramiranga	CE	1	control,ND1,ND2	hap1	G	3	3	1	1	1	2	2
CFBH-T10833	Guaramiranga	CE	1	control,ND1,ND2	hap2	G	1	2	1	1	2	2	3
CFBH-T10834	Guaramiranga	CE	1	control,ND1,ND2	hap1	G	1	2	1	1	2	2	4
CFBH-T10841	Guaramiranga	CE	1	control,ND1,ND2	hap1	G	-	-	-	-	-	-	-
PHV1450	São Desidério	BA	2	ND1,ND2	hap289	P	-	-	150	150	20	20	-
PHV1451	São Desidério	BA	2	ND1,ND2	hap290	P	-	-	150	150	2	24	-
MTJ26	Januária	MG	3	control,ND1,ND2	hap285	P	48	49	150	150	20	11	5
MTJ27	Januária	MG	3	control,ND1,ND2	hap286	P	-	-	150	150	2	24	-
MTJ53	Januária	MG	3	control,ND1,ND2	hap285	P	9	9	150	150	20	11	6
MTJ54	Januária	MG	3	control,ND1,ND2	hap287	P	1	9	150	150	2	24	7
MTJ55	Januária	MG	3	control,ND1,ND2	hap286	P	-	-	150	150	2	24	-
MTJ56	Januária	MG	3	control,ND1,ND2	hap285	P	50	48	-	-	-	-	-
MTJ71	Januária	MG	3	control,ND1,ND2	hap285	P	48	48	150	150	2	24	8
MTJ72	Januária	MG	3	control,ND1,ND2	hap288	P	48	48	150	150	7	7	9
MTJ84	Januária	MG	3	control,ND1,ND2	hap287	P	48	49	150	150	20	20	10
MTJ85	Januária	MG	3	control,ND1,ND2	hap285	P	48	48	150	150	20	20	11
CFBH-T3642	Areia	PB	4	control,ND1,ND2	hap222	N	37	36	106	107	8	8	12

				mtDNA			nuclear						
							a.polypep.		crystallin		rhodopsin		STR
number	municipality	state	L	fragments	Hap.	clade	al.1	al.2	al.1	al.2	al.1	al.2	
CFBH-T3645	Recife	PE	5	control,ND1,ND2	hap223	N	-	-	108	109	11	11	-
CFBH-T3650	Recife	PE	5	control,ND1,ND2	hap223	N	-	-	110	111	2	2	-
CFBH-T3658	Recife	PE	5	control,ND1,ND2	hap223	N	-	-	1	1	2	2	-
CFBH-T3663	Recife	PE	5	control,ND1,ND2	hap223	N	-	-	112	113	2	2	-
CFBH-T9161	Itacaré	BA	6	control,ND1,ND2	hap248	N	5	37	129	129	11	11	13
MNRJ35341	Itacaré	BA	6	control,ND1,ND2	hap273	N	-	-	141	142	11	11	-
MNRJ35342	Itacaré	BA	6	ND1,ND2	hap274	N	37	35	-	-	11	11	-
CFBH-T9233	Aurelino Leal	BA	7	control,ND1,ND2	hap280	N	-	-	117	146	11	11	-
CFBH-T4151	Uruçuca	BA	8	control,ND1,ND2	hap232	N	35	35	118	119	11	21	14
CFBH-T9129	Uruçuca	BA	8	control,ND1,ND2	hap244	N	5	36	-	-	2	2	-
CFBH-T9131	Uruçuca	BA	8	ND1,ND2	hap245	N	42	35	126	105	-	-	-
CFBH-T9132	Uruçuca	BA	8	control,ND1,ND2	hap246	N	37	39	128	5	2	22	15
CFBH-T9140	Uruçuca	BA	8	control,ND1,ND2	hap247	N	35	36	-	-	2	11	-
CFBH-T9141	Uruçuca	BA	8	control,ND1,ND2	hap248	N	42	37	-	-	11	11	-
CFBH-T9147	Uruçuca	BA	8	control,ND1,ND2	hap249	N	5	36	-	-	1	22	-
CFBH-T9176	Uruçuca	BA	8	ND1,ND2	hap245	N	1	35	-	-	11	11	-
CFBH-T9181	Uruçuca	BA	8	control,ND1,ND2	hap249	N	36	39	-	-	11	11	-
CFBH-T3572	Itabuna	BA	9	ND1,ND2	hap214	N	35	36	-	-	11	11	-
CFBH-T3577	Itabuna	BA	9	control,ND1,ND2	hap215	N	-	-	-	-	11	11	-
CFBH-T3578	Itabuna	BA	9	control,ND1,ND2	hap216	N	36	36	-	-	11	11	-
CFBH-T3586	Itabuna	BA	9	ND1	hap217	N							-
CFBH-T3591	Itabuna	BA	9	control,ND1,ND2	hap218	N	36	36	-	-	11	11	-
CFBH-T3596	Itabuna	BA	9	control,ND1,ND2	hap219	N	35	36	-	-	2	11	-
CFBH-T3605	Itabuna	BA	9	control,ND1,ND2	hap220	N	-	-	105	105	11	11	-
CFBH-T3609	Itabuna	BA	9	control,ND1,ND2	hap221	N	5	36	-	-	11	11	-
MRT 5806	Jussari	BA	10	control,ND1,ND2	hap268	N	38	35	-	-	2	11	-
MRT 5975	Jussari	BA	10	control,ND1,ND2	hap267	N	38	36	139	105	11	11	16
MTR 13599	Camacan	BA	11	control,ND1,ND2	hap260	N	29	39	134	135	11	11	17
MTR 13608	Camacan	BA	11	control,ND1,ND2	hap261	N	9	9	114	84	11	11	18
MTR 13609	Camacan	BA	11	control,ND1,ND2	hap262	N	5	9	-	-	2	11	-
MTR 13610	Camacan	BA	11	control,ND1,ND2	hap261	N	9	39	136	137	11	11	19
MTR 13620	Camacan	BA	11	control,ND1,ND2	hap263	N	-	-	-	-	11	11	-
MTR 13621	Camacan	BA	11	control,ND1,ND2	hap264	N	5	29	-	-	-	-	-
MTR16007	Camacan	BA	11	control,ND1,ND2	hap277	N	35	9	144	119	-	-	-
MTR16011	Camacan	BA	11	control,ND1,ND2	hap278	N	38	9	-	-	-	-	-
MTR16045	Camacan	BA	11	control,ND1,ND2	hap260	N	38	35	105	145	-	-	-
CFBH-T12560	Camacan	BA	11	control,ND1,ND2	hap281	N	5	1	114	147	11	11	20
CFBH-T12561	Camacan	BA	11	control,ND1,ND2	hap282	N	5	35	148	121	11	11	21
CFBH-T12564	Camacan	BA	11	control,ND1,ND2	hap283	N	36	36	-	-	11	11	-
CFBH-T12565	Camacan	BA	11	control,ND1,ND2	hap284	N	5	39	117	149	11	11	22
PUCMG2	Sta. Maria do Salto	MG	12	control,ND1,ND2	hap258	N	5	38	-	-	-	-	-
MNRJ38920	Porto Seguro	BA	13	control,ND1,ND2	hap275	N	38	36	143	105	2	11	23
MNRJ38921	Porto Seguro	BA	13	control,ND1,ND2	hap265	N	46	5	131	117	11	11	24
CFBH-T2389	Grão Mogol	MG	14	control,ND1,ND2	hap207	N	1	1	99	81	11	11	25
PUCMG1	Cristália	MG	15	ND1,ND2	hap279	N	1	47	81	140	-	-	-
MTR 13384	Trancoso	BA	16	control,ND1,ND2	hap255	N	38	38	-	-	2	11	-
MTR 13385	Trancoso	BA	16	control,ND1,ND2	hap256	N	5	43	131	117	-	-	-
MTR 13472	Trancoso	BA	16	control,ND1,ND2	hap257	N	5	38	-	-	11	11	-
MTR 13482	Trancoso	BA	16	control,ND1,ND2	hap258	N	29	44	132	133	11	11	26
MTR 13594	Trancoso	BA	16	control,ND1,ND2	hap259	N	5	44	66	99	11	11	27
MTR 13595	Trancoso	BA	16	control,ND1,ND2	hap230	N	-	-	-	-	2	11	-
MTR 13646	Trancoso	BA	16	control,ND1,ND2	hap265	N	29	35	-	-	11	11	-

				mtDNA			nuclear						
							a.polypep.		crystallin		rhodopsin		STR
number	municipality	state	L	fragments	Hap.	clade	al.1	al.2	al.1	al.2	al.1	al.2	
CFBH-T4062	Caraíva	BA	17	control,ND1,ND2	hap224	N	38	38	114	115	2	11	28
CFBH-T4063	Caraíva	BA	17	control,ND1,ND2	hap225	N	5	39	116	115	11	11	29
CFBH-T4064	Caraíva	BA	17	control,ND1,ND2	hap226	N	5	38	99	82	11	11	30
CFBH-T4065	Caraíva	BA	17	control,ND1,ND2	hap227	N	35	9	-	-	11	11	-
CFBH-T4066	Caraíva	BA	17	control,ND1,ND2	hap228	N	5	8	-	-	11	11	-
CFBH-T4067	Caraíva	BA	17	control,ND1,ND2	hap229	N	-	-	-	-	11	11	-
CFBH-T4068	Caraíva	BA	17	control,ND1,ND2	hap224	N	-	-	-	-	11	11	-
CFBH-T4069	Caraíva	BA	17	control,ND1,ND2	hap227	N	5	1	114	117	11	8	31
CFBH-T4070	Caraíva	BA	17	control,ND1,ND2	hap230	N	38	38	-	-	11	11	-
CFBH-T4071	Caraíva	BA	17	control,ND1,ND2	hap231	N	38	5	-	-	11	11	-
CFBH-T7689	Prado	BA	18	control,ND1,ND2	hap239	N	5	9	66	127	2	11	32
AF402	Santa Bárbara	MG	19	control,ND1,ND2	hap266	N	20	9	138	121	11	11	33
CFBH-T7652	Nova Lima	MG	20	control,ND1,ND2	hap238	N	1	27	125	125	2	11	-
CFBH-T7660	Nova Lima	MG	20	control,ND1,ND2	hap94	C	-	-	126	126	11	11	-
CFBH-T7664	Nova Lima	MG	20	control,ND1,ND2	hap237	N	5	5	-	-	2	11	34
JC 794	Mariana	MG	21	control,ND1,ND2	hap153	C	8	9	80	81	11	11	35
CFBH-T7482	Ouro Branco	MG	22	control,ND1,ND2	hap92	C	20	8	65	66	2	11	36
CFBH-T7492	Ouro Branco	MG	22	control,ND1,ND2	hap93	C	-	-	67	66	11	11	-
CFBH-T7532	Ouro Branco	MG	22	control,ND1,ND2	hap94	C	5	8	68	69	11	11	37
MNRJ38327	Catas Altas	MG	23	control,ND1,ND2	hap190	C	29	30	87	87	2	2	39
MNRJ38329	Catas Altas	MG	23	ND1,ND2	hap191	C	31	32	88	89	2	11	38
CFBH-T10842	Belo Horizonte	MG	24	control,ND1,ND2	hap116	C	8	9	-	-	7	8	-
MTR 11543	Santa Marta	MG	25	control,ND1,ND2	hap157	C	-	-	83	83	10	10	-
MTR 11548	Santa Marta	MG	25	control,ND1,ND2	hap158	C	-	-	84	85	11	11	-
MTR15751	Espera Feliz	ES	26	control,ND1,ND2	hap193	C	5	5	90	91	2	11	40
MTR15756	Espera Feliz	ES	26	control,ND1,ND2	hap276	N	-	-	-	-	2	11	-
MTR 11588	Córrego Calçado	ES	27	control,ND1,ND2	hap159	C	-	-	84	84	11	11	-
MRT 1252	Ibitirama	ES	28	control,ND1,ND2	hap141	C	1	1	77	77	2	2	41
MRT 1253	Ibitirama	ES	28	control,ND1,ND2	hap142	C	1	24	-	-	2	11	-
MTR 12585	Ibitirama	ES	28	control,ND1,ND2	hap146	C	24	25	78	79	11	11	42
MTR 12609	Ibitirama	ES	28	control,ND1,ND2	hap141	C	-	-	-	-	2	11	-
MTR 10732	Caparaó	MG	29	control,ND1,ND2	hap269	N	27	8	82	82	23	23	44
MTR 10733	Caparaó	MG	29	control,ND1,ND2	hap156	C	26	27	82	67	11	11	43
MTR15775	Dores do Rio Preto	ES	30	control,ND1,ND2	hap194	C							-
CFBH-T9177	Sooretama	ES	31	control,ND1,ND2	hap291	N	5	35	97	98	11	11	45
CFBH-T5935	Sooretama	ES	31	control,ND1,ND2	hap233	N	1	1	120	121	-	-	-
CFBH-T5936	Sooretama	ES	31	control,ND1,ND2	hap234	N	5	1	122	123	1	2	47
CFBH-T5945	Sooretama	ES	31	control,ND1,ND2	hap235	N	9	9	114	124	1	2	46
CFBH-T10466	Linhares	ES	32	control,ND1,ND2	hap250	N	5	1	-	-	11	11	-
CFBH-T9043	Linhares	ES	32	control,ND1,ND2	hap242	N	-	-	-	-	11	11	-
CFBH-T9046	Linhares	ES	32	control,ND1,ND2	hap243	N	5	5	129	129	11	11	48
MTR 12132	Linhares	ES	32	control,ND1,ND2	hap270	N	5	1	-	-	11	11	-
MTR 12200	Linhares	ES	32	control,ND1,ND2	hap271	N	5	5	140	131	11	11	49
MTR 12248	Linhares	ES	32	control,ND1,ND2	hap272	N	5	9	99	105	11	11	50
CFBH-T3362	Aracruz	ES	33	control,ND1,ND2	hap208	N	5	1	-	-	11	11	-
CFBH-T3367	Aracruz	ES	33	control,ND1,ND2	hap41	C	1	14	43	43	11	8	51
CFBH-T3370	Aracruz	ES	33	control,ND1,ND2	hap209	N	1	9	-	-	11	11	-
CFBH-T3373	Aracruz	ES	33	control,ND1,ND2	hap210	N	24	9	82	101	11	11	52
CFBH-T9008	Santa Leopoldina	ES	34	control,ND1,ND2	hap240	N	5	1	128	5	11	11	53
CFBH-T9009	Santa Leopoldina	ES	34	control,ND1,ND2	hap240	N	40	41	-	-	19	11	-
CFBH-T9014	Santa Leopoldina	ES	34	control,ND1,ND2	hap241	N	5	1	-	-	11	11	-
CFBH-T9080	Santa Leopoldina	ES	34	control,ND1,ND2	hap110	C	-	-	-	-	2	11	-

				mtDNA			nuclear						
							a.polypep.		crystallin		rhodopsin		STR
number	municipality	state	L	fragments	Hap.	clade	al.1	al.2	al.1	al.2	al.1	al.2	
CFBH-T9178	Santa Tereza	ES	35	control,ND1,ND2	hap206	N	5	35	99	100	2	11	54
CFBH-T5953	Santa Tereza	ES	35	control,ND1,ND2	hap236	N	-	-	-	-	1	2	-
MNRJ34956	Santa Tereza	ES	35	control,ND1,ND2	hap236	N	24	45	-	-	2	2	-
CFBH-T3393	Vila Velha	ES	36	control,ND1,ND2	hap206	N	-	-	-	-	2	11	-
CFBH-T3397	Vila Velha	ES	36	control,ND1,ND2	hap211	N	24	9	102	103	11	11	55
CFBH-T3400	Vila Velha	ES	36	control	hap212	N							-
CFBH-T3407	Vila Velha	ES	36	control,ND1,ND2	hap213	N	24	24	104	104	2	11	56
LSH1	Guarapari	ES	37	ND1	hap251	N							-
LSH38	Guarapari	ES	37	control,ND1,ND2	hap252	N	5	1	130	100	-	-	-
LSH39	Guarapari	ES	37	control,ND1,ND2	hap253	N	5	5	95	96	2	2	57
LSH60	Anchieta	ES	38	ND1,ND2	hap254	N							-
MTR 1237	S. José do Calçado	ES	39	control,ND1,ND2	hap140	C	5	1	75	76	2	2	58
CFBH-T9324	Teresópolis	RJ	40	control,ND1,ND2	hap204	C	1	1	-	-	2	20	-
CFBH-T9325	Teresópolis	RJ	40	control,ND1,ND2	hap204	C	1	1	-	-	2	20	-
Tfreitas	Itaipava	RJ	41	control,ND1,ND2	hap203	C	1	34	45	45	2	11	59
CFBH-T4380	Petrópolis	RJ	42	control,ND1,ND2	hap68	C	-	-	54	55	11	13	-
CFBH-T9331	Guapimirim	RJ	43	control,ND1,ND2	hap205	C	1	6	4	94	2	13	60
CFBH-T10355	Seropédica	RJ	44	control,ND1,ND2	hap112	C	1	6	-	-	-	-	-
CFBH-T3377	Itaguaí	RJ	45	control,ND1,ND2	hap44	C	-	-	-	-	11	11	-
CFBH-T3381	Itaguaí	RJ	45	control,ND1,ND2	hap208	N	1	6	-	-	2	2	-
CFBH-T3385	Itaguaí	RJ	45	control,ND1,ND2	hap42	C	6	6	-	-	2	2	-
CFBH-T3387	Itaguaí	RJ	45	control,ND1,ND2	hap43	C	-	-	44	45	2	2	-
CFBH-T3422	Itaguaí	RJ	45	control,ND1,ND2	hap44	C	-	-	45	46	2	2	-
CFBH-T2317	Cristina	MG	46	control,ND1,ND2	hap13	C	5	5	6	20	2	2	61
CFBH-T5086	Cristina	MG	46	control,ND1,ND2	hap72	C	5	5	56	57	-	-	-
CFBH-T5087	Cristina	MG	46	control,ND1,ND2	hap73	C	5	5	58	13	2	-	62
CFBH-T3099	S. R. Passa Quatro	SP	47	control,ND1,ND2	hap21	C	5	5	30	59	-	2	-
CFBH-T7699	Bauru	SP	48	control,ND1,ND2	hap95	C	5	5	-	-	2	12	-
CFBH-T7705	Bauru	SP	48	control,ND1,ND2	hap96	C	5	5	13	13	1	2	63
CFBH-T7712	Bauru	SP	48	control,ND1,ND2	hap95	C	5	10	11	20	-	-	-
CFBH-T7717	Bauru	SP	48	control,ND1,ND2	hap95	C	-	-	-	-	10	12	-
CFBH-T7727	Bauru	SP	48	control,ND1,ND2	hap97	C	5	5	70	11	-	-	-
CFBH-T0867	Sto. Ant. do Pinhal	SP	49	control,ND1,ND2	hap5	C	6	6	6	6	2	2	64
CFBH-T0868	Sto. Ant. do Pinhal	SP	49	control,ND1,ND2	hap5	C	5	6	7	7	2	2	65
CFBH-T1278	S. Luís Paraitinga	SP	50	control,ND1,ND2	hap8	C	5	5	16	17	2	2	66
CFBH-T6526	S. Luís Paraitinga	SP	50	control,ND1,ND2	hap86	C	5	5	22	6	2	2	67
CFBH-T3170	Nat. da Serra	SP	51	control,ND1,ND2	hap22	C	-	-	6	4	2	2	-
CFBH-T3174	Nat. da Serra	SP	51	control,ND1,ND2	hap4	C	5	5	32	25	2	2	68
CFBH-T0336	Ubatuba	SP	52	control,ND1,ND2	hap4	C	5	5	4	5	2	2	69
CFBH-T2588	Ubatuba	SP	52	control,ND1,ND2	hap16	C	5	6	24	25	2	11	70
CFBH-T3628	Ubatuba	SP	52	control,ND1,ND2	hap47	C	14	15	4	5	2	2	71
CFBH-T3633	Ubatuba	SP	52	control,ND1,ND2	hap48	C	6	6	45	22	2	2	72
CFBH-T3638	Ubatuba	SP	52	control,ND1,ND2	hap49	C	6	6	49	49	2	2	73
CFBH-T3659	Ubatuba	SP	52	control,ND1,ND2	hap50	C	6	6	49	49	2	2	74
CFBH-T3803	Caraguatatuba	SP	53	control,ND1,ND2	hap53	C	5	16	22	22	2	2	75
CFBH-T3806	Caraguatatuba	SP	53	control,ND1,ND2	hap13	C	5	6	41	9	2	2	76
CFBH-T3834	Caraguatatuba	SP	53	control,ND1,ND2	hap54	C	5	5	13	28	2	2	77
CFBH-T3836	Caraguatatuba	SP	53	control,ND1,ND2	hap55	C	5	6	22	41	2	2	78
CFBH-T8867	São Sebastião	SP	54	ND1,ND2	hap107	C	5	17	-	-	2	2	-
CFBH-T8890	São Sebastião	SP	54	ND1,ND2	hap108	C	5	6	-	-	1	2	-
CFBH-T8891	São Sebastião	SP	54	control,ND1,ND2	hap109	C	5	5	-	-	2	2	-
CFBH-T8900	São Sebastião	SP	54	control,ND1,ND2	hap109	C	5	6	6	22	2	2	79

