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Diversidade, Distribuição e Diversificação do gênero *Brachycephalus* Fitzinger, 1826 (Anura: Brachycephalidae)

THAIS HELENA CONDEZ

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

Julho - 2014

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TÍTULO: Diversidade, Distribuição e Diversificação do gênero Brachycephalus Fitzinger, 1826 (Anura: Brachycephalidae)

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RESUMO

O gênero *Brachycephalus* é endêmico da Mata Atlântica e atualmente compreende 21 espécies, em sua maioria com distribuição restrita a áreas de altitude ao longo das principais cadeias de montanhas do bioma. Estas espécies exibem tamanho reduzido e compartilham características morfológicas especializadas devido ao processo evolutivo de miniaturização. Apesar da recente descoberta de novas espécies para o gênero, pouco se conhece acerca de sua diversidade, distribuição e história evolutiva. Neste trabalho estudamos a variação de caracteres morfológicos, ecológicos e genéticos das populações de *Brachycephalus* ao longo de sua distribuição geográfica. No primeiro capítulo descrevemos a variação morfométrica do gênero com base em uma análise de mais de mil indivíduos, pertencentes a diversas populações representativas da diversidade de espécies ao longo de sua distribuição. Identificamos dois grupos de espécies baseados no tamanho do corpo e largura do focinho, que são congruentes com variações em altitude e complexidade topográfica nas localidades de ocorrência. Entre as espécies de altitude elevada comprovamos a relação entre latitude e tamanho do corpo; aquelas com maior tamanho do corpo foram encontradas em ambientes com temperaturas mais elevadas. Entre as espécies de ampla distribuição altitudinal, conhecidas popularmente como sapos-pulga, encontramos pouca variação morfométrica. No segundo capítulo avaliamos a hipótese taxonômica atual, mapeamos e reconstruímos os estados de caracteres ancestrais para coloração, tamanho do corpo, canto de anúncio e distribuições altitudinal e geográfica. Nossos resultados confirmam que *Brachycephalus* é monofilético e não encontramos evidências para distinção entre os sapos-pulga (antigamente chamados de *Psyllophryne*) e as demais espécies do gênero. Os caracteres analisados variaram entre os clados identificados e sugerem que as espécies atuais tiveram um ancestral de coloração críptica, sem hiperossificação de elementos do esqueleto, com ampla distribuição altitudinal e canto de anúncio curto, composto por notas de alta frequência com um a três pulsos. No terceiro capítulo utilizamos uma amostragem populacional mais completa para inferir a relação entre as espécies e descrever a diversidade genética do gênero. As análises realizadas com os três fragmentos de DNA mitocondrial revelaram um tempo de divergência antigo para as principais linhagens de *Brachycephalus* e a influência de barreiras geográficas e áreas históricas de estabilidade durante do Oligoceno, Mioceno e Quaternário. Adicionalmente, evidenciamos que resistências climáticas e da paisagem influenciam o fluxo gênico entre as populações, causando maior isolamento reprodutivo e maiores oportunidades de diversificação na porção tropical da Mata Atlântica.

Palavras-chave: anfíbios, diversificação, *Brachycephalus,* Mata Atlântica, altitude