				mtDNA			nuclear						
							a.polypep.		crystallin		rhodopsin		STR
number	municipality	state	L	fragments	Hap.	clade	al.1	al.2	al.1	al.2	al.1	al.2	
CFBH-T2664	Ilha Bela	SP	55	control,ND1,ND2	hap17	C	5	6	22	22	2	2	80
CFBH-T4008	Ilha Bela	SP	55	control,ND1,ND2	hap63	C	5	5	-	-	2	2	-
CFBH-T4009	Ilha Bela	SP	55	control,ND1,ND2	hap64	C	5	6	-	-	2	2	-
CFBH-T4010	Ilha Bela	SP	55	control,ND1,ND2	hap65	C	5	5	22	22	2	2	81
CFBH-T6033	Ilha Bela	SP	55	control,ND1,ND2	hap17	C	5	5	22	22	2	2	82
CFBH-T6048	Ilha Bela	SP	55	control,ND1,ND2	hap63	C	5	6	-	-	2	2	-
CFBH-T6053	Ilha Bela	SP	55	control,ND1,ND2	hap85	C	6	6	22	22	2	2	83
CFBH-T6054	Ilha Bela	SP	55	control,ND1,ND2	hap13	C	5	5	22	22	2	2	84
H-466	Bertioga	SP	56	control,ND1,ND2	hap185	C	6	6	5	9	-	-	-
H-478	Bertioga	SP	56	control,ND1,ND2	hap183	C	5	17	-	-	2	2	-
H-485	Bertioga	SP	56	control,ND1,ND2	hap109	C	5	5	-	-	2	2	-
H-488	Bertioga	SP	56	control,ND1,ND2	hap184	C	5	5	6	22	-	-	-
UF94/18	Bertioga	SP	56	control,ND1,ND2	hap109	C	6	6	-	-	2	2	-
UF94/30	Bertioga	SP	56	control,ND1,ND2	hap183	C	5	12	22	22	2	2	85
CFBH-T1956	Cubatão	SP	57	control,ND1,ND2	hap10	C	5	10	6	19	2	2	86
CFBH-T2516	Cubatão	SP	57	control,ND1,ND2	hap14	C	5	5	21	22	10	2	87
CFBH-T2517	Cubatão	SP	57	control,ND1,ND2	hap15	C	5	5	23	22	2	2	88
CFBH-T3491	Cubatão	SP	57	control,ND1,ND2	hap45	C	-	-	47	9	2	2	-
CFBH-T3896	Cubatão	SP	57	control,ND1,ND2	hap57	C	5	5	-	-	2	2	-
CFBH-T3897	Cubatão	SP	57	control,ND1,ND2	hap58	C	5	5	23	41	5	2	89
CFBH-T3910	Cubatão	SP	57	control,ND1,ND2	hap59	C	5	17	-	-	2	2	-
CFBH-T5522	Cubatão	SP	57	control,ND1,ND2	hap75	C	5	1	63	37	2	2	90
AF1430	Santos	SP	58	control,ND1,ND2	hap179	C	5	1	22	22	2	2	93
AF1435	Santos	SP	58	control,ND1,ND2	hap180	C	5	17	-	-	2	2	-
AF1443	Santos	SP	58	control,ND1,ND2	hap181	C	5	5	6	22	12	19	94
CFBH-T3953	Santos	SP	58	control,ND1,ND2	hap61	C	5	18	22	41	2	2	91
CFBH-T3954	Santos	SP	58	control,ND1,ND2	hap62	C	5	5	41	22	2	2	92
MTR 16675	Santo André	SP	59	control,ND1,ND2	hap13	C	5	5	46	22	2	2	95
MTR10400	Santo André	SP	59	control,ND1,ND2	hap154	C	5	10	35	22	-	-	-
MTR10402	Santo André	SP	59	control,ND1,ND2	hap155	C	5	5	-	-	2	2	-
IT-H0023	São Bernardo	SP	60	control,ND1,ND2	hap163	C	10	10	22	22	2	2	96
IT-H0025	São Bernardo	SP	60	control,ND1,ND2	hap164	C	5	10	22	22	2	2	97
CFBH-T10514	Itanhaém	SP	61	control,ND1,ND2	hap113	C	5	5	11	12	2	2	100
CFBH-T3856	Itanhaém	SP	61	control,ND1,ND2	hap56	C	5	5	52	53	2	2	98
CFBH-T3912	Itanhaém	SP	61	control,ND1,ND2	hap60	C	5	10	20	35	2	2	99
CFBH-T5869	Peruíbe	SP	62	control,ND1,ND2	hap83	C							-
CFBH-T5870	Peruíbe	SP	62	control,ND1,ND2	hap83	C	5	5	26	29	2	2	101
CFBH-T5871	Peruíbe	SP	62	control,ND1,ND2	hap84	C	4	5	28	8	2	2	102
AF122	Iguape	SP	63	control,ND1,ND2	hap143	C	4	16	-	-	2	2	-
AF147	Iguape	SP	63	control,ND1,ND2	hap144	C	5	5	-	-	2	2	-
AF151	Iguape	SP	63	control,ND1,ND2	hap145	C	5	5	11	11	2	2	106
AF160	Iguape	SP	63	control,ND1,ND2	hap147	C	5	16	9	9	2	2	107
AF169	Iguape	SP	63	control,ND1,ND2	hap90	C	5	5	9	11	-	-	-
CFBH-T2246	Iguape	SP	63	control,ND1,ND2	hap11	C	5	5	-	-	9	9	-
CFBH-T2248	Iguape	SP	63	control,ND1,ND2	hap12	C	5	5	-	-	2	2	-
CFBH-T4031	Iguape	SP	63	control,ND1,ND2	hap66	C	5	5	8	20	2	2	103
CFBH-T4032	Iguape	SP	63	control,ND1,ND2	hap67	C	5	1	20	11	2	2	104
CFBH-T5144	Iguape	SP	63	control,ND1,ND2	hap74	C	1	16	60	61	2	2	105
CFBH-T6957	Iguape	SP	63	control,ND1,ND2	hap88	C	19	19	-	-	2	2	-
CFBH-T6976	Iguape	SP	63	control,ND1,ND2	hap89	C	5	5	-	-	2	2	-
CFBH-T7034	Iguape	SP	63	control,ND1,ND2	hap90	C	5	5	9	27	-	-	-
CFBH-T6952	Pariquera Açú	SP	64	control,ND1,ND2	hap87	C	10	16	-	-	2	11	-

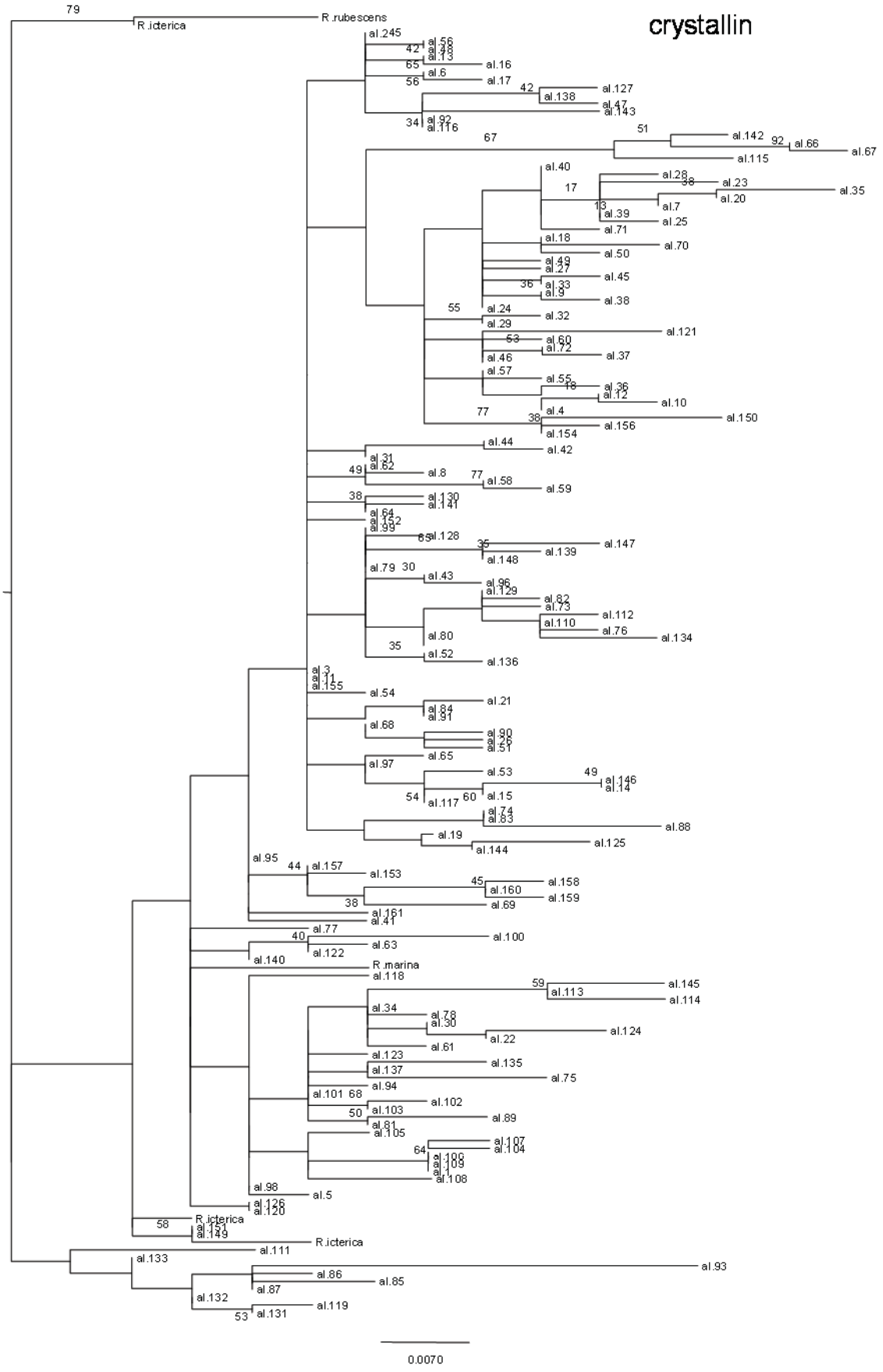
				mtDNA			nuclear						
							a.polypep.		crystallin		rhodopsin		STR
number	municipality	state	L	fragments	Hap.	clade	al.1	al.2	al.1	al.2	al.1	al.2	
CFBH-T7322	Ilha Comprida	SP	65	control,ND1,ND2	hap91	C	5	5	13	13	2	2	108
CFBH-T10697	Cananéia	SP	66	control,ND1,ND2	hap103	C	5	5	13	13	2	2	111
CFBH-T8433	Cananéia	SP	66	control,ND1,ND2	hap103	C	5	5	-	-	2	2	-
CFBH-T8434	Cananéia	SP	66	control,ND1,ND2	hap104	C	5	5	13	11	1	2	109
CFBH-T8547	Cananéia	SP	66	control,ND1,ND2	hap105	C	5	23	13	13	2	2	110
CFBH-T8548	Cananéia	SP	66	control,ND1,ND2	hap104	C	5	5	-	-	2	2	-
CFBH-T8683	Cananéia	SP	66	control,ND1,ND2	hap106	C	5	5	-	-	2	2	-
CFBH-T5155	Ilha do Cardoso	SP	67	control,ND1,ND2	hap32	C	5	5	62	22	2	2	112
AF403	São Paulo	SP	68	control	hap149	C	5	10	20	22	2	2	113
MTR 9957	São Paulo	SP	68	control,ND1,ND2	hap113	C	5	5	41	22	2	2	115
MTR 9958	São Paulo	SP	68	control,ND1,ND2	hap188	C	5	5	-	-	2	2	-
MTR 9959	São Paulo	SP	68	control,ND1,ND2	hap189	C	5	5	41	41	2	2	116
MTR 9960	São Paulo	SP	68	control,ND1,ND2	hap5	C	4	5	-	-	2	2	-
MTR 9963	São Paulo	SP	68	control,ND1,ND2	hap182	C	5	5	6	11	2	2	114
CFBH-T3108	Cotia	SP	69	control,ND1,ND2	hap13	C	5	5	28	32	-	-	-
CFBH-T8274	Biritiba Mirim	SP	70	control,ND1,ND2	hap29	C	5	5	6	9	-	-	-
CFBH-T8275	Biritiba Mirim	SP	70	control,ND1,ND2	hap101	C	17	17	14	26	2	2	117
CFBH-T8276	Biritiba Mirim	SP	70	control,ND1,ND2	hap102	C	5	5	-	-	-	-	-
Uniban2555	Biritiba Mirim	SP	70	control,ND1,ND2	hap5	C	28	17	6	6	2	11	118
CFBH-T3180	Santa Isabel	SP	71	control,ND1,ND2	hap5	C	5	6	33	22	2	2	119
CFBH-T3184	Santa Isabel	SP	71	control,ND1,ND2	hap23	C	5	5	22	4	2	2	120
CFBH-T3189	Santa Isabel	SP	71	control,ND1,ND2	hap24	C	5	5	6	6	-	-	-
CFBH-T3190	Santa Isabel	SP	71	control,ND1,ND2	hap24	C	5	5	20	6	12	6	124
CFBH-T3194	Santa Isabel	SP	71	control,ND1,ND2	hap25	C	5	11	4	22	-	-	-
CFBH-T3195	Santa Isabel	SP	71	control,ND1,ND2	hap26	C	5	5	34	22	2	2	121
CFBH-T3202	Santa Isabel	SP	71	control,ND1,ND2	hap27	C	-	-	35	22	2	2	-
CFBH-T3204	Santa Isabel	SP	71	control,ND1,ND2	hap28	C	12	11	36	37	-	-	-
CFBH-T3206	Santa Isabel	SP	71	control,ND1,ND2	hap29	C	6	6	6	6	1	2	122
CFBH-T4426	Santa Isabel	SP	71	control,ND1,ND2	hap69	C	5	5	35	9	14	2	123
CFBH-T4427	Santa Isabel	SP	71	control,ND1,ND2	hap70	C	5	5	41	11	-	-	-
MTR15559	Iperó	SP	72	ND1,ND2	hap192	C	5	5	-	-	12	12	-
IT-H0437	Juquitiba	SP	73	control,ND1,ND2	hap167	C	5	5	-	-	12	6	-
IT-H0438	Juquitiba	SP	73	control,ND1,ND2	hap168	C	5	10	22	11	2	18	125
IT-H0439	Juquitiba	SP	73	control,ND1,ND2	hap45	C	5	10	-	-	-	-	-
IT-H0459	Juquitiba	SP	73	control,ND1,ND2	hap169	C	5	5	-	-	2	2	-
IT-H0461	Juquitiba	SP	73	control,ND1,ND2	hap66	C	17	10	20	11	-	-	-
IT-H0462	Juquitiba	SP	73	control,ND1,ND2	hap113	C	5	5	35	35	1	2	127
IT-H0463	Juquitiba	SP	73	control,ND1,ND2	hap45	C	5	5	6	41	12	12	128
IT-H0468	Juquitiba	SP	73	control,ND1,ND2	hap170	C	5	5	9	11	2	2	126
IT-H0538	Juquitiba	SP	73	ND1,ND2	hap171	C	5	5	-	-	-	-	-
AF303	Carapicuíba	SP	74	control,ND1,ND2	hap45	C	5	5	9	11	6	6	129
AF304	Carapicuíba	SP	74	control,ND1,ND2	hap5	C	5	5	-	-	-	-	-
AF316	Carapicuíba	SP	74	control,ND2	hap148	C	5	5	20	20	-	-	-
AF423	Carapicuíba	SP	74	control,ND2	hap150	C	5	17	34	41	-	-	-
ALC86/77	Piedade	SP	75	control,ND1,ND2	hap5	C	5	5	28	28	1	12	130
ALC86/80	Piedade	SP	75	control,ND1,ND2	hap113	C	5	17	-	-	2	2	-
ALC86/81	Piedade	SP	75	control,ND1,ND2	hap113	C	5	5	41	41	12	12	131
ALC87/1	Piedade	SP	75	control,ND1,ND2	hap160	C	5	5	28	28	10	2	132
ALC87/3	Piedade	SP	75	control,ND1,ND2	hap161	C	5	5	-	-	-	-	-
ALC87/5	Piedade	SP	75	control,ND1,ND2	hap162	C	5	5	-	-	2	2	-
H023	Piedade	SP	75	control,ND1,ND2	hap113	C	5	5	64	20	12	6	135
H035	Piedade	SP	75	control,ND1,ND2	hap186	C	5	17	41	22	-	-	-

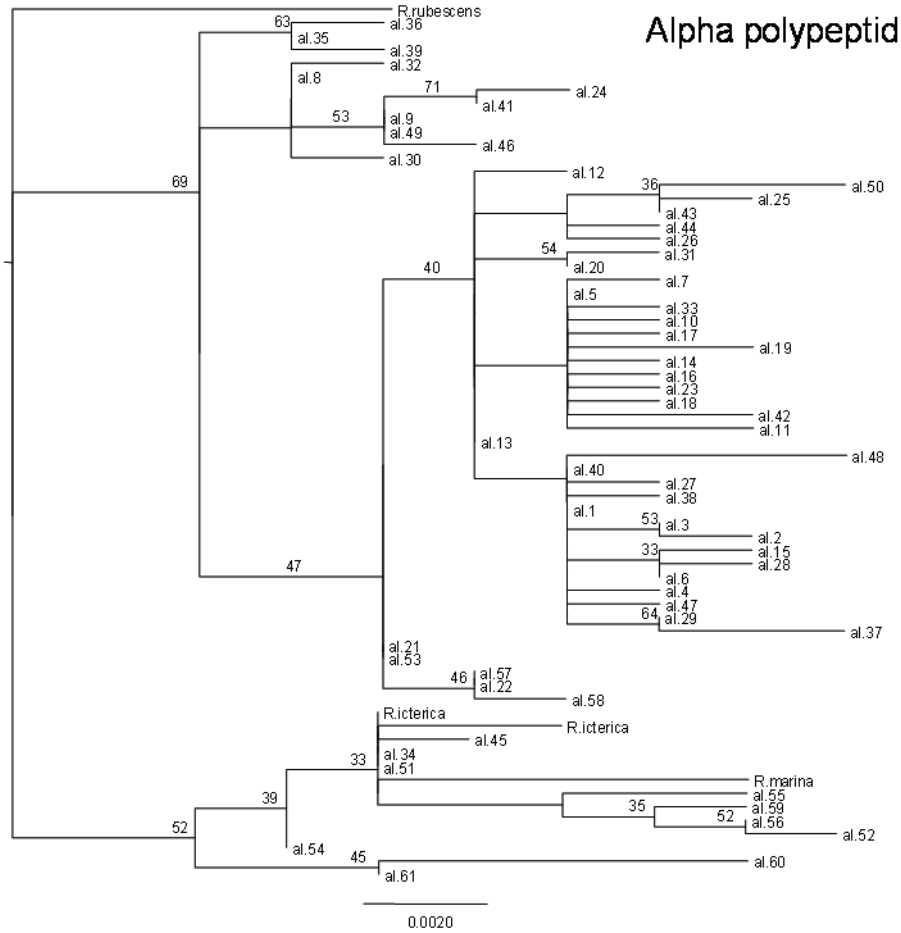
				mtDNA			nuclear						
							a.polypep.		crystallin		rhodopsin		STR
number	municipality	state	L	fragments	Hap.	clade	al.1	al.2	al.1	al.2	al.1	al.2	
IT-H0207	Piedade	SP	75	control,ND1,ND2	hap165	C	5	5	6	41	12	12	133
IT-H0208	Piedade	SP	75	control,ND1,ND2	hap166	C	5	5	-	-	2	2	-
IT-H0209	Piedade	SP	75	control,ND1,ND2	hap66	C	17	10	20	11	-	-	-
UF88/60	Piedade	SP	75	control,ND1,ND2	hap113	C	5	5	64	20	12	6	134
H0300	Buri	SP	76	control,ND1,ND2	hap174	C	5	5	-	-	10	1	-
H0355	Buri	SP	76	control,ND1,ND2	hap151	C	5	5	20	29	1	1	141
H0356	Buri	SP	76	control,ND1,ND2	hap187	C	5	5	10	11	1	1	140
H0366	Buri	SP	76	control,ND1,ND2	hap152	C	5	5	20	11	10	1	139
IT-H0573	Buri	SP	76	control,ND1,ND2	hap173	C	5	5	29	29	1	12	137
IT-H0580	Buri	SP	76	control,ND1,ND2	hap80	C	5	10	-	-	10	10	-
IT-H0581	Buri	SP	76	control,ND1,ND2	hap174	C	5	5	-	-	10	1	-
IT-H0583	Buri	SP	76	control,ND1,ND2	hap175	C	10	10	10	26	1	1	138
IT-H0610	Buri	SP	76	control,ND1,ND2	hap151	C	5	10	9	11	-	-	-
IT-H0612	Buri	SP	76	control,ND1,ND2	hap152	C	5	5	26	27	12	12	136
IT-H0613	Buri	SP	76	control,ND1,ND2	hap151	C	5	5	-	-	1	1	-
CFBH-T5679	Ribeirão Grande	SP	77	control,ND1,ND2	hap76	C	5	5	11	29	10	1	142
CFBH-T5680	Ribeirão Grande	SP	77	control,ND1,ND2	hap77	C	5	5	64	9	1	12	143
CFBH-T5681	Ribeirão Grande	SP	77	control,ND1,ND2	hap78	C	5	10	28	11	12	12	144
CFBH-T5682	Ribeirão Grande	SP	77	control,ND1,ND2	hap79	C	5	5	-	-	10	12	-
CFBH-T5683	Ribeirão Grande	SP	77	control,ND1,ND2	hap80	C	5	5	-	-	1	12	-
CFBH-T5684	Ribeirão Grande	SP	77	control,ND1,ND2	hap81	C	5	10	-	-	1	12	-
CFBH-T5685	Ribeirão Grande	SP	77	control,ND1,ND2	hap82	C	5	5	26	26	1	12	145
CFBH-T5686	Ribeirão Grande	SP	77	control,ND1,ND2	hap79	C	5	10	-	-	2	12	-
CFBH-T5687	Ribeirão Grande	SP	77	control,ND1,ND2	hap80	C	5	5	29	9	1	2	146
CFBH-T5688	Ribeirão Grande	SP	77	control,ND1,ND2	hap80	C	5	5	-	-	1	12	-
CFBH-T5041	Guapiara	SP	78	control,ND1,ND2	hap71	C	5	10	40	11	1	12	147
CFBH-T1034	Ribeirão Branco	SP	79	control,ND1,ND2	hap6	C	4	5	10	10	-	-	-
CFBH-T1035	Ribeirão Branco	SP	79	control,ND1,ND2	hap7	C	5	5	9	10	1	2	148
IIH-149	Wenceslau Brás	PR	80	control,ND1,ND2	hap176	C	5	1	10	26	12	12	149
IIH-172	Wenceslau Brás	PR	80	control,ND1,ND2	hap177	C	5	5	10	26	10	12	150
IIH-224	Wenceslau Brás	PR	80	control,ND1,ND2	hap178	C	5	5	10	10	10	12	151
II - H046	Pinhalão	PR	81	control,ND1,ND2	hap172	C	5	1	10	26	10	12	152
II - H114	Ortigueira	PR	82	control,ND1,ND2	hap151	C	5	5	86	13	-	-	-
CFBH-T11188	Ortigueira	PR	82	control,ND1,ND2	hap121	C	5	1	9	9	12	12	153
CFBH-T9476	Teodoro Sampaio	SP	83	control,ND1,ND2	hap199	C	5	1	10	26	12	12	154
CFBH-T7881	Teodoro Sampaio	SP	83	control,ND1,ND2	hap200	C	5	1	93	26	10	12	155
CFBH-T7897	Teodoro Sampaio	SP	83	control,ND1,ND2	hap201	C	5	5	10	26	10	12	156
CFBH-T7898	Teodoro Sampaio	SP	83	control,ND1,ND2	hap201	C	5	1	-	-	10	12	-
CFBH-T9512	Teodoro Sampaio	SP	83	control,ND1,ND2	hap202	C	1	1	10	26	10	10	157
CFBH-T7899	Teodoro Sampaio	SP	83	control,ND1,ND2	hap202	C	5	1	-	-	10	10	-
CFBH-T9513	Teodoro Sampaio	SP	83	control,ND1,ND2	hap200	C	5	5	-	-	10	12	-
CFBH-T9514	Teodoro Sampaio	SP	83	control,ND1,ND2	hap200	C	5	5	10	26	10	12	158
IIBPH1333	Canindeyú	PAR	84	control,ND1,ND2	hap119	C	5	1	10	9	10	12	159
IIBPH1173	Itapúa	PAR	85	control,ND1,ND2	hap117	C	5	5	9	9	12	12	160
IIBPH1191	Itapúa	PAR	85	control,ND1,ND2	hap118	C	5	5	9	9	10	12	161
IIBPH1336	Itapúa	PAR	85	ND1	hap120	C	-	-	9	9	10	12	-
IIBPH1342	Itapúa	PAR	85	control,ND1,ND2	hap121	C	5	1	9	9	12	12	162
IIBPH1353	Itapúa	PAR	85	control,ND1,ND2	hap113	C	5	5	20	9	10	12	163
IIBPH1356	Itapúa	PAR	85	control,ND1,ND2	hap113	C	5	5	9	9	10	10	164
MVS243	Adrianópolis	PR	86	control,ND1,ND2	hap196	c1	4	5	28	13	-	-	-
MVS244	Adrianópolis	PR	86	control,ND1,ND2	hap197	C	33	33	92	26	10	12	165
MVS242	Ribeira	SP	87	control,ND1,ND2	hap195	C	4	5	28	13	-	-	-

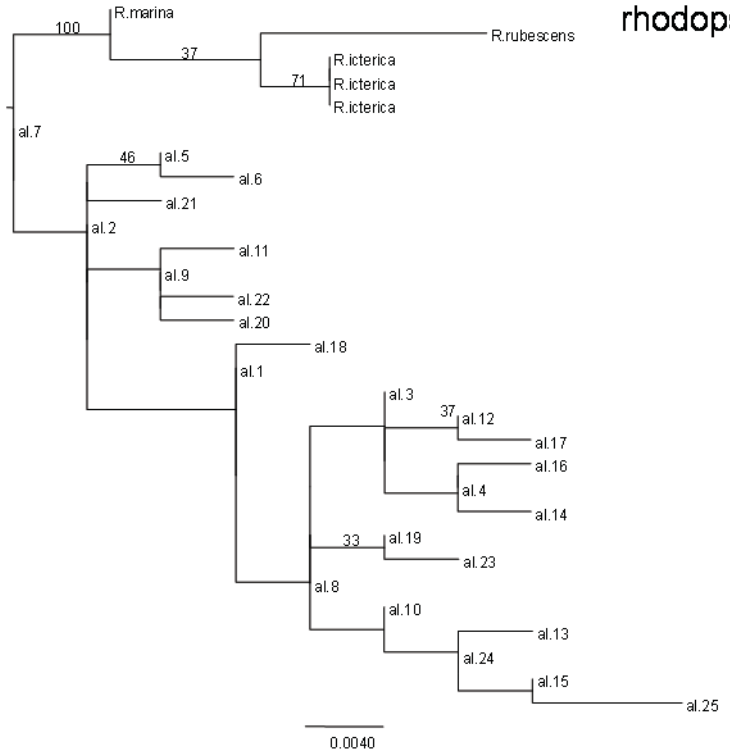
				mtDNA			nuclear						
							a.polypep.		crystallin		rhodopsin		STR
number	municipality	state	L	fragments	Hap.	clade	al.1	al.2	al.1	al.2	al.1	al.2	
CFBH-T3619	Quatro Barras	PR	88	control,ND1,ND2	hap46	c1	-	-	48	11	5	6	-
CFBH-T9193	Quatro Barras	PR	88	control,ND1,ND2	hap124	c1	4	4	41	41	6	17	166
CFBH-T12598	Antonina	PR	89	control,ND1,ND2	hap3	c1	4	5	-	-	1	2	-
CFBH-T3084	Antonina	PR	89	control,ND1,ND2	hap18	C	5	5	26	27	-	-	-
CFBH-T3085	Antonina	PR	89	control,ND1,ND2	hap19	c1	4	7	28	26	10	1	167
CFBH-T3086	Antonina	PR	89	control,ND1,ND2	hap20	C	-	-	29	27	1	12	-
CFBH-T3304	Morretes	PR	90	control,ND1,ND2	hap30	c1	5	7	11	38	-	-	-
CFBH-T3306	Morretes	PR	90	control,ND1,ND2	hap31	c1	4	5	11	9	2	2	168
CFBH-T3310	Morretes	PR	90	control,ND1,ND2	hap32	C	5	1	27	11	1	12	169
CFBH-T3312	Morretes	PR	90	control,ND1,ND2	hap33	c1							-
K970	São José dos Pinhais	PR	91	control,ND1,ND2	hap123	c1	5	1	-	-	10	2	-
CFBH-T10261	Guaratuba	PR	92	control,ND1,ND2	hap111	c1	4	7	8	9	-	-	-
CFBH-T3665	Guaratuba	PR	92	control,ND1,ND2	hap51	c1	4	5	8	11	-	-	-
CFBH-T3666	Guaratuba	PR	92	control,ND1,ND2	hap51	c1	4	5	8	11	-	-	-
CFBH-T3672	Guaratuba	PR	92	control,ND1,ND2	hap52	c1	-	-	50	51	-	-	-
CFBH-T3679	Guaratuba	PR	92	control,ND1,ND2	hap33	c1	5	7	22	11	-	-	-
CFBH-T3314	Itapoá	SC	93	control,ND1,ND2	hap34	c1	5	5	27	26	1	2	170
CFBH-T9201	Massaranduba	SC	94	control,ND1,ND2	hap131	c1	5	7	9	11	-	-	-
CFBH-T9202	Massaranduba	SC	94	control,ND1,ND2	hap132	c1	4	1	-	-	1	5	-
CFBH-T9202	Massaranduba	SC	94	control,ND1,ND2	hap133	c1	4	5	9	9	-	-	-
CFBH-T9204	Massaranduba	SC	94	control,ND1,ND2	hap134	c1	7	7	74	9	1	1	171
CFBH-T9205	Massaranduba	SC	94	control,ND1,ND2	hap135	c1	1	7	73	9	5	6	172
CFBH-T9206	Massaranduba	SC	94	control,ND1,ND2	hap136	c1	4	4	38	41	10	12	173
CFBH-T9207	Massaranduba	SC	94	control,ND1,ND2	hap134	c1	7	7	-	-	5	5	-
CFBH-T9208	Massaranduba	SC	94	control,ND1,ND2	hap136	c1	1	7	9	11	-	-	-
CFBH-T9209	Massaranduba	SC	94	control,ND1,ND2	hap137	c1	4	1	26	73	12	6	174
CFBH-T9210	Massaranduba	SC	94	control,ND1,ND2	hap138	c1	7	7	-	-	5	5	-
CFBH-T9211	Massaranduba	SC	94	control,ND1,ND2	hap139	c1	7	7	15	9	5	5	175
CFBH-T3316	Rio dos Cedros	SC	95	control,ND1,ND2	hap35	c1	-	-	39	11	1	1	-
CFBH-T3320	Rio dos Cedros	SC	95	control,ND1,ND2	hap36	c1	-	-	40	41	1	5	-
CFBH-T3323	Rio dos Cedros	SC	95	control,ND1,ND2	hap37	c1	4	4	42	11	1	12	176
CFBH-T3343	Blumenau	SC	96	control,ND1,ND2	hap38	c1	-	-	14	9	-	-	-
CFBH-T3348	Blumenau	SC	96	control,ND1,ND2	hap39	c1	4	4	11	15	-	-	-
CFBH-T3351	Blumenau	SC	96	control,ND1,ND2	hap40	c1	13	7	15	9	1	5	177
CFBH-T9194	Blumenau	SC	96	control,ND1,ND2	hap125	c1	4	7	15	15	6	6	178
CFBH-T9195	Blumenau	SC	96	control,ND1,ND2	hap126	c1	4	1	72	11	1	1	179
CFBH-T9196	Blumenau	SC	96	control,ND1,ND2	hap126	c1	4	7	15	9	6	6	180
CFBH-T9197	Blumenau	SC	96	control,ND1,ND2	hap127	c1	4	1	73	9	-	-	-
CFBH-T9198	Blumenau	SC	96	control,ND1,ND2	hap128	c1	7	7	15	73	10	5	181
CFBH-T9199	Blumenau	SC	96	control,ND1,ND2	hap129	c1	1	7	9	72	1	1	182
CFBH-T9200	Blumenau	SC	96	control,ND1,ND2	hap130	c1	4	7	26	41	-	-	-
CFBH-T10731	Gov. Celso Ramos	SC	97	control,ND1,ND2	hap114	c1	4	1	11	14	5	6	183
CFBH-T10732	Gov. Celso Ramos	SC	97	control,ND1,ND2	hap115	c1	1	1	11	15	1	2	184
CFBH-T1735	Itapema	SC	98	control,ND1,ND2	hap9	c1	4	4	18	11	-	-	-
PUCRS	S.. Am. Imperatriz	SC	99	control,ND1,ND2	hap198	c1	4	1	26	11	12	6	185
CFBH-T8092	Anitápolis	SC	100	control,ND1,ND2	hap98	c1	21	22	-	-	3	15	-
CFBH-T8093	Anitápolis	SC	100	control,ND1,ND2	hap295	S							-
CFBH-T8093	Anitápolis	SC	100	control,ND1,ND2	hap100	c1	-	-	154	155	3	25	-
CFBH-T8094	Anitápolis	SC	100	control,ND1	hap99	c1	1	1	11	11	-	-	-
CFBH-T8096	Anitápolis	SC	100	control,ND1,ND2	hap100	c1	22	22	155	155	25	16	-
CFBH-T8097	Anitápolis	SC	100	control,ND1,ND2	hap100	c1	1	1	-	-	6	16	-
CFBH-T8098	Anitápolis	SC	100	control,ND1,ND2	hap296	S	1	1	71	11	-	-	193

				mtDNA			nuclear						
							a.polypep.		crystallin		rhodopsin		STR
number	municipality	state	L	fragments	Hap.	clade	al.1	al.2	al.1	al.2	al.1	al.2	
CFBH-T8099	Anitápolis	SC	100	control,ND1,ND2	hap297	S	-	-	155	155	3	3	-
ELMG453	Ponte Serrada	SC	101	control,ND1,ND2	hap299	S							-
CFBH-T11195	Ponte Serrada	SC	101	control,ND1,ND2	hap122	C	5	5	28	28	12	12	186
CFBH-T11196	Fernandes Pinheiro	PR	102	control,ND1,ND2	hap292	S	21	22	159	160	3	3	187
MVS398	Salete	SC	103	ND1,ND2	hap304	S	-	-	-	-	10	5	-
CFBH-T10793	Xavantina	SC	104	control,ND1,ND2	hap293	S	51	52	151	151	1	2	189
CFBH-T10794	Xavantina	SC	104	control,ND1,ND2	hap294	S	53	54	152	153	2	2	190
CFBH-T9184	Xavantina	SC	104	control,ND1,ND2	hap299	S	-	-	156	156	3	15	-
CFBH-T9186	Xavantina	SC	104	control,ND1,ND2	hap299	S	53	56	156	156	3	4	188
CFBH-T9188	Xavantina	SC	104	control,ND1,ND2	hap299	S	-	-	155	156	3	25	-
EMLG234	Guatambú	SC	105	control,ND1,ND2	hap299	S	57	56	156	155	-	-	-
EMLG235	Guatambú	SC	105	control,ND1,ND2	hap299	S	21	22	157	5	-	-	-
EMLG236	Guatambú	SC	105	control,ND1,ND2	hap300	S	-	-	158	5	-	-	-
EMLG237	Guatambú	SC	105	control,ND1,ND2	hap299	S							-
EMLG253	Guatambú	SC	105	control,ND1,ND2	hap299	S	58	59	156	156	25	25	191
EMLG453	Guatambú	SC	105	-	-	-	21	22	-	-	3	25	-
flona062007	Guatambú	SC	105	control,ND1,ND2	hap292	S	-	-	159	160	3	3	-
flona1603	Guatambú	SC	105	control,ND1,ND2	hap292	S	21	21	156	156	3	25	192
MNRJ33006	Mato Castelhano	RS	106	control,ND1,ND2	hap302	S	13	22	162	163	3	25	194
MNRJ33012	Mato Castelhano	RS	106	control,ND1,ND2	hap303	S	61	22	163	163	25	25	195
CFBH-T8298	Cotiporã	RS	107	control,ND1,ND2	hap298	S	55	55	-	-	25	25	-
UFRGST667	Nova Roma do Sul	RS	108	control,ND1,ND2	hap305	S	-	-	163	163	25	25	-
CFBH-T9213	Bento Gonçalves	RS	109	control,ND1,ND2	hap301	S	21	60	155	161	3	15	196
	outgroups												
CFBH-T1338	*R. arenarum*			control,ND1,ND2									
CFBH-T3051	*R. icterica*			control,ND1,ND2									
CFBH-T3354	*R. icterica*			control,ND1									
CFBH-T3361	*R. granulosa*			control,ND1									
CFBH-T5092	*R. merianae*			control,ND1,ND2									
CFBH-T5731	*R.merianae*			control,ND1,ND2									
CFBH-T8885	*R. icterica*			control,ND1									
CFBH-T8933	*R. icterica*			ND1									

Appendix 2– nuclear gene genealogies. Numbers before clades indicate support values. (values under 30 are not shown).


crystallin
R.rubescens
R.icterica
R.marina
0.0070


Alpha polypeptide
R.rubescens
al.36
al.35
al.39
al.32
al.8
al.24
al.41
al.9
al.49
al.46
al.30
al.12
al.50
al.25
al.43
al.44
al.26
al.31
al.20
al.7
al.5
al.33
al.10
al.17
al.19
al.14
al.16
al.23
al.18
al.42
al.11
al.13
al.48
al.40
al.27
al.38
al.1
al.3
al.2
al.15
al.28
al.6
al.4
al.47
al.29
al.37
al.21
al.53
al.57
al.22
al.58
R.icterica
R.icterica
al.45
al.34
al.51
R.marina
al.55
al.59
al.56
al.52
al.54
al.60
al.61
0.0020


rhodopsin
R.marina
R.rubescens
R.icterica
R.icterica
R.icterica
al.7
al.5
al.6
al.21
al.2
al.11
al.9
al.22
al.20
al.18
al.1
al.3
al.12
al.17
al.16
al.4
al.14
al.19
al.23
al.8
al.10
al.13
al.24
al.15
al.25
0.0040

CAPÍTULO 3

Phylogeography of the *Rhinella crucifer* group: using model-based approaches to test diversification of endemic toads in the Brazilian Atlantic Forest

Phylogeography of the *Rhinella crucifer* group: using model-based approaches to test diversification of endemic toads in the Brazilian Atlantic Forest

Maria Tereza Chiarioni Thomé, Kelly Raquel Zamudio, Célio Fernando Baptista Haddad, João Alexandrino

ABSTRACT

We here investigate diversification in the *Rhinella crucifer* species group, a widely distributed group of closely related toads endemic to the Brazilian Atlantic Forest biome. We combine nuclear and mitochondrial sequence data collected at fine scale with both traditional and model-based approaches to specifically test previously published hypotheses and alternative population diversification scenarios. We found no signature of higher levels of genetic diversity in putative refuge areas according to three different refuge scenarios. The demographic history of the *Rhinella crucifer* group supported regional differences within the biome with moderate demographic fluctuations in the northern region, more prominent changes in the central region and stability in southern Atlantic Forests. Tests of concurrent demographic scenarios using coalescent simulations allowed us to reject hypotheses of refugial vicariance associated to extensive demographic expansions, confirming the idea that large scale habitat disruption has not determined the diversification in this group. Coalescent simulations supported instead a joint effect of geographic barriers and modest demographic expansions. The available data on the demographic dynamics of Atlantic Forest taxa make global refuge hypotheses based on massive forest fragmentation unlikely for this biome. Rather, geographic discontinuities across complex landscapes appear equally or more importantly associated with diversification, although it remains unclear the detailed spatio-temporal mechanics of putative barriers. Our data provide further support to the idea of idiosyncratic mechanisms interacting to promote diversity in the Atlantic Forests.