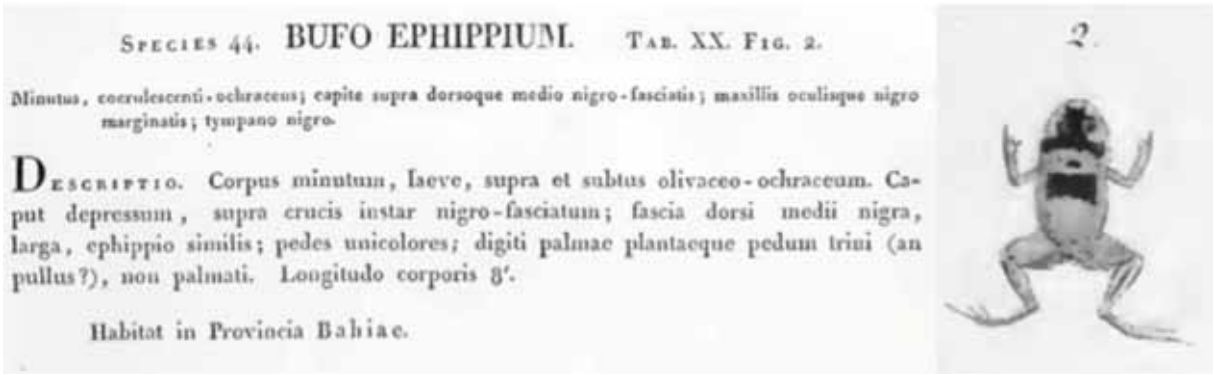
ABSTRACT

The genus *Brachycephalus* is endemic to the Atlantic forest of Brazil and currently comprises 21 species. Most of them are restricted to high elevation areas along the main mountain ranges of the biome. These species exhibit extremely small adult body size and share morphological features due to evolutionary process of miniaturization. Despite of the recent description of new species, some characteristics, such as diversity, species distribution, and evolutionary history, are still poor understood. Here, we addressed the variation of morphological, ecological, and genetic traits of *Brachycephalus* populations along their distribution. In the first chapter we described morphometric variation within more than one thousand individuals from several populations and species across the genus distribution. We identified two main groups of species based on body size and snout width variation. These groups are congruent with localities variation in altitude and topographic complexity. We evidenced a relationship between latitude and body size in species associated to high elevation. Higher temperatures were correlated with larger body sizes. Within the flea-toads, the altitudinal widespread species, we found little morphometric variation. In the second chapter we evaluated the current taxonomic hypotheses, mapped and reconstructed the ancestral states for body color, body size, advertisement call, and altitudinal and geographic distributions. Our data confirmed that the genus is monophyletic and we found no molecular evidence of distinction between flea-toads (the formerly called *Psyllophryne*) and other *Brachycephalus* species. The analyzed traits were variable across the main clades and our data suggested that the extant species evolved from a dull colored ancestor, without hyperossification in skull and postcranial skeleton, with wide altitudinal range, and short advertisement call composed by one to four high frequency notes with 1-3 pulses. In the third chapter we used more data on populations to infer species relationships and describe the genetic diversity of the genus. The mtDNA analysis showed relatively older times of diversification for *Brachycephalus*, being influenced by geographic barriers and historical areas of stability during the Oligocene, Miocene, and Quaternary. Additionally, we found that climate and landscape resistances to gene flow increase reproductive isolation and consequently the opportunities for diversification in northern montane Atlantic forest.

Key-words: amphibians, diversification, *Brachycephalus,* Atlantic forest, altitude.

INTRODUÇÃO GERAL

O gênero *Brachycephalus* foi descrito pelo zoólogo austríaco Leopold Joseph Franz Johann Fitzinger em 1826 para contemplar a primeira e até então única espécie de *Brachycephalus* conhecida (FROST, 2014). *Brachycephalus ephippium* foi originalmente descrito em 1824, como *Bufo ephippium*, por Johann Baptist von Spix (Figura 1). O zoólogo Spix e o botânico Karl Friedrich Philip von Martius vieram pela primeira vez ao Brasil em 1817, enviados pela Academia de Ciências de Munique, Alemanha, com o principal objetivo de pesquisar e catalogar espécies da fauna e flora brasileira (FITTKAU, 2001). Como resultado desta viagem, e menos de seis anos depois, Spix descreveu cerca de 500 espécies e seus trabalhos constituem importantes referências históricas para a zoologia brasileira.



SPECIES 44. BUFO EPHIPPIUM. TAB. XX. FIG. 2.

Minutus, coerulescenti-ochraceus; capite supra dorsoque medio nigro-fasciatis; maxillis oculisque nigro marginatis; tympano nigro.

DESCRIPTIO. Corpus minutum, laeve, supra et subtus olivaceo-ochraceum. Caput depressum, supra crucis instar nigro-fasciatum; fascia dorsi medii nigra, larga, ephippio similis; pedes unicolores; digiti palmae plantaeque pedum trini (an pullus?), non palmati. Longitudo corporis 8'.

Habitat in Provincia Bahiae.

2.

Figura 1. Descrição original de *Bufo ephippium* de Johann von Spix (1824). Dois anos depois a espécie foi atribuída ao gênero *Brachycephalus* por Leopold Fizinger (1986).

A localidade-tipo descrita para *Brachycephalus ephippium* no trabalho de Spix é a província da Bahia, criada em 1821. Entretanto, a viagem de Spix e Martius percorreu diversas províncias brasileiras, com destaque para a província da Amazônia e do Rio de Janeiro. Esta atribuição da localidade tipo de *B. ephippium* como "Provincia Bahiae" é duvidosa e pode ser decorrente de um erro de rotulagem do material tipo coletado, uma vez que este é o único *Brachycephalus ephippium* supostamente proveniente desta região (POMBAL et al., 1998). Além disso, Spix e Martius passaram pela região da Serra dos Órgãos, Teresópolis, Rio de Janeiro, onde atualmente a ocorrência da espécie é comum. O Rio de Janeiro é por essa razão indicado como provável localidade de origem do material descrito por Spix (POMBAL et al., 1998). Alguns anos depois, em 1835, Cocteau coletou exemplares de *Brachycephalus ephippium* no Rio de Janeiro, os quais acreditava serem

espécies diferentes da descrita por Spix, por serem totalmente amarelos em vida (COCTEAU, 1835). Alguns anos mais tarde, o brasileiro Alípio de Miranda-Ribeiro comprovou tratar-se de uma diferença de fixação do material, e foi o primeiro pesquisador a avaliar as "variedades" da espécie descrita por Spix (MIRANDA-RIBEIRO, 1920). Além da descrição de *Brachycephalus nodoterga*, as contribuições de Miranda-Ribeiro para o estudo do gênero foram recentemente avaliadas e resultaram na revalidação de outras três espécies: *B. atelopoide*, *B. bufonoides* e *B. garbeanus* (POMBAL, 2010).

As espécies *Psyllophryne didactylus* e *Psyllophryne hermogenesi* foram descritas em 1971 e 1998, respectivamente (IZECKSOHN, 1971; GIARETTA & SAWAYA, 1998). Além destas espécies, *Brachycephalus pernix* foi também descrito, ampliando a distribuição geográfica do gênero ao estado do Paraná, no sul do país (POMBAL et al., 1998). Apesar das semelhanças, *Brachycephalus* e *Psyllophryne* foram considerados gêneros distintos por anos, com base na presença de *omosternum* na cintura peitoral de *Psyllophryne* e ausência em *Brachycephalus*. Atualmente, o gênero *Psyllophryne* é considerado sinônimo de *Brachycephalus*, baseado na presença de *omosternum* reduzido em *Brachycephalus ephippium