1. Introduction

The understanding of evolutionary processes in the Neotropics is a challenging science as many species remain undiscovered and only a fraction has been studied using molecular tools. The Brazilian Atlantic Forest (AF) is no exception to this rule; with a original distribution that follows the eastern coast line of South America for about 30 latitudinal degrees, it harbors a highly endemic but poorly known biota (Myers et al. 2000, Tabareli et al. 2003). Human pressure over the last 500 years has severely fragmented the AF and reduced this continental distribution to only 7–16% (Morellato & Haddad 2000, Ribeiro et al. 2009), stressing the need for conservation of its biodiversity and underlying processes.

The number of evolutionary studies on AF taxa has increased over the past ten years, allowing for progress towards understanding evolutionary mechanisms that promote genetic diversity throughout the biome (Carnaval 2002, Leite, 2003, Pellegrino et al. 2005, Grazziotin et al. 2006, Moraes–Barros et al. 2006, Francisco et al. 2007, Cabanne et al. 2008, Lara–Ruiz et al. 2008, Fitzpatrick et al. 2009, Mata et al. 2009, Carnaval et al. 2009, Brunes et al. 2010, Colombi et al. 2010, Rezende et al. 2010, Thomé et al. 2010, Álvarez-Presas et al. 2011, Horta et al. 2011, Pavan et al. 2011). Vicariance is mostly invoked to explain lineage or species diversification but the relative contribution of allopatric isolation via geographic barriers versus isolation due to habitat changes during Pleistocene climatic cycles (e.g. Pleistocene Refuges Hypothesis, Haffer 1969) remains a controversial issue.

Studies across AF species distributions show recurrent patterns associated to geographic barriers (Lynch 1979, Silva et al. 2004, Pinto-da-Rocha et al. 2005, Sigrist & Carvalho, 2008) such as the Doce river. Although this area coincides with phylogeographic breaks for many organisms (Pellegrino et al. 2005, Cabanne et al. 2007, Lara-Ruiz et al. 2008, Brunes et al. 2010, Resende et al. 2010), the timing of divergence varies across taxa raising doubts on the role of the Doce river as a promoter of vicariance or as a barrier associated to secondary contact (Cabanne et al. 2008, Colombi et al. 2010). Other examples of barriers associated with species distributions limits and/or genetic breaks include the Mucuri river (Resende et al. 2010), Paraíba do Sul (Silva & Straube 1996, Pellegrino et al. 2005, Grazziotim et al. 2006, Cabanne et al. 2007, 2008, Sigrist & Carvalho, 2008), Paranapanema (Grazziotin et al. 2006) and Ribeira do Iguape (Marcelino et al. 2009) river valleys, among

others. These putative barriers remain to be investigated in a rigorous comparative phylogeography framework to elucidate their role in generating these generalized patterns.

On the other hand, a hypothesis of AF refuges inferred by palaeomodeling of species distributions (Carnaval & Moritz 2008) was formally tested for three widespread amphibian congeners using a model–based approach (Carnaval et al. 2009). The authors corroborated predictions that stable areas (refuges) were restricted to the northern AF region while severe forest contractions produced unstable habitats at higher latitudes (Carnaval & Moritz 2008, Carnaval et al. 2009). This hypothesis has gained some support from a number of phylogeographic studies (Moraes–Barros et al. 2006, Cabanne et al. 2007, Lara–Ruiz et al. 2008, Fitzpatrick et al. 2009, Mata et al. 2009, Brunes et al. 2010, Horta et al. 2011) while others showed genetic patterns that supported population (an therefore AF habitats) persistence outside those putative refuges (Grazziotin et al. 2006, Cabanne et al. 2008, Fitzpatrick et al. 2009, Thomé et al. 2010, Álvarez–presas et al. 2011). Because only Carnaval et al. (2009) used model-based approaches to formally test distinct population histories across AF, other studies should not be taken as definitive evidence in favor or against refuge diversification hypotheses.

We here pursue both traditional and model-based approaches to specifically test alternative population diversification scenarios in the *Rhinella crucifer* species group, a widely distributed group of closely related toads. The group is endemic to the AF and its distribution spans the whole of this biome. These organisms show the typical biological traits associated to an optimal range-expansion phenotype (Van Bocxlaer et al. 2010), but a broad scale phylogeography revealed deep and rich mitochondrial structure within the group, supporting a Plio–Pleistocene timeframe for lineage divergences (Thomé et al. 2010). Phylogeographic breaks were fairly coincident with known geographic barriers (e.g., Rio Doce), and palaeomodeling predicted severe forest fragmentation. However, the association of putative refuges to mtDNA lineages was unclear and the rough geographic sampling prevented a more rigorous examination of distinct allopatric models of diversification. A more recent survey of population genetic structure within the group supported the existence of five phylogeographic units based on multilocus sequence data, with the core area of the groups' range encompassing three genetic units distributed in the northern, central and southern regions of the AF (Thomé et al., in prep.). We take advantage of this fine scale

sampling and previously defined genetic structure to investigate the evolutionary processes underlying the diversification of the *R. crucifer* group, and integrating several sources of information to explicitly test hypotheses of population diversification at both the biome and regional scales. We specifically compared the genetic diversity from samples collected at putative refuge and non–refuge areas defined from distinct previously published forest change scenarios. The expectations are that areas where populations persisted at least during the Last Glacial Maximum will have retained higher genetic diversity than areas where populations have gone extinct and that were recently colonized from nearby refugial sources. To explicitly examine alternative scenarios of population diversification and demographic history we performed coalescent simulations using an approximate Bayesian statistical framework.

2. Methods

Sampling

Data for this study was obtained from Thomé et al. (in prep.) and included 398 individuals from 109 localities distributed across the AF (fig. 1 A). We conducted analyses at different spatial scales: at the biome scale we included all sampled individuals from the five genetic units defined for the *Rhinella crucifer* group (sensu Thomé et al. in prep.), for the analyses conducted at more regional scale we included only the genetic units N, C and S (units G and P were excluded due to limited sampling) (fig. 1 A). We used the described genetic breaks among these units to define three distinct regions: northern AF, central AF, and southern AF (corresponding to units N, C and S respectively) (fig. 1 A). We used two nuclear fragments (the β-crystallin gene and the intron 1 of the A alpha polypeptide) and a single mitochondrial fragment (a partial sequence of the NADH dehydrogenase subunit 2, hereafter called ND2), for which there is a nucleotide substitution rate available (Crawford 2003). Samples from the putative hybrid zones between units N and C, and between C and S (sensu Thomé et al. in prep.), were eliminated from the nuclear loci alignments in analyses performed at regional scales, except when more than one region was involved (see below).

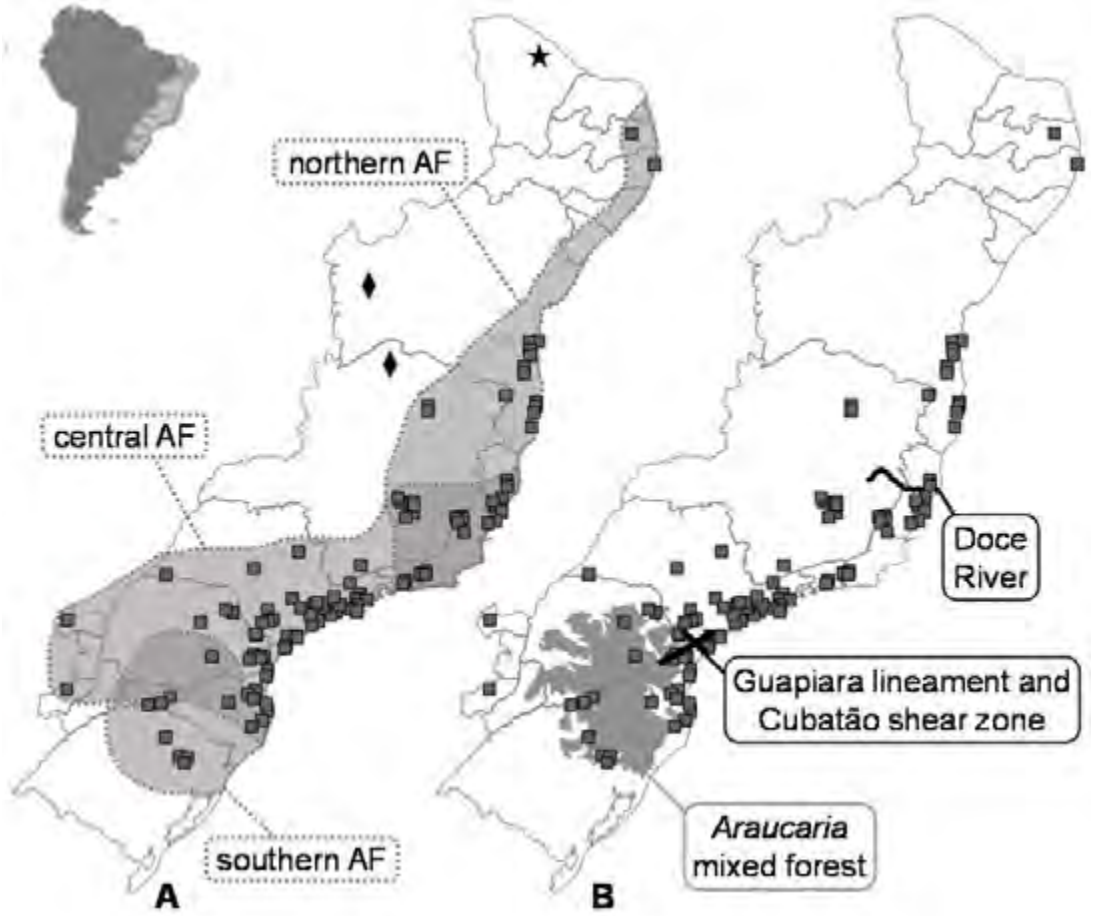


Figure 1: A, localities sampled in this study. Diamonds and star mark the distribution of genetic units P and G, respectively; squares mark the distribution of the core area of the group, with areas delimited in grey corresponding to the AF regions as considered in this study (see methods). B, geographic barriers considered in this study and distribution of the *Araucaria* mixed forest.

Divergence versus gene flow

We inferred times of divergence and levels of gene flow restriction associated with the diversification of genetic units applying an isolation with migration model to multilocus data as implemented in the program IMa (Hey & Nielsen 2007). At the biome level, we investigated the genetic breaks between the northern and central, and between the central and southern AF regions (fig. 1B). At a more regional scale, we investigated substructure within the central region, more specifically the genetic break isolating populations of the southeastern states of Paraná and Santa Catarina from remaining populations in the central region (Thomé et al. in prep.). Because there are transition zones between genetic units where

it becomes difficult to assign individuals to specific genetic units, perhaps resulting from secondary contact and hybridization (Thomé et al. in prep), we set boundaries between units within transition zones that coincided with associated putative barriers or forest ecotones. These boundaries were i) the Doce River (between northern and central), ii) the transition between wet rainforest and wet *Araucaria* mixed forest (between central and southern units), and iii) the Guapiara lineament (in the Ribeira river valley) and Cubatão shear zone (between São Paulo and Paraná populations), hereafter referred as Guapiara fault (fig. 1B).

All six parameters available in the model were estimated for all analyses (population-split time, effective population size of the ancestral and current populations, and migration rates in both directions). The upper bounds for the prior distributions were set after preliminary analyses so that posterior distributions for each parameter were well accommodated within the prior distributions. We used the HKY mutation model (Hasegawa et al. 1985) for the three loci and performed two runs of 6–8 million steps following a burnin of 500,000 steps with 15–25 chains each. The population-split parameter (t) was converted to divergence time in years using the ND2 mutation rate in Crawford (2003) and the respective mutation rate scalar, and values of 2Nm were calculated independently of the mutation rate.

Demographic history

To examine if genetic diversity is compatible with recent demographic expansions from historical refuges we i) compared nucleotide diversity contrasting localities inside and outside refuges, according to three distinct refuge hypotheses, and ii) investigated patterns of demographic growth within regions. For diversity comparisons from inside and outside refuges we first considered the putative refuges inferred from palaeomodeling of the *Rhinella crucifer* group (Thomé et al. 2010) and, secondly, from palaeomodeling of the AF itself (Carnaval et al. 2008). Thirdly, because independent evidence from paleoindicators suggests that fragmentation might have been associated to certain types of forest (i.e. semi–deciduous; see discussion in Thomé et al. 2010), we also compared diversity between rainforest areas (putative refuges) and semi–deciduous forest areas. Nucleotide diversity measures were estimated for the ND2 fragment using DNAsp 5.0 (Librado & Rozas 2009).

Patterns of demographic change were evaluated through Fu's *Fs* (Fu 1997) and R_2 (Ramos–Onsins & Rozas 2002) following Ramos–Onsins & Rozas (2002). We calculated

both statistics for the ND2 fragment in DnaSP 5.0 (Librado & Rozas 2009) with 10,000 coalescent simulations to assess statistical significance. To estimate growth and current effective population sizes we used the multilocus Bayesian version of LAMARC 2.1.3 (Kuhner 2006). For the southern region, we analyzed the whole dataset while for northern and central regions we produced smaller datasets with 25 sequences for each loci following recommendations by the program author. We selected samples at random using the list randomizer available at www.random.org/. We checked the consistency of results by performing three runs with different starting points (seeds) for the southern region, and one run for three different small datasets both for the northern and central regions. The estimates and respective 99% confidence intervals from distinct runs were averaged across runs. The F84 model was used in all analyses for all loci. We set Bayesian prior intervals for theta (1^{-10} – 10,000) and growth (-2,000 – 2,000) after preliminary analyses. We used three replicates with one final chain of 4,000,000 steps long sampling at each 40 interactions, and kept 90,000 genealogies after a burnin of 10,000. We checked effective sample size values (ESS) in Tracer 1.5.0 (Rambaut & Drummond 2007) and compared the curve files for all loci in all runs (and replicates) to confirm unimodality of probability densities. To investigate changes in population size over time we generated GMRF skyride plots (Minin et al. 2008) for the ND2 fragment in BEAST 1.6.1 (Drummond & Rambaut 2007) using time-aware smoothing. We used the SRD06 model suggested by Shapiro et al. (2006) that allows 3rd positions to have a different relative rate of substitution, transition–transversion ratio, and gamma distribution. We ran two analyses for 100 million generations sampling at each 10,000, and combined the results after checking ESS values in Tracer 1.5.0 (Rambaut & Drummond 2007).

Testing explicit scenarios of diversification and demographic history

We explicitly tested the ability of alternative population diversification hypotheses to explain observed genetic diversity using an approach of approximate Bayesian computation (Beaumont et al. 2002). First, we designed concurrent demographic scenarios and used them to perform single locus coalescent simulations. The scenarios were simulated with prior parameter intervals (ancestral population sizes, growth, time of divergence) compatible with the hypotheses being tested (fig. 2). Equal numbers of genealogies were generated for each concurrent scenario and nucleotide diversity statistics were directly calculated from each

simulated genealogy. Statistics from models being compared were pooled in a single dataset that was used to select a fixed proportion of genealogies that best fitted nucleotide diversity calculated from observed data. The relative number of genealogies produced from each concurrent scenario in the pool of selected genealogies was interpreted as a direct measure of its posterior probability (see Pritchard 2000).

We specifically investigated the genetic breaks between northern and central AF, and within central AF (between populations of the southeastern states of Paraná and Santa Catarina and the rest of central AF, see *Divergence versus gene flow*). For the genetic break between northern and central AF, we contrasted a scenario of population divergence determined by a putative geographic barrier (barrier scenario A, fig. 2A) against a scenario of differentiation involving isolation in two refuges (refuge scenario B, fig. 2B). Because we found evidence for demographic growth in the northern and central regions (see results) we allowed for population size changes in both scenarios. The barrier scenario A assumed a history of moderate bottlenecks (70– 90% of current population sizes remained prior to demographic expansion). The refuge scenario B assumed that populations suffered more drastic size reductions (only 1–20% of their current effective size remained prior to demographic expansion). To verify the sensitivity of the method to priors of ancestral population size, we performed separate runs using wide (Θ_{AW}=5–40) and restricted (Θ_{AR}=5–15) priors for ancestral population sizes. We restricted priors of ancestral population size following results from IMa analysis (the 95% highest posterior density intervals).

To examine the origins of the genetic break between populations of the southeastern states of Paraná and Santa Catarina from the rest of the the central region, we contrasted a scenario of two populations that diverged due to a putative barrier (barrier scenario C, fig. 2C) with a scenario of recent colonization from a larger refuge population (scenario D, fig. 2D). The barrier scenario C assumed that both populations suffered moderate reductions in effective size (50– 90% of current population sizes remained). The refuge scenario D assumed a very strong bottleneck (population reduction to of 1–5% of current size) in one of the diverging populations. We also investigated the sensitivity of the method by performing separate runs using wide (Θ_{AW}=2–20) and restricted (Θ_{AR}=2.5–6) priors for ancestral population. We restricted priors of ancestral population size following results from IMa analysis (the 95% highest posterior density intervals).

We used the program ms (Hudson 2002) to generate 100,000 genealogies for each test and a modified version of SampleStats (Hudson 2002) to obtain diversity statistics from all simulated genealogies. We automated this process using *perl* scripts. We used the msReject algorithm (Hickerson et al. 2007) to select simulated genealogies that best fitted observed data according to three statistics: nucleotide diversity within population one, nucleotide diversity within population two, and average nucleotide diversity between populations one and two. These statistics were calculated for observed data in DnaSP 5.0 (Librado & Rozas 2009). We additionally checked the fit of accepted genealogies to observed data by comparing the distributions of "simulated" nucleotide diversity statistics with the value estimated from real data. Principal component analysis was used to produce ordination plots using diversity statistics as variables. High goodness of fit was visually inferred if estimates calculated from observed data appeared near the centroid of the distribution of scores representing the combined nucleotide diversity statistics (i.e., synthetic variables from PCA) used in the selection of simulated genealogies.

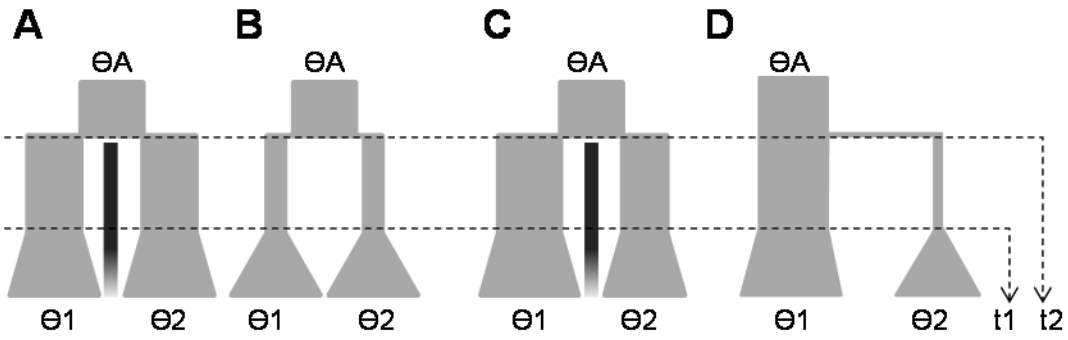


Figure 2: Demographic scenarios tested for diversification in the *Rhinella crucifer* group. Arrows show time parameters that varied according to prior intervals. Scenarios A and B were used to test for the genetic breaks between northern and central regions, and between central and southern regions. Scenarios C and D were used to test a genetic break within the central region (see text for details).

3. Results

Divergence versus gene flow

Maximum likelihood estimates (MLE) and high posterior distribution (HPD) intervals of all parameters in IMa analyses were consistent across independent runs. Posterior probability distributions for all parameters were unimodal in all analyses. ESS values for the Doce river analysis were relatively low after eight million interactions (between 25 and 90). Divergence times indicated a population split occurring ~800,000 years ago (fig. 3). Population sizes of the northern region (Θ1) and central (Θ2) regions were not significantly different. Migration rates indicated that gene flow was much larger into central AF from the northern region (table 1). For the Guapiara fault ESS values varied between 45 and 7731. The inferred divergence time was ~600,000 years (fig. 3) and populations were smaller at eastern states of Paraná and Santa Catarina than in the rest of central AF. Migration rates were slightly higher for migrants coming into populations at eastern states of Paraná and Santa Catarina. For the *Araucaria* mixed forest, ESS values after 6 million interactions were very low (between 7 and 229) indicating poor mixing. Time of divergence was estimated at ~2.7 million years (MYR) (fig. 3) with a wide HPD interval. Current population sizes differed, with larger populations in non-mixed forests (Θ1) than in mixed forests (Θ2). Migration rates also varied and indicated intense movement of genes into mixed forest populations (table 1).

Table 1: Demographic parameters used to evaluate divergence and gene flow between populations of toads within the *Rhinella crucifer* group. Maximum-likelihood estimates (MLE) and 90% Highest Posterior Density (HPD) intervals were obtained with IMa (see methods).

	Doce river		**Guapiara fault**		**Araucaria mixed forest**	
	MLE	**HPD**	**MLE**	**HPD**	**MLE**	**HPD**
t(tμ)	1.5489	(1.1575–1.9475)	0.9165	(0.5385–1.2825)	3.7892	(1.1220–11.9580)
t(years)	833,243	(622,686–1,047,673)	598,623	(276,821–1,064,325)	2,705,099	(632,430–10,682,666)
Θ1	32.4530	(25.7974–38.959)	25.6865	(20.2927–30.9418)	26.5815	(21.8901–31.205)
Θ2	36.6892	(31.0488–42.3393)	4.3711	(2.6436–6.0692)	10.7292	(7.2967–14.0241)
ΘA	10.6303	(6.1047–15.0321)	4.6763	(1.6011–7.6331)	17.8694	(4.5022–29.963)
m1	0.0284	(0.0025–0.0725)	0.1492	(0.0250–0.2850)	0.0131	(0.005–0.065)
m2	0.2009	(0.1125–0.2925)	1.4381	(0.6150–2.2350)	0.8887	(0.515–1.245)
2N1m1	0.4608		1.9162		0.1741	
2N2m2	3.6854		3.1430		4.7675	

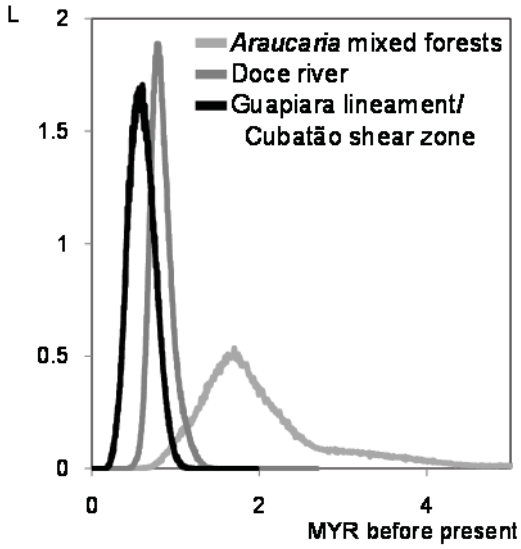


Figure 3: Posterior probabilities for divergence times estimated for genetic breaks using IMa (see text for details).

Demographic history

At the biome level, 139 (42 localities), 295 (75 localities), and 90 (18 localities) samples belonged to refugial areas according to, respectively, palaeodistribution modeling the of the *Rhinella crucifer* group (Thomé et al. 2010), considering rainforest areas as putative refuges, and palaeodistribution modeling of the biome (Carnaval et al. 2008). At a regional scale, we included 94 samples from 28 localities in the northern region, 264 samples from 76 localities in the central region, and 23 samples from 10 localities in the southern region. The number of samples/ localities in refugial areas according to the three aforementioned hypotheses were, respectively: 11/6, 85/21, and 75/16 for the northern region; 119/35, 197/53, and 15/4 for the central region; 9/2, 0/0, 13/6 for the southern region. At the biome scale, nucleotide diversity was lower in refugial areas than in non-refugial areas for all refuge hypotheses. At the regional scale, we found similar results for the northern region only, with refuges based on palaeodistribution modeling of the biome showing lower diversity. Other comparisons show similar values of nucleotide diversity for refuge and non-refuge areas (fig. 4). Total nucleotide diversity decreased from the northern region to the central and southern regions (table 2).

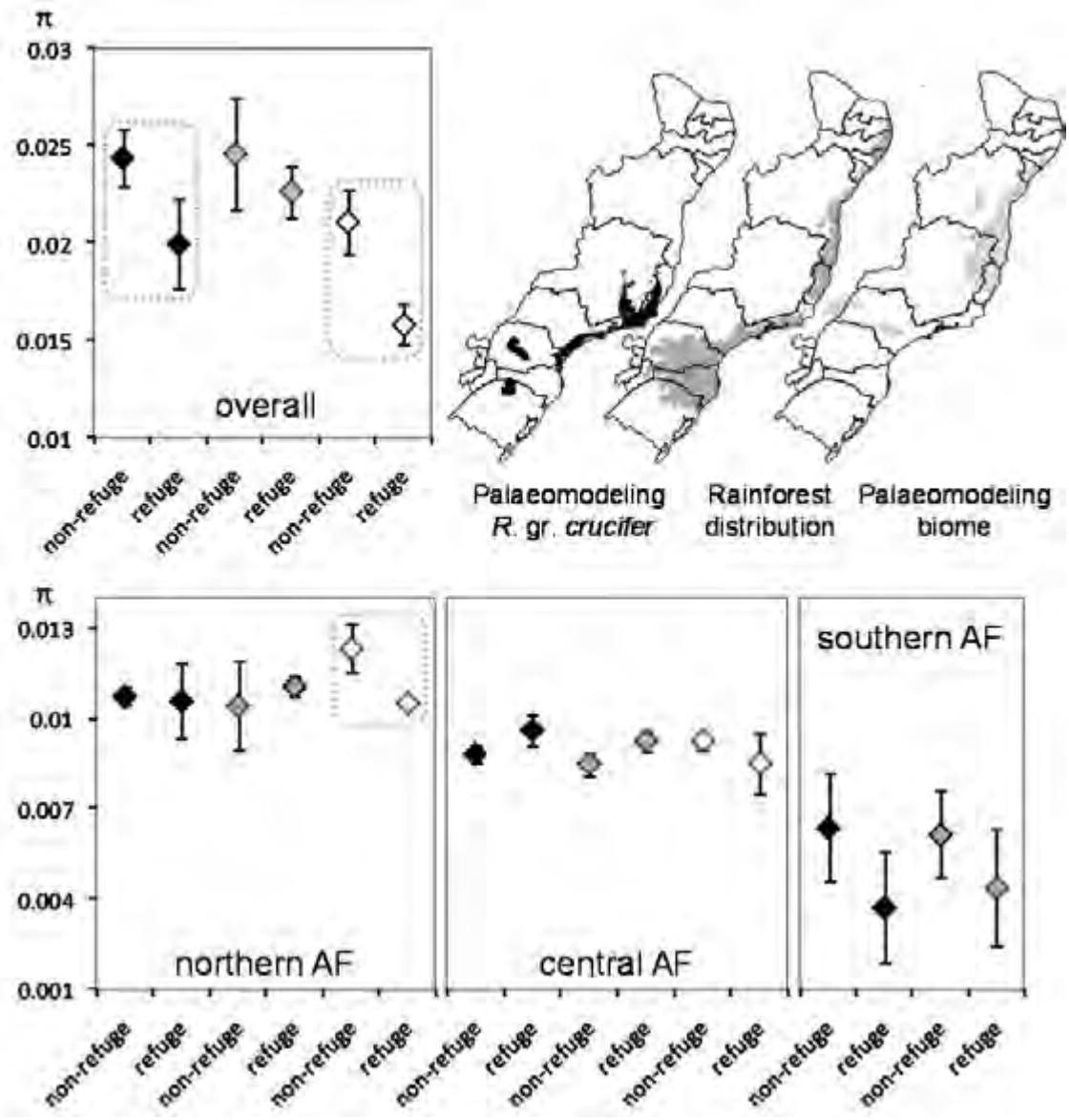


Figure 4: Nucleotide diversity comparisons at biome scale (overall) and regional scales. Maps illustrate refuge areas according to the three considered hypotheses. Color in the plots code for the hypotheses represented on the maps. Bars indicate standard deviation.

Values of Fs were significantly low for the northern AF while R_2 was only marginally significant in this region. The two statistics were significantly low for central region and non significant for southern AF (table 2). Growth estimates obtained in LAMARC (table 2) and skyride plots (fig. 5) showed compatible results. There was positive growth within 99% confidence intervals for the northern and central AF (moderate average values). Population size changes through time were found for the same regions with smooth but significant

growth. The time to the most recent common ancestor differed among regions and was smaller for the central region. The degree of population size changes also varied with growth being more abrupt at the central region than in northern AF (fig. 5).

Table 2: Expansion tests (Fs and R_2 statistics) with statistical significance, growth (δ) estimated with 99% confidence intervals, and nucleotide diversity (π) with standard deviations for the northern, central and southern regions of the AF. Bold values indicate statistical significance.

	Northern AF		**Central AF**		**Southern AF**	
ND2						
Fs	-21.611	**P<0.05**	-75.506	**P<0.05**	-0.545	P>0.10
R_2	0.053	**0.10 >P >0.05**	0.039	**P<0.05**	0.091	P>0.10
π	0.01129	(0,00031)	0.00926	(0.00027)	0.00526	(0.00135)
all fragments						
δ	**354.22**	**(140.0–420.87)**	**193.33**	**(13.0–489.85)**	95.62	(-167.41–453.21)

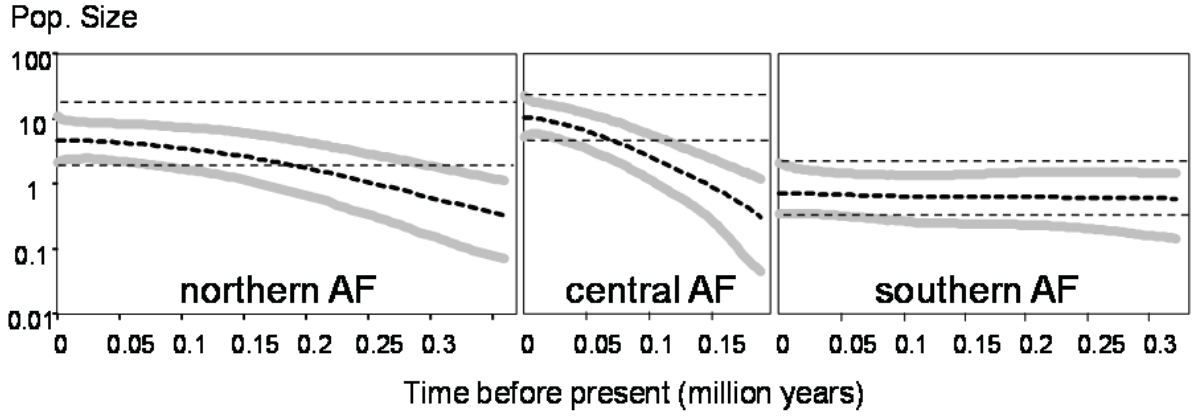


Figure 5: GMRF skyride plots produced in BEAST for the three regions of the AF. Black bold dashed lines indicate median values of relative population size. Gray lines indicate 95% confidence intervals and black thin dashed lines indicate significance.

Testing diversification and demographic scenarios

Scenarios A and B had relative posterior probabilities of 0.604 and 0.396, respectively, using a wide prior for ancestral population size, but simulations using a more restricted prior

for ancestral size showed that the posterior probability for scenario A increased to 0.993. We obtained similar results for comparisons between C and D when alternatively using wide (probabilities of 0.998 and 0.002) and restricted (probabilities of 1.0 and 0.0) priors for ancestral population sizes. The results of PCA of nucleotide diversity statistics used in the rejection step showed that the estimate for observed data was centrally placed within the distribution of values from the accepted genealogies, indicating a good fit of simulated data for all analyses (fig. 6). The distributions of scenarios A and B overlapped when using wide priors to contrast the scenarios, with scenario A showing a better fit to observed values. For other comparisons, the observed data occupies the central position within the distribution of simulated values under one of the scenarios being compared (either A or C; fig. 6). The variance explained by the first two axis were high for all analyses, with axis 1 responding for almost all the variation in the comparisons between scenarios A and B (table 3). Variable loadings indicate that this axis is highly positively related to the average nucleotide diversity between populations, which points for a strong participation of this statistics in the rejection step (table 3).

Table 3: results of principal component analyses used to evaluate the fit of the accepted genealogies simulated for the comparison of demographic scenarios A and B, and C and D (see text for details).

	Scenarios A and B				**Scenarios C and D**			
	Wide Θ		**Restricted Θ**		**Wide Θ**		**Restricted Θ**	
	Axis 1	**Axis 2**	**Axis 1**	**Axis 2**	**Axis 1**	**Axis 2**	**Axis 1**	**Axis 2**
Eigenvalues	81.436	2.07	26.144	0.96	6.123	0.802	3.033	0.483
Percentage	95.519	2.428	93.471	3.432	86.444	11.32	77.026	12.271
Cum. Percentage	95.519	97.947	93.471	96.902	86.444	97.764	77.026	89.297
PCA variable loadings:								
π pop. 1	-0.009	0.559	-0.001	0.998	0.075	0.997	0.071	-0.203
π pop. 2	-0.021	0.829	-0.002	0.064	-0.002	0.01	0.01	0.979
π pop.1 x pop.2	1	0.023	1	0.001	0.997	-0.075	0.997	0.005

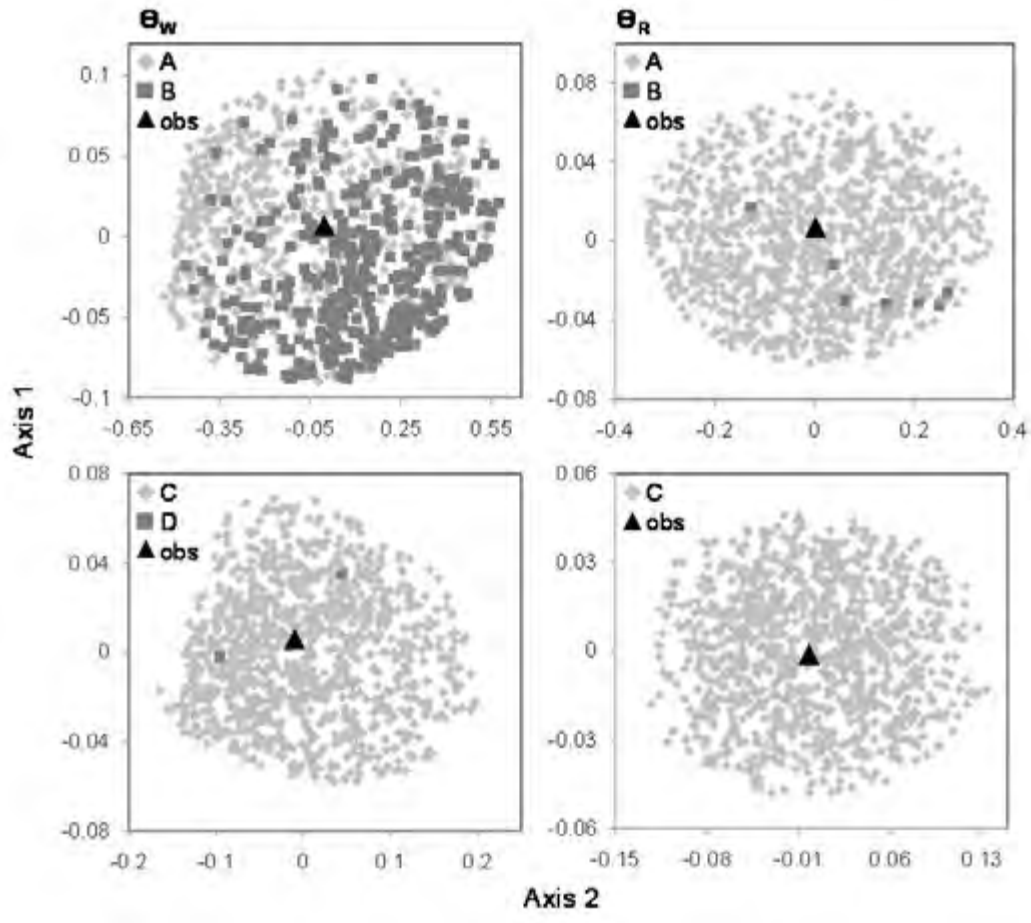


Figure 6: Plots of principal component analyses showing the fit of the accepted genealogies simulated for the comparison of demographic scenarios A and B, and C and D (see text for details).

4. Discussion

Divergence times estimated for the main genetic breaks were placed within the Plio–Pleistocene periods, as in previous analyses (Thomé et al. 2010). Migration rates varied between studied barriers: asymmetric migration rates across the Doce river suggest this river is not completely coincident with the center of the genetic transition zone between northern and central units; higher migration rates into *Araucaria* mixed forest are compatible with inferred demographic expansion patterns and suggesting that forest type may be a week ecological barrier; and migration across the Guapiara fault was proportional to relative population sizes. We found no signal of higher diversity retention in putative refuge areas according to three distinct refuge hypotheses for the AF. Thus, differential genetic diversity

retention due to massive habitat fragmentation was rejected overall. The demographic history of the group was revealed to be distinct for each of the three regions of the AF with a moderate increase in population size in the northern region, more prominent growth for the central region, and population demographic stability in the southern AF. Other studies inferring demographic changes in AF taxa are only partially in agreement with our results, both spatially and temporally. Coalescent simulations under concurrent demographic scenarios rejected hypotheses that involved strong population demographic fluctuations associated to vicariance in very small refuges or recent colonization from refugial areas. Models of vicariance combined with modest demographic expansions were instead supported in all cases.

Population divergence and gene flow

By using isolation-with-migration models with multilocus data we inferred the time of divergence between northern and central regions of the AF (as divided by the Doce river) to be around 800,000 years. The same splitting age was found for the mitochondrial clades N and C in Thomé et al. (2010) with a single locus and a simple distance-based calibration (*Da*). However, the method used in the present study did allow us to estimate two different migration rates across this barrier, with migrants being far more common dispersing from the northern region southwards than in the opposite direction (table 2). We have no reason to believe that this result is biologically meaningful, i.e., that overwater dispersal occurred preferentially in that direction, especially because the pattern of past demographic expansions inferred for both regions would cause migration in the opposite direction to be more prominent (considering that growth was more intense at the central region as inferred by skyride plots, fig. 5). The discrepancy in migration rates could be an artifact of our analysis if the river did not coincide with the center of the transition zone between northern and central groups. A more careful look at the region indeed indicates that the Doce river, at its current position, does not coincide to the central part of the overlapping zone between northern and central AF populations (fig. 1). The river is far north and closer to the northern limits of the putative contact zone between units N and C, which might have produced a signal of unbalanced migration. Alternatively, asymmetric migration could be associated with distinct population densities in areas contiguous to the contact zone. Our study cannot specifically discern between these hypothesis, for which formal studies describing the genetic structure of

the transition zone would be needed. Therefore, we are still uncertain about the role of this river in isolating populations of *R. crucifer*.

The mean divergence time of 2,7 MYR estimated for the break between populations presently distributed in distinct types of forest (wet rainforest and wet *Araucaria* mixed forest) is more recent than the ~4 MYR previously estimated for this genetic break (Thomé et al. 2010), but the wide confidence intervals of the present analysis include this value. Relative population sizes were in agreement with the relative geographic ranges of genetic units (fig. 1), with the southern region presenting a lower effective population size than the central region. Migration into the southern region was much higher than in the opposite direction, which could have been associated with higher densities resulting from the demographic expansion inferred for the central region (fig. 4). Extensive migration across an ecological transition (i.e., distinct forest types) suggest habitat preference or divergent ecological selection do not constitute absolute barriers for gene flow. But again, addressing these specific hypotheses regarding the genetic interaction between diverged population units would need more detailed and formal descriptions of the contact zone, which was clearly not the goal here and must be postponed to future studies.

The mean divergence time inferred for the genetic break along the Guapiara fault was ~600,000 years. Both the Guapiara lineament and Cubatão shear zone are among the list of tectonic faults with Quaternary surface rupture (Saadi et al. 2002), but there is no information available on the timing and extent of these ruptures.

Refuge hypotheses and demographic history

We compared genetic diversity from samples collected at putative refuge areas and non–refuge areas to test for differential diversity retention caused by habitat stability. We tested three different refuge configurations derived from the literature (fig. 4) and found no positive relationship between putative stable areas and nucleotide diversity, thus rejecting all refuge hypotheses. Conversely, we found the inverse pattern to be statistically significant in some cases; nucleotide diversity was higher for non–refugial areas as inferred by palaeodistribution modeling of both the AF biome and of the *R. crucifer* species group. The same tendency was apparent in regional analyses only for comparisons between non-refuge

and refuge areas within northern AF as inferred by palaeodistribution modeling of the whole biome.

There are two possible explanations for this contradictory pattern, the first being that statistical significance is a spurious result caused by random effects of sampling. In this case, fragmentation may have not occurred, or have occurred in a way that is not represented at all by both palaeomodels. It is very unlikely that massive habitat fragmentation as predicted by these hypotheses would have occurred without affecting the genetic diversity of *R. crucifer* toads since all forms within the complex are undoubtedly forest dependent. Landscape ecology studies reveal however that small patches of forest can sustain large populations of *Rhinella ornata* in fragmented AF landscapes, apparently because this species is able to cross more open habitat between fragments at least 100 meters apart (Dixo 2005). Furthermore, Dixo (2005) found that *R. ornata* is more abundant in large fragments than in continuous forest or small fragments, and suggested that this species can benefit from moderate habitat disturbance. Indeed, genetic erosion in *R. ornata* in a fragmented landscape was only detected as a slight reduction in the number of haplotypes at smaller fragments; nucleotide diversity and genetic connectivity remained unaffected (Dixo et al. 2009). These results bring insight to another possible explanation; habitat disturbance may have occurred to some extent at putative non–refuge areas, but not enough to fragment the forest extensively (e.g. as predicted by palaeomodels). Under this scenario, gene flow could have prevented the negative effects of genetic drift (Bennet & Provan 2008) allowing for genetic diversity maintenance, while mild habitat disturbance would be compatible with relatively larger population sizes, explaining the increase in nucleotide diversity in non–refuge areas. Such behavior would not be surprising considering that the group presents all the life history traits required by an optimal range-expansion phenotype (sensu Van Boaxlaer et al. 2009).

The results of all methods applied to examine the demographic history of the group were concordant in revealing distinct histories for the three AF regions (table 2, fig. 5). The dynamics of population sizes through time indicate a moderate increase in population size for the northern region since the last 350,000 years, and a more prominent growth for the central region for the last 150,000 years. In both cases growth seems continuous back in the coalescent to the time for the most recent common ancestor, with no abrupt changes. Extreme demographic population stability marked the history of the southern region of the AF. These

results are not straightforward to interpret when considering that nucleotide diversity is lower at the southern region (table 2), but a possible explanation rely on a smaller population size despite of habitat stability, as estimated in IMa analyses (table 1). Also, the southern region occupies a relatively smaller area (fig. 1) and the animals seemed less abundant at this region than in other areas of the AF during field incursions. With respect to nucleotide diversity in the other regions, it is hard to confirm whether the relatively higher diversity in the north is due to moderate habitat instability (as explained above) or simply relates to a slightly longer history of populations in this region (as shown in skyride plots, fig. 5). Because the northern AF unit appears less marked by population size changes than the central AF unit, and considering that a more prominent fragmentation would eventually cause some diversity loss, it seems fair to speculate that habitat was more stable in the former region.

Studies reporting demographic inferences of other AF taxa are only partially concordant with our results. In central AF, demographic history of the frogs genus *Thoropa* showed early population stability followed by demographic expansion within the same timeframe as in *Rhinella* gr. *crucifer* (Fitzpatrick et al. 2009). The same pattern was apparent for the pitvipers *Bothrops jararaca*, but expansion seems to have started slightly later for these snakes (Grazziotin et al. 2006). Other studies showed growth trends for populations in central and northern regions but lack temporal concordance. This is the case for populations of the birds *Xyphorhynchus fuscus fuscus* and *X. f. tenuirostris* with demographic signatures of expansion apparent for both regions (e.g. CAF and SAF in Cabanne et al. 2008, 2007). Growth in northern region was not dated but in central AF demographic expansions would have started at ~57,000 or 19,000 years ago, depending on the lineage. In the central AF, distinct times of expansion for distinct lineages of the same organism were also observed within *B. jaracaca* and *Thoropa* (Grazziotin et al. 2006, Fitzpatrick et al. 2009). Because those lineages are mostly allopatric, the temporal variation in demography implies that past habitat conditions across the central region were not homogeneous, suggesting a complex history of populations in this region. There were studies reporting results contradictory to ours, at least apparently. A scenario of past population stability in northern AF and recent (Holocene) dramatic expansions in central and, especially, southern AF were proposed for three species of treefrogs genus *Hypsiboas* (Carnaval et al. 2009), while long term stability of land planarian populations was inferred for the central region (Álvarez–presas et al. 2011).

Testing alternative demographic scenarios

Approximate Bayesian computation (ABC) was successfully applied in testing alternative population diversification and demographic scenarios in *Rhinella* gr. *crucifer*. Nucleotide diversity statistics derived from simulated genealogies accepted by ABC showed a good fit to observed data (fig. 6). Acceptance of population models was therefore never a result of choosing the best between population models that poorly reproduce the observed data. Clear rejection of one alternative scenario was sometimes only possible when the prior for ancestral population size was restricted, revealing that the method can be quite sensitive to changes in this parameter. Such sensitivity may result in lack of power of coalescent simulations in distinguishing between divergence and demographic scenarios in extremely large populations. Setting prior values for ancestral population size should always be guided by previous knowledge. Here, intervals for ancestral population size were estimated using isolation-with migration models with multilocus data, which appears more appropriate than using completely arbitrary intervals for population scenario comparisons (fig. 6).

The first comparison examined the processes driving the genetic break between northern and central AF regions. A scenario of a geographic barrier causing divergence with moderate expansions superimposed on older structure was contrasted against a scenario where lineages have differentiated in small isolated habitat patches with the current pattern of geographical distribution being caused by large demographic expansions (fig. 2 A and B). Under this second scenario of refugial vicariance, the Doce river would correspond to a secondary barrier as speculated by Cabanne et al. (2008) for *Xyphorhynchus fuscus* birds. The absence of an obvious barrier other than the Doce river, the relatively recent age of the split, and the presence of growth at both sides of this putative barrier suggests the existence of a suture zone for *X. fuscus* (Cabanne et al. 2008) and *Rhinella* gr. *crucifer* (present study), but the model comparison approach allowed us to clearly reject a model of refugial vicariance for the toads. We found strong support for the existence of a barrier behind this genetic split, which is not unexpected given this region coincides with biogeographic breaks known for several organisms. It is unclear how the Doce River would have posed such a restriction to gene flow to organisms that may well move across water bodies. A factor that has been quite neglected is the disruption in climate caused by the synergic effect of a cold oceanic upwelling north of the Rio de Janeiro (Araujo 1997) and the increased distance of mountain

ranges from the coast in northern AF, starting north of the Doce river. Both changes affect the climate by decreasing rainfall amounts and increasing seasonality, which correlates well with strong floristic differentiation between northern and southern coastal rain forests (Oliveira–Filho & Fontes 2000). Because reproductive periods in amphibians are strongly influenced by such environmental variables (amount of rainfall and length of the rainy season, Wells 2007), this climate disruption might constitute a cryptic barrier between populations in northern and central AF regions.

To investigate the genetic break separating populations of the eastern states of Paraná and Santa Catarina from other populations at the central region we tested a scenario of geographic barrier against a scenario of recent colonization associated to a small founder population (fig. 2 C and D). The results are unequivocal, regardless of priors for ancestral population size, in supporting the case of genetic isolation driven by a barrier, with relatively stable demographies and moderate growth since the Holocene.

Conclusions

We found no signature of higher levels of genetic diversity in putative refuge areas according to three different refuge scenarios, which clearly argues for rejecting massive habitat fragmentation and large scale recent colonization. Limited habitat disturbance may be however compatible with geographical patterns of nucleotide diversity in the group. The demographic history of the *Rhinella crucifer* group supported regional differences within the AF with moderate demographic fluctuations in the northern region, more prominent changes in the central region and stability in southern AF. Other studies of AF taxa are in partial agreement with our results, supporting regionalization in processes of genetic diversification at the biome level. Tests of concurrent demographic scenarios using coalescent simulations allowed us to reject hypotheses of refugial vicariance associated to extensive demographic expansions, confirming the idea that large scale habitat disruption has not determined the diversification in *R.* gr. *crucifer*. Coalescent simulations supported instead a joint effect of geographic barriers and modest demographic expansions in generating genetic diversity within this group of toads.

Scenarios of dramatic biological responses to recent past climate changes were inferred for temperate and tropical zones in Europe (Hewitt 1999) and Australia (Williams &

Pearson 1997). However, the available data on the demographic dynamics of AF taxa make global refuge hypotheses based on massive forest fragmentation unlikely for this biome. Rather, there is now growing support for differential responses of organisms to Pleistocene climatic changes depending on their life histories, as seen in other tropical regions of the world (Bridle et al. 2004). Geographic discontinuities across the complex AF landscapes appear equally or more importantly associated with diversification, although it remains unclear the detailed spatio-temporal mechanics of putative barriers. Pleistocene climatic shifts may modulate the effectiveness of Pliocene geographic barriers to gene flow (Cabanne et al. 2008), corroborating the idea of idiosyncrasy of mechanisms producing diversity in an interactive way (Leite 2003). Additional complexity is brought by the fact that habitat throughout this widespread biome is by no means homogeneous, it encompasses different climates, vegetation types, and several geologic formations with highly complex topography (Oliveira–Filho & Fontes 2000), breaking up the biome into many habitats, and perhaps also driving speciation by niche specialization (Haddad & Prado 2005). Due to these environmental discontinuities, the study of diversification in widespread AF taxa might benefit from considering global and regional scales to detect evolutionary processes acting at different scales while the scarcity of fine scale geologic data and palaeoindicators encompasses the need of multidisciplinary data in recovering the histories of these complex landscapes.

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Capítulo 4

Variação da forma no grupo de *Rhinella crucifer* (Anura, Bufonidae)

Variação da forma no grupo de *Rhinella crucifer* (Anura, Bufonidae)

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1. Introdução

O grupo de *Rhinella crucifer* apresenta uma distribuição ampla que coincide com o domínio da Mata Atlântica Brasileira (Baldissera et al. 2004). O grupo foi originalmente descrito como monotípico (Duelmann & Schulte 1992), mas variações morfológicas vêm confundindo autores ao longo de muitos anos. Como testemunho, existem na literatura diversas observações e descrições antigas destas variações, que resultaram em um histórico vasto de nomes propostos e sinonímias (Baldissera 2001, Frost 2011). Muitas dessas variações parecem ser baseadas em caracteres morfológicos aparentemente sem sinal evolutivo, como o padrão de coloração dorsal em “cruz” que denominou o epíteto (Baldissera et al. 2004).

Baldissera et al. publicaram em 2004 uma revisão taxonômica do grupo *Rhinella crucifer* baseada em um enorme esforço amostral, representando toda sua distribuição. Nessa revisão cinco espécies foram delimitadas com base em caracteres da morfologia externa e com o auxílio de análise morfométrica tradicional. Para três dessas 'morfoespécies' já haviam nomes disponíveis, sendo que *R. crucifer* Wied–Neuwied, 1821, foi mantida e *R. ornata* Spix, 1824, e *R. henseli* A. Lutz, 1924 foram revalidadas. Ainda, duas novas espécies, *R. abei* e *R. pombali*, foram descritas (Baldissera et al. 2004). Distribuições foram estimadas para cada uma das cinco morfoespécies (Baldissera et al. 2004) com algumas modificações posteriores (Lima et al. 2005, Silveira et al. 2009).

A taxonomia atual do grupo baseada na revisão de Baldissera et al. (2004) continua válida, mas a existência de cinco espécies foi questionada recentemente em um estudo filogeográfico feito com uma amostragem relativamente grosseira (Thomé et al. 2010). Nesse estudo, dados genéticos suportam que o grupo estaria estruturado em três linhagens mitocondriais cuja correspondência com as cinco morfoespécies é limitada. A única morfoespécie claramente associada a uma linhagem mitocondrial é *R. henseli*, correspondendo ao clado mais divergente do grupo, ocupando o extremo sul da Mata Atlântica. Foi possível uma associação precária de *R. crucifer* e *R. ornata* com clados

distribuídos nas regiões norte e central do bioma, com *R. abei* sendo associado ao sub–clado mais derivado do clado central, restrito ao extremo leste dos estados do Paraná e Santa Catarina. Não foi possível associar *R. pombali* a nenhum clado, sendo levantada a hipótese desta morfoespécie ter sido descrita a partir de híbridos entre *R. ornata* e *R. crucifer* (Thomé et al. 2010). Em um segundo estudo genético, dessa vez com amostragem mais refinada, foi feito um novo levantamento da estrutura genética no grupo. As linhagens que ocupam o norte (N), centro (C) e sul (S) da Mata atlântica foram melhor delimitadas, e ganharam o suporte de dados de DNA nuclear. Novamente, alguma sub–estrutura foi encontrada na unidade C e duas outras unidades genéticas localizadas nos extremos norte (G) e oeste (P) da distribuição do grupo foram descritas. Foram também encontradas evidências de uma larga zona de hibridação entre as unidades N e C, e de hibridação entre C e S para algumas localidades, corroborando a hipótese proposta anteriormente de *R. pombali* ser uma 'morfoespécie híbrida' (capítulo 2 desta tese).

Uma das possíveis explicações para a disparidade entre estrutura genética e morfométrica no grupo de *R. crucifer* está nos resultados encontrados na análise morfométrica de Baldissera et al. (2004), onde os próprios autores mencionam que grande parte da variação contida nos dois primeiros eixos canônicos foi explicada pelo tamanho dos animais. Ainda, contrastando–se os valores médios de comprimento rostro–cloacal encontrados pelos autores com a distribuição das morfoespécies, nota–se que existe alguma relação entre o tamanho destes animais e a latitude, indicando a possível existência de variação clinal (Baldissera 2001). Nesse caso, torna–se clara a necessidade de uma análise morfométrica onde o tamanho seja corretamente desconsiderado.

A morfometria geométrica permite a quantificação da forma pura através da definição de marcos anatômicos homólogos. Coordenadas são obtidas para cada marco, e o conjunto de coordenadas de cada indivíduo é alinhado de forma que diferenças de tamanho, posição e orientação sejam eliminadas, restando apenas a informação relacionada à forma (Monteiro & Reis 1999). Neste estudo utilizamos esta técnica para fazer uma releitura ainda que preliminar da variação morfométrica no grupo de *Rhinella crucifer*, levando em consideração a estrutura genética e história evolutiva do grupo descritas nos capítulos anteriores. As questões abordadas são: Existe alguma variação de forma no grupo como um todo? A variação existente está relacionada com a estruturação genética? A história evolutiva do grupo prevê a

variação na forma desses animais? Nossas expectativas são que i) exista variação de forma no grupo, ii) que essa variação esteja estruturada em unidades concordantes com as unidades genéticas encontradas anteriormente e que iii) indivíduos de unidades geneticamente mais distantes apresentem variação de forma mais discrepante, enquanto que indivíduos de unidades mais próximas sejam mais parecidos.

2. Métodos

Nós analisamos 271 machos adultos de 71 localidades distribuídas pelo bioma (fig.1, apêndice 1). Foram incluídos membros das cinco unidades genéticas definidas no capítulo 2 desta tese.

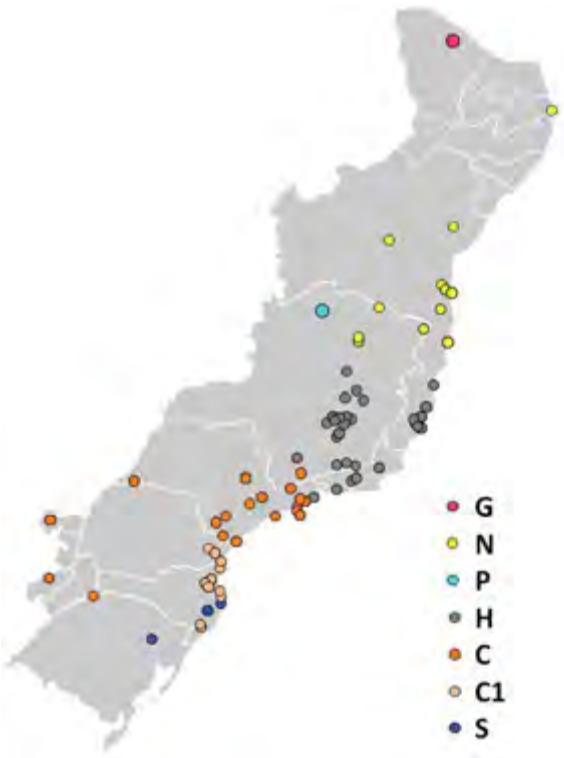

Figura 1: Localidades amostradas nesse estudo. Cores codificam para categorias consideradas (ver métodos).

Fizemos fotografias digitais da região anterior do corpo de cada espécimen com o auxílio de uma mesa estativa, mantendo sempre a mesma distância entre a câmera e o espécimen a ser fotografado e utilizando sempre uma escala. Posicionamos os exemplares de

forma padronizada, mantendo a linha da maxila inferior do animal a ser fotografado paralela à base da mesa. Definimos doze marcos anatômicos localizados na região dorsal dos espécimens (cabeça e glândulas parotóides, fig. 2A). Para o alinhamento utilizamos um procedimento de superposição (Procrustes) onde as coordenadas dos marcos anatômicos provenientes das diferentes fotos são reposicionadas e re–escaladas de forma que a soma dos quadrados das distâncias entre marcos seja minimizada, gerando uma configuração consenso (fig. 2B) a partir das medidas centróides das coordenadas alinhadas (Rohlf & Slice 1990). Para as análises da variação de forma classificamos os espécimens em categorias correspondentes às unidades genéticas G, P, N, C, e S, sendo que indivíduos da zona híbrida putativa entre N e C foram classificados em uma categoria à parte (H), assim como indivíduos do extremo leste dos estados de Santa Catarina e Paraná (sub–unidade c1), correspondentes a estruturação genética da unidade C.

Para verificar se existe variação de forma no grupo como um todo e para uma inspeção exploratória da quantidade relativa de variação existente produzimos uma matriz de covariância para que pudesse ser feita uma análise de componentes principais. Para avaliar se mudanças de forma são localizadas ou não utilizamos uma técnica de interpolação onde grades de transformação são construídas representando a variação entre os marcos (Bookstein 1991). Nessa análise é possível visualizar a 'energia de dobramento' envolvida nas mudanças de forma, que indica o grau de localização das mudanças, e a principal direção da variação na forma. Para verificar se a variação encontrada está relacionada com sua estruturação genética utilizamos análises de variação canônica onde testes de permutação pareados baseados na distância de Mahalanobis e de Procrustes foram feitos para averiguar se existem mudanças de forma suficientes para diferenciar as categorias. Para investigar se a história evolutiva do grupo condiz com a variação na forma fizemos análises de variação canônica hierárquicas, onde diferentes categorias foram consideradas. Também consideramos a história filogenética do grupo mapeando componentes principais sobre uma filogenia utilizando o método de Maddison (1991). Devido a divergências nas hipóteses filogenéticas propostas no capítulo 2, utilizamos duas árvores, uma com a topologia do DNA mitocondrial e uma segunda árvore simplificada onde as unidades genéticas mais próximas, cujo relacionamento é de alguma forma controverso, foram incluídas em um único clado (capítulo 2 dessa tese). Um teste de permutação foi aplicado para verificar a hipótese nula de ausência de sinal filogenético nas duas análises (Klingenberg & Gidaszewski 2010). Todas as análises foram feitas no programa

MorphoJ 1.02 (Klingenberg 2011), com exceção da obtenção das coordenadas, onde utilizamos o programa TPSdig2 (http: / / life.bio.sunysb.edu/morph). Testes de permutação foram feitos com 10,000 réplicas.

3. Resultados e discussão

Após a superposição das coordenadas dos espécimens amostrados (fig. 2B), é possível visualizar a configuração consenso produzida e a gama de variação nas coordenadas de todos os espécimens para os marcos anatômicos. Na grade de transformação (fig. 2C) observa–se a mudança total na forma a partir da configuração de consenso. A mudança total pode ser dividida em um componente uniforme, que descreve a variação global, e um componente não–uniforme, que engloba as variações locais nas mudanças de forma. O componente uniforme inclui apenas a variação que é comum a todas as partes da configuração de marcos, que pode ser visto aqui como a deformação da grade em uma forma parecida a de um losango, formado pelo afastamento das extremidades superior direita e inferior esquerda. O componente não–uniforme é responsável pelas 'deformações' do losango (i.e., se esse componente não existisse, o losango seria formado apenas por linhas retas). Na grade de transformação é possível notar uma área de maior dobramento indicando mudança de forma localizada, mais especificamente nas imediações dos marcos 9 e 10, que variam em direções diagonais à grade.

Na análise dos componentes principais (fig. 3) os dois primeiros componentes (eixos) explicaram 53,1% da variância total encontrada. Diagramas associando os marcos com a variação explicada por estes dois eixos novamente indicam grande participação dos marcos 9 e 10 (fig. 4), associados anteriormente a uma área onde a variação parece estar localizada. Estes marcos estão posicionados de forma a delimitar cristas cefálicas (fig 2A), estruturas geralmente bem ossificadas em bufonídeos e amplamente utilizadas para definição de grupos fenéticos (Duelmann & Schulte 1992, Narvaes & Trefaut 2009). Baldissera et al (2004) também utilizam o grau de desenvolvimento destas estruturas para diferenciar algumas morfoespécies, como por exemplo *R. henseli*, cujo grau de ossificação das cristas cefálicas é mínimo, e *R. crucifer*, com ossificações extremamente conspícuas nessa região do crânio.

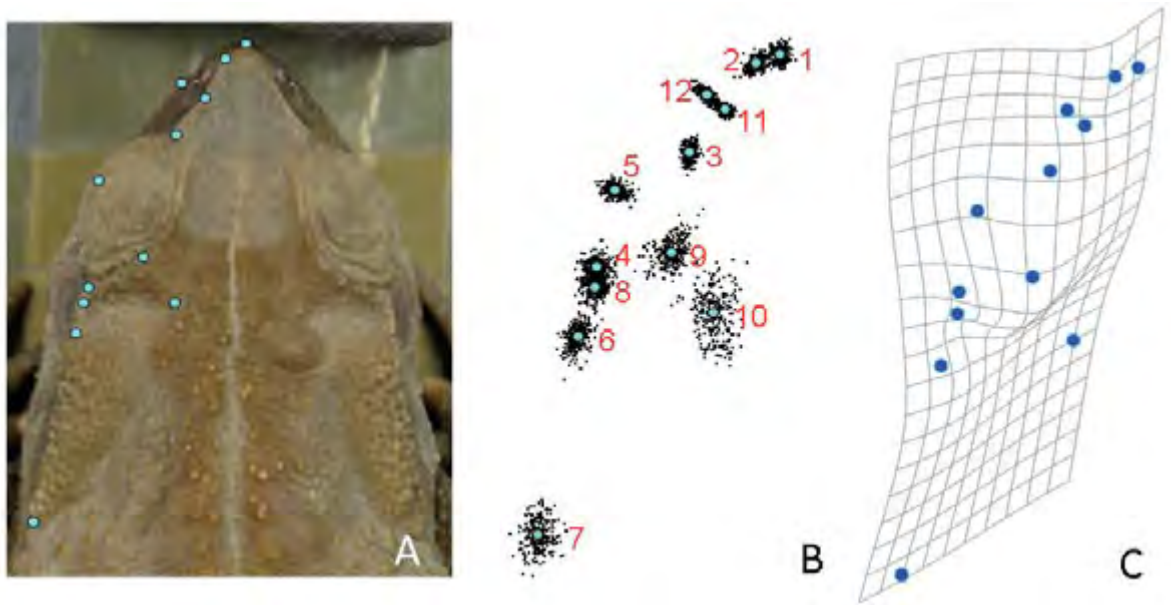

Figura. 2: marcos anatômicos definidos nesse estudo. A, exemplo de marcos posicionados em um espécimen, B, configuração consenso após superposição de Procrustres com as coordenadas referentes a cada espécimen em preto e a posição média dos marcos em azul claro e C, grade de transformação de forma (re–escalada por fator 2 para melhor visualização).

A ordenação dos escores dos espécimens nestes dois componentes principais não revela padrões óbvios de variação condizentes com as categorias consideradas neste estudo (unidades genéticas) (fig. 3). Esse resultado revela uma quantidade de variação moderada que dificulta a diferenciação de grupos sem que haja alguma informação preliminar. Este resultado não é surpreendente por duas razões, a primeira sendo a recente diversificação no grupo (Thomé et al. 2010), e em segundo lugar porque o crânio dos bufonídeos em geral é extremamente conservado (Martin 1972, Pramuk 2006). De fato, a maior parte dos marcos anatômicos utilizados nesse estudo foi definida sobre características do crânio. É possível ainda que a baixa variação encontrada seja fruto do método utilizado, que pode ter sido pouco eficiente na recuperação dos padrões de forma nestes animais. Entretanto, Vieira et al. (2008) obtiveram sucesso no uso de fotografias para extração de marcos anatômicos da morfologia externa de outro anuro. O fato destes autores terem sido capazes de discernir entre morfotipos dentro de uma mesma população sugere que esta hipótese seja menos provável.

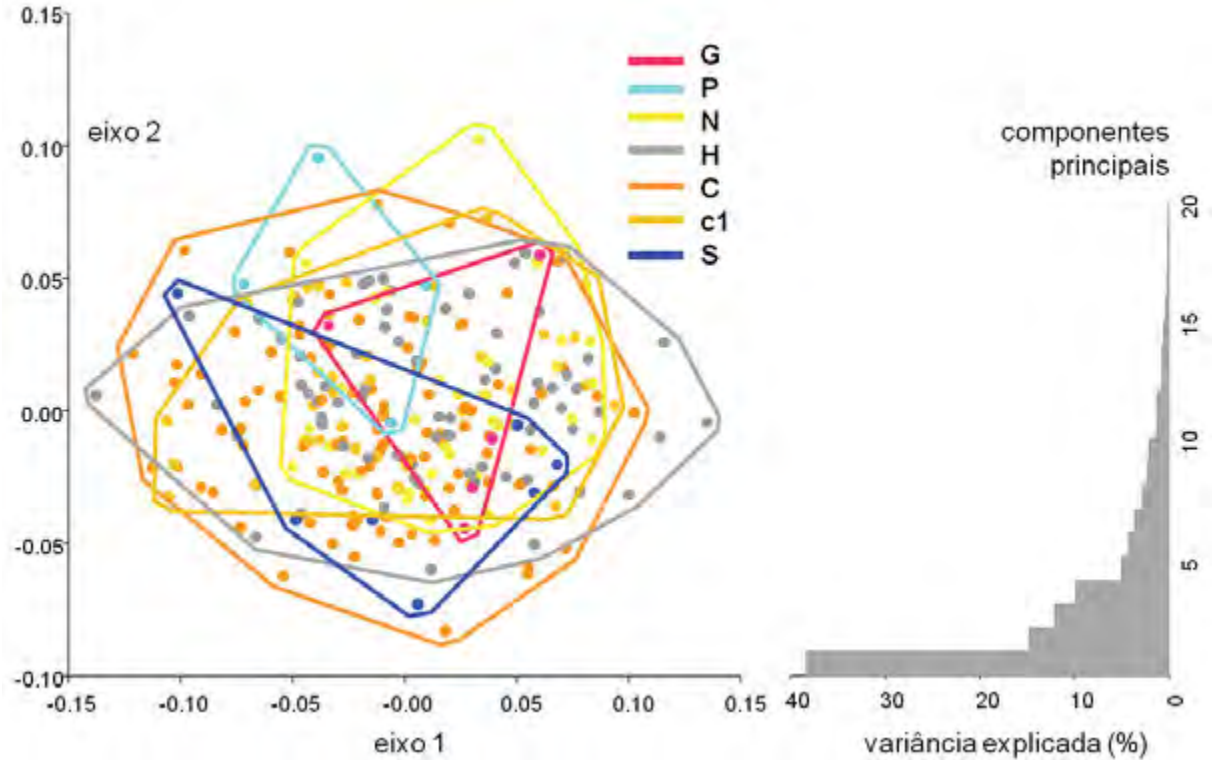

Figura. 3: Análise dos componentes principais representando os escores de cada espécimen amostrado relativamente aos dois primeiros eixos, e a quantidade de variância explicada por cada eixo.

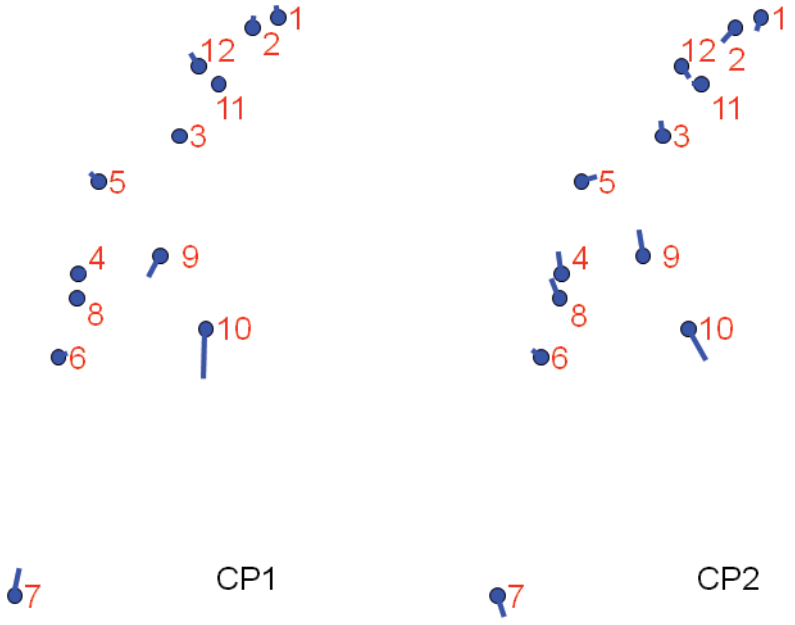

Figura 4: diagramas mostrando as mudanças de forma associadas ao componente principal 1 (CP1) e componente principal 2 (CP2). Círculos marcam a posição média dos marcos na configuração consenso e barras associadas marcam o comprimento e direção das mudanças explicada em cada eixo.

Nas análises de variação canônica consideramos primeiramente todas as unidades genéticas como categorias separadas (fig. 5A). Em seguida, fizemos a mesma análise classificando espécimens de unidades genéticas mais próximas em uma categoria única (fig. 5B). Na primeira análise as duas primeiras variáveis canônicas acomodaram 66,8% da variância, enquanto que na segunda análise essas variáveis acomodaram 77,2%. Por fim, fizemos uma terceira análise incluindo apenas os espécimens das unidades genéticas mais próximas (fig. 6), onde as duas primeiras variáveis canônicas responderam a 77,7% da variância. Graficamente, o resultado das duas primeiras análises é parecido, havendo o confinamento dos escores dos espécimens da unidade S a regiões mais periféricas do espaço multivariado, com exceção de um espécimen proveniente de Anitápolis, SC. Dados genéticos analisados no capítulo 2 dessa tese suportam que hibridação entre membros das unidades C e S ocorre com certa freqüência nessa localidade. A possibilidade deste espécimen constituir um híbrido poderia explicar a sua morfologia intermediária, evidenciada por seu posicionamento relativo nas análises canônicas.

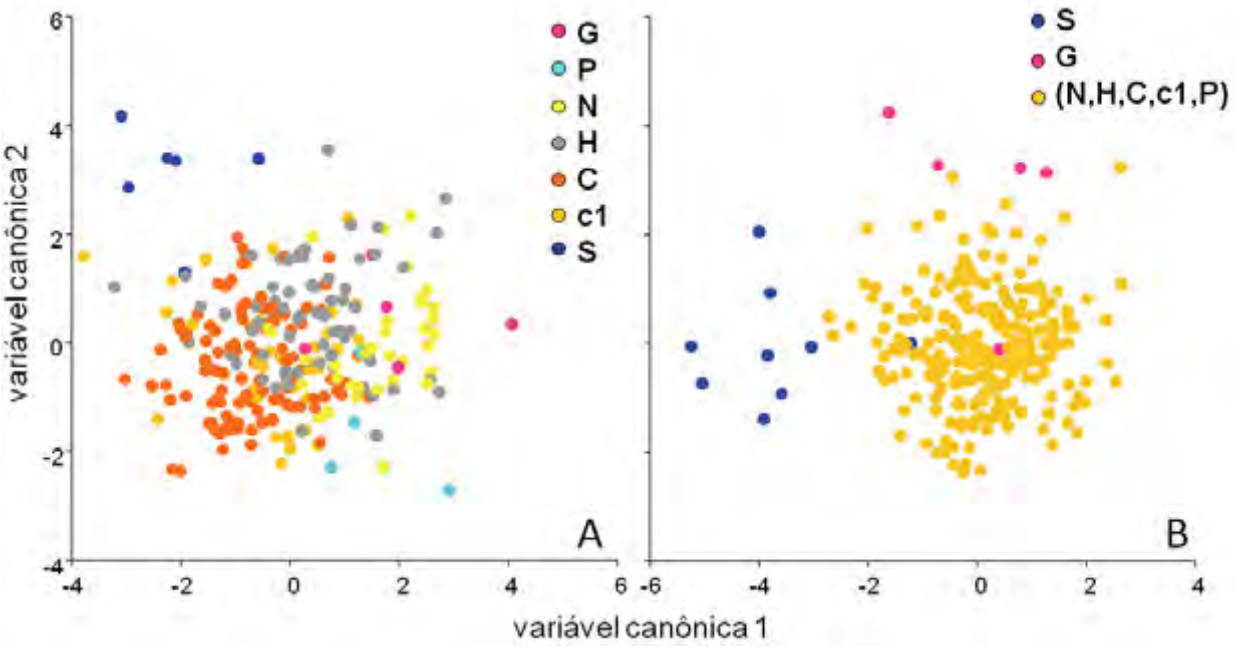

Figura 5: Análises de variação canônica. A, análise considerando todas as categorias separadamente e B, análise considerando categorias correspondentes a unidades genéticas próximas conjuntamente.

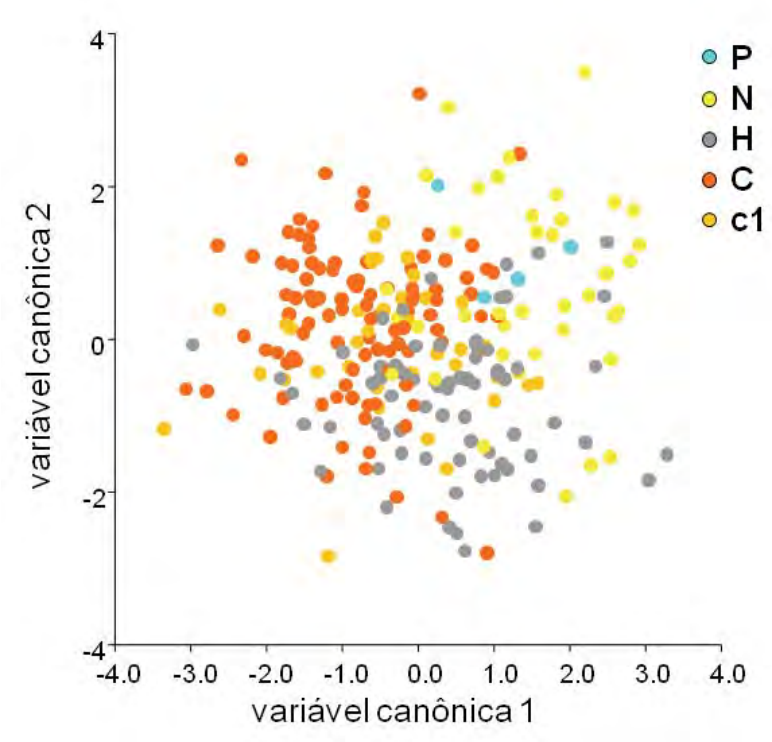

Figura 6: Análise de variação canônica das unidades genéticas mais próximas.

Na análise considerando as unidades genéticas mais próximas dentro de uma mesma categoria há uma melhor separação da unidade genética G, embora com alguma sobreposição. Já na análise considerando apenas as unidades genéticas mais próximas existe uma evidente sobreposição dos escores de todas as categorias. Testes de permutação indicaram que, de uma maneira geral, a variação de forma encontrada é suficiente para diferenciar significativamente indivíduos das unidades genéticas analisadas na maioria dos casos. As comparações onde essa diferenciação não foi significativa frequentemente envolveram a unidade G e, em menor grau, a unidade P. Estas duas unidades são as menos representadas nesse estudo (apêndice 1), o que enfraquece qualquer argumento para explicar este padrão. Ainda assim, a semelhança entre as unidades N e G é um padrão que se repete nas diversas análises (ver abaixo).

Foi encontrada ausência de variação significativa entre a unidade C e a sub–unidade c1 (tabela 2). Resultado semelhante foi encontrado na análise morfométrica de Baldissera et al. (2004), onde o polígono referente aos espécimes de *R. abei* (associado a c1 no presente

estudo) se encontrava aninhado no polígono de *R. ornata* (associado a C no presente estudo). De fato, a diagnose destas morfoespécies é extremamente difícil e subjetiva.

Ao mapear as variáveis de forma (nesse caso os dois primeiros componentes principais) sobre as duas filogenias disponíveis encontramos resultados diferentes. Seguindo a filogenia mitocondrial, a hipótese nula de ausência de sinal filogenético sobre a forma não pode ser rejeitada (fig. 7A, p = 0,27). Entretanto, ao considerar as unidades genéticas mais próximas conjuntamente temos um efeito claro da filogenia sobre a forma desses animais (fig 7B, p < 0,0001). As médias dos escores sobre os dois primeiros componentes principais na primeira análise novamente sugerem semelhança na forma dos espécimens das unidades genéticas N e G.

Tabela 2: testes de permutação referentes a análises canônicas supracitadas. Valores de p menores que 0,005 estão em negrito.

Distância de Mahalanobis							Distância de Procrustes					
	c1	**N**	**G**	**S**	**H**	**C**	**c1**	**N**	**G**	**S**	**H**	**C**
N	**<.0001**						**0.0002**					
G	**0.001**	0.1379					0.0618	0.6097				
S	**<.0001**	**<.0001**	**0.0011**				**0.0115**	**<.0001**	**0.0056**			
H	**<.0001**	**<.0001**	**0.0025**	**<.0001**			**0.0253**	**0.0231**	0.2666	**0.0044**		
C	**0.0217**	**<.0001**	**<.0001**	**<.0001**	**<.0001**		0.1309	**<.0001**	**0.0203**	**0.0069**	**<.0001**	
P	**0.0084**	**0.0081**	0.2433	**0.0005**	**0.0009**	**<.0001**	0.0649	**0.0044**	0.081	**0.0044**	**0.0169**	**0.0077**
	G	**S**					**G**	**S**				
S	**0.0003**						**0.0052**					
P,C,c1,N,H	**0.0067**	**<.0001**					0.1566	**0.0028**				
	c1	**N**	**H**	**C**			**c1**	**N**	**H**	**C**		
N	**<.0001**						**0.0001**					
H	**0.0001**	**<.0001**					**0.0266**	**0.0206**				
C	**0.019**	**<.0001**	**<.0001**				0.1319	**<.0001**	**<.0001**			
P	**0.0067**	**0.0076**	**0.0002**	**<.0001**			0.0621	**0.0056**	**0.0175**	**0.0101**		

Considerando as questões levantadas inicialmente na introdução, nossas análises preliminares de variação da forma em espécimens de *Rhinella* gr. *crucifer* indicam que existe variação de forma no grupo como um todo, sendo que todas as nossas expectativas foram contempladas. Entretanto, a variação encontrada é sutil, já que não foram encontrados padrões

de separação conspícuos na análise dos componentes principais. A análise de variação canônica considerando espécimens de unidades genéticas mais próximas apresentou poder de discriminação consideravelmente maior do que a análise onde todas as unidades estavam representadas como categorias. Esse resultado já é sugestivo de que a variação de forma no grupo está relacionada com a estruturação genética. Outros resultados que suportam a existência de sinal filogenético na forma das *Rhinella* gr. *crucifer* resultante das análises canônicas é a maior diferenciação da unidade S, seguida da unidade G, que são as unidades subseqüentemente mais divergentes segundo dados genéticos (capítulo 2 dessa tese), e a ausência de variação significativa entre as unidades C e c1. Ainda, os resultados da análise canônica excluindo as unidades S e G mostram um enorme grau de sobreposição nos escores dos espécimens das unidades P, C, N, dos possíveis híbridos e da sub–unidade c1.

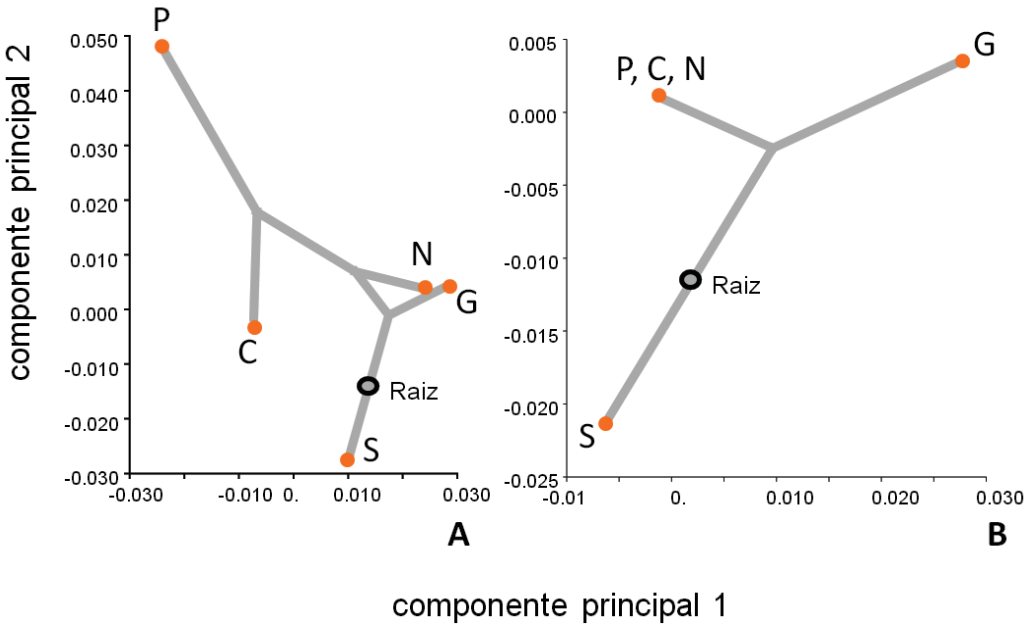

Figura 7: Médias dos escores de cada unidade genética sobre os dois primeiros eixos principais.

A história evolutiva do grupo prevê, em certo grau, a variação na forma desses animais, e maior confirmação desse padrão foi feita com otimização de variáveis de forma na árvore filogenética simplificada onde a influência da filogenia foi formalmente testada. De forma análoga ao que encontramos nas análises anteriores, esse efeito não é detectado quando unidades geneticamente próximas são consideradas separadamente. Entretanto, existem ainda

muitas questões a serem investigadas futuramente a partir dos dados produzidos neste capítulo. Um exemplo é verificar a participação relativa da filogenia e de variáveis ambientais (p.e. variáveis climáticas), já que cada unidade genética está restrita a regiões geográficas diferentes (capítulo 2 dessa tese). Esclarecer essa relação será um desafio, visto que as unidades mais divergentes se encontram geograficamente separadas em aproximadamente 25 graus de latitude, ocorrendo nos extremos norte e sul do bioma, onde as condições climáticas muito provavelmente diferem daquelas encontradas nas regiões mais centrais da Mata Atlântica.

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Apêndice 1– espécimens estudados

Tombo	Unidade	Município	UF
CFBH28170	G	Guaramiranga	CE
CFBH28171	G	Guaramiranga	CE
CFBH28172	G	Guaramiranga	CE
CFBH28174	G	Guaramiranga	CE
CFBH28175	G	Guaramiranga	CE
CFBH18717	N	Aurelino Leal	BA
CFBH24628	N	Camacan	BA
CFBH24629	N	Camacan	BA
CFBH24630	N	Camacan	BA
CFBH13361	N	Caraíva	BA
CFBH13365	N	Caraíva	BA
CFBH13363	N	Caraíva	BA
CFBH13364	N	Caraíva	BA
CFBH13365	N	Caraíva	BA
CFBH13366	N	Caraíva	BA
CFBH13367	N	Caraíva	BA
CFBH13368	N	Caraíva	BA
CFBH13370	N	Caraíva	BA
CFBH13371	N	Caraíva	BA
CFBH13372	N	Caraíva	BA
CFBH13374	N	Caraíva	BA
CFBH13399	N	Caraíva	BA
CFBH2583	N	Ilhéus	BA
CFBH2898	N	Ilhéus	BA
CFBH2901	N	Ilhéus	BA
CFBH2903	N	Ilhéus	BA
CFBH2904	N	Ilhéus	BA
CFBH27907	N	Palmeiras	BA
CFBH27996	N	Santa Bárbara	BA
CFBH21056	N	Uruçuca	BA
CFBH21058	N	Uruçuca	BA
CFBH21113	N	Uruçuca	BA
CFBH23368	N	Uruçuca	BA
PUC7534	N	Cristalia	MG
PUC7535	N	Cristalia	MG
PUC7536	N	Cristalia	MG
PUC7537	N	Cristalia	MG
PUC7538	N	Cristalia	MG
PUC7539	N	Cristalia	MG
PUC7540	N	Grão Mogol	MG
PUC4023	N	Santa Maria do Salto	MG
PUC4024	N	Santa Maria do Salto	MG
PUC6178	N	Santa Maria do Salto	MG
PUC9705	N	São João do Paraíso	MG
CFBH23404	N	João Pessoa	PB
CFBH2834	H	Aracruz	ES
CFBH2865	H	Aracruz	ES

Tombo	Unidade	Município	UF
CFBH2866	H	Aracruz	ES
CFBH2867	H	Aracruz	ES
PUC2841	H	Cariacica	ES
PUC2842	H	Cariacica	ES
CFBH23211	H	Conceição da Barra	ES
CFBH2427	H	Conceição da Barra	ES
CFBH18090	H	Linhares	ES
CFBH18091	H	Linhares	ES
CFBH958	H	Linhares	ES
CFBH959	H	Linhares	ES
CFBH18001	H	Santa Leopoldina	ES
CFBH4177	H	Santa Teresa	ES
CFBH2874	H	Vila Velha	ES
CFBH2875	H	Vila Velha	ES
CFBH2876	H	Vila Velha	ES
CFBH2877	H	Vila Velha	ES
CFBH23210	H	Vitória	ES
CFBH22816	H	Belo Horizonte	MG
PUC3525	H	Belo Horizonte	MG
PUC3526	H	Belo Horizonte	MG
PUC3527	H	Belo Horizonte	MG
PUC3528	H	Belo Horizonte	MG
PUC3530	H	Belo Horizonte	MG
PUC3534	H	Belo Horizonte	MG
PUC3538	H	Belo Horizonte	MG
PUC5143	H	Belo Horizonte	MG
PUC5144	H	Belo Horizonte	MG
PUC5176	H	Belo Horizonte	MG
PUC5572	H	Belo Horizonte	MG
PUC6003	H	Belo Horizonte	MG
PUC3150	H	Brumadinho	MG
PUC11671	H	Caeté	MG
PUC762	H	Caeté	MG
PUC863	H	Caeté	MG
PUC4977	H	Conceição do Mato Dentro	MG
PUC6947	H	Conselheiro Lafaiete	MG
PUC962	H	Guanhães	MG
PUC1384	H	Joanésia	MG
PUC6064	H	Juíz de Fora	MG
PUC11666	H	Lima Duarte	MG
PUC11667	H	Lima Duarte	MG
PUC12565	H	Mar de Espanha	MG
PUC11940	H	Nova Lima	MG
PUC11941	H	Nova Lima	MG
PUC11942	H	Nova Lima	MG
PUC11943	H	Nova Lima	MG
PUC11944	H	Nova Lima	MG
PUC9445	H	Nova Lima	MG
PUC6959	H	Ouro Branco	MG
PUC7551	H	Ouro Branco	MG
PUC7552	H	Ouro Branco	MG

Tombo	Unidade	Município	UF
PUC7553	H	Ouro Branco	MG
PUC12035	H	Rio Piracicaba	MG
PUC11911	H	São Gonçalo do Rio Abaixo	MG
PUC1914	H	São Gonçalo do Rio Abaixo	MG
PUC11707	H	São Gonçalo do Rio Preto	MG
PUC1966	H	Serra do Caraça	MG
PUC5693	H	Varginha	MG
PUC5694	H	Varginha	MG
CFBH2869	H	Itaguaí	RJ
CFBH2870	H	Itaguaí	RJ
CFBH2871	H	Itaguaí	RJ
CFBH1377	H	Parati	RJ
CFBH5066	H	Parati	RJ
CFBH5786	H	Parati	RJ
CFBH13938	H	Petrópolis	RJ
CFBH28097	H	Santa Maria Madalena	RJ
CFBH28098	H	Santa Maria Madalena	RJ
CFBH28099	H	Santa Maria Madalena	RJ
CFBH28100	H	Santa Maria Madalena	RJ
CFBH18815	H	Teresópolis	RJ
CFBH18816	H	Teresópolis	RJ
MZUSP142100	P	Januária	MG
MZUSP142105	P	Januária	MG
MZUSP142362	P	Januária	MG
MZUSP142101	P	Januária	MG
CFBH14647	C	Cristina	MG
CFBH9937	C	Cristina	MG
IIBPH1191	C	Itapúa	Paraguai
IIBPH1333	C	Canindeyú	Paraguai
IIBPH1342	C	Itapúa	Paraguai
IIBPH1563	C	Itapúa	Paraguai
IIBPH1564	C	Itapúa	Paraguai
IIBPH1565	C	Itapúa	Paraguai
IIBPH1342	C	Itapúa	Paraguai
IIBPH1567	C	Itapúa	Paraguai
CFBH24271	C	Derrubadas	RS
CFBH6371	C	Barra do Turvo	SP
CFBH6372	C	Barra do Turvo	SP
CFBH6364	C	Cananéia	SP
CFBH6366	C	Cananéia	SP
CFBH6368	C	Cananéia	SP
CFBH6369	C	Cananéia	SP
CFBH1689	C	Capão Bonito	SP
CFBH3727	C	Caraguatatuba	SP
CFBH15345	C	Ilha Bela	SP
CFBH15349	C	Ilha Bela	SP
CFBH15351	C	Ilha Bela	SP
CFBH15352	C	Ilha Bela	SP
CFBH15353	C	Ilha Bela	SP
CFBH15354	C	Ilha Bela	SP
CFBH15356	C	Ilha Bela	SP

Tombo	Unidade	Município	UF
CFBH15358	C	Ilha Bela	SP
CFBH15359	C	Ilha Bela	SP
CFBH15360	C	Ilha Bela	SP
CFBH15361	C	Ilha Bela	SP
CFBH15362	C	Ilha Bela	SP
CFBH15363	C	Ilha Bela	SP
CFBH15364	C	Ilha Bela	SP
CFBH15365	C	Ilha Bela	SP
CFBH15366	C	Ilha Bela	SP
CFBH15368	C	Ilha Bela	SP
CFBH15369	C	Ilha Bela	SP
CFBH15379	C	Ilha Bela	SP
CFBH15383	C	Ilha Bela	SP
CFBH15385	C	Ilha Bela	SP
CFBH15386	C	Ilha Bela	SP
CFBH15390	C	Ilha Bela	SP
CFBH17400	C	Ilha Bela	SP
CFBH7753	C	Ilha Bela	SP
CFBH7754	C	Ilha Bela	SP
CFBH7755	C	Ilha Bela	SP
CFBH6336	C	Iporanga	SP
CFBH725	C	Jundiaí	SP
CFBH726	C	Jundiaí	SP
CFBH727	C	Jundiaí	SP
CFBH6902	C	Ribeirão Branco	SP
CFBH6903	C	Ribeirão Branco	SP
CFBH1373	C	Rio Claro	SP
CFBH5081	C	Rio Claro	SP
CFBH5082	C	Rio Claro	SP
CFBH5083	C	Rio Claro	SP
CFBH8353	C	Rio Claro	SP
CFBH8354	C	Rio Claro	SP
CFBH8355	C	Rio Claro	SP
CFBH8358	C	Rio Claro	SP
CFBH8359	C	Rio Claro	SP
CFBH8360	C	Rio Claro	SP
CFBH7193	C	Santo Antônio dos Pinhais	SP
CFBH7194	C	Santo Antônio dos Pinhais	SP
CFBH23919	C	Santos	SP
CFBH14812	C	São Luís do Paraitinga	SP
CFBH16310	C	São Luís do Paraitinga	SP
CFBH16311	C	São Luís do Paraitinga	SP
CFBH8911	C	São Luís do Paraitinga	SP
CFBH7665	C	São Sebastião	SP
CFBH7666	C	São Sebastião	SP
CFBH18382	C	Teodoro Sampaio	SP
CFBH18387	C	Teodoro Sampaio	SP
CFBH118	C	Ubatuba	SP
CFBH1206	C	Ubatuba	SP
CFBH1208	C	Ubatuba	SP
CFBH1210	C	Ubatuba	SP

Tombo	Unidade	Município	UF
CFBH1216	C	Ubatuba	SP
CFBH1218	C	Ubatuba	SP
CFBH1219	C	Ubatuba	SP
CFBH1220	C	Ubatuba	SP
CFBH12545	C	Ubatuba	SP
CFBH12547	C	Ubatuba	SP
CFBH12549	C	Ubatuba	SP
CFBH12551	C	Ubatuba	SP
CFBH12552	C	Ubatuba	SP
CFBH12553	C	Ubatuba	SP
CFBH12554	C	Ubatuba	SP
CFBH12558	C	Ubatuba	SP
CFBH12559	C	Ubatuba	SP
CFBH12563	C	Ubatuba	SP
CFBH12564	C	Ubatuba	SP
CFBH16518	C	Ubatuba	SP
CFBH1683	C	Ubatuba	SP
CFBH1748	C	Ubatuba	SP
CFBH1749	C	Ubatuba	SP
CFBH1750	C	Ubatuba	SP
CFBH2907	C	Ubatuba	SP
CFBH2908	C	Ubatuba	SP
CFBH2909	C	Ubatuba	SP
CFBH326	C	Ubatuba	SP
CFBH327	C	Ubatuba	SP
CFBH328	C	Ubatuba	SP
CFBH329	C	Ubatuba	SP
CFBH22897	c1	Morretes	PR
CFBH23165	c1	Morretes	PR
CFBH23166	c1	Morretes	PR
CFBH2837	c1	Morretes	PR
CFBH18141	c1	Quatro Barras	PR
CFBH20282	c1	Anitápolis	SC
CFBH20283	c1	Anitápolis	SC
CFBH20284	c1	Anitápolis	SC
CFBH20279	c1	Anitápolis	SC
CFBH9965	c1	Anitápolis	SC
CFBH9966	c1	Anitápolis	SC
CFBH18163	c1	Blumenau	SC
CFBH18164	c1	Blumenau	SC
CFBH18166	c1	Blumenau	SC
CFBH18168	c1	Blumenau	SC
CFBH18169	c1	Blumenau	SC
CFBH2858	c1	Blumenau	SC
CFBH22659	c1	Governador Celso Ramos	SC
CFBH21317	c1	Guaratuba	SC
CFBH2916	c1	Guaratuba	SC
CFBH2918	c1	Guaratuba	SC
CFBH8458	c1	Itapema	SC
CFBH2841	c1	Itapoá	SC
CFBH18175	c1	Massaranduba	SC

Tombo	Unidade	Município	UF
CFBH18176	c1	Massaranduba	SC
CFBH18177	c1	Massaranduba	SC
CFBH18178	c1	Massaranduba	SC
CFBH18179	c1	Massaranduba	SC
CFBH18180	c1	Massaranduba	SC
CFBH18181	c1	Massaranduba	SC
CFBH18182	c1	Massaranduba	SC
CFBH2842	c1	Rio dos Cedros	SC
CFBH2843	c1	Rio dos Cedros	SC
CFBH2844	c1	Rio dos Cedros	SC
CFBH23728	c1	Siderópolis	SC
CFBH23729	c1	Siderópolis	SC
CFBH9851	c1	Treviso	SC
CFBH18238	S	Bento Gonçalves	RS
CFBH20277	S	Anitápolis	SC
CFBH20278	S	Anitápolis	SC
CFBH20279	S	Anitápolis	SC
CFBH20280	S	Anitápolis	SC
CFBH20281	S	Anitápolis	SC
CFBH21230	S	Florianópolis	SC

CONCLUSÕES GERAIS

i. A diversidade genética no grupo de *Rhinella crucifer* é profundamente estruturada segundo marcadores mitocondriais. Fragmentos de DNA nuclear são marcados por sorteamento incompleto de linhagens, sendo sua estrutura melhor observada com análises de freqüência alélica.

ii. Os dois tipos de marcadores suportam a existência de cinco espécies delimitáveis geneticamente, aqui referidas como unidades genéticas. Duas destas apresentam distribuições restritas e periféricas, enquanto três unidades genéticas constituem a área nuclear de distribuição para o grupo. Encontramos evidência de hibridação para dois pares de unidades, com desequilíbrio cito-nuclear para um dos pares.

iii. A correspondência entre unidades genéticas e morfoespécies é limitada: existe clara associação entre a unidade mais ao sul e *R. henseli*, mas não entre outras unidades e espécies. *R. pombali* coincide com uma das 2 zona híbridas encontradas, e sugerimos que esta espécie deve ser invalidada.

iv. Segundo a distribuição das três unidades genéticas centrais, o bioma pode ser dividido nas regiões norte, centro e sul. A divergência mais antiga, datada do Plioceno, separa as populações da unidade mais ao sul do bioma. O restante das quebras genéticas entre unidades e sub–estruturação apresentam divergências Pleistocênicas.

v. Algumas quebras genéticas coincidem geograficamente com as barreiras geográficas putativas e zonas de intergradação ecológica, associadas algumas vezes a fluxo gênico assimétrico. O papel de barreiras específicas ainda precisa ser investigado.

vi. Nossos modelos de distribuição paleoecológica suportam fragmentação de habitat associada a ciclos glaciais. A diversificação em refúgios Plio–Pleistocênicos segundo este modelo e segundo duas outras configurações de possíveis refúgios da literatura foi testada com dados genéticos. Todos os modelos foram rejeitados. A diversidade foi algumas vezes maior para as áreas de não-refúgio.

vii. Análises demográficas revelaram histórias diferentes para 3 regiões do bioma, com expansão populacional moderada na região norte, crescimento um pouco mais acentuado na região central e estabilidade demográfica nas populações do sul.

viii. Simulações coalescentes desenhadas para testar cenários demográficos alternativos permitiram rejeitar hipóteses de diversificação envolvendo vicariância associada a refúgios e colonizações recentes, suportando a influência de barreiras geográficas na diversificação genética observada. De maneira geral, os dados suportam uma história complexa para o grupo e para o bioma.

ix. Análises preliminares da variação de forma no grupo indicam que existe variação no grupo como um todo, mas a variação é sutil e está relacionada a história evolutiva do grupo, mas a influência de variáveis ambientais ainda precisa ser testada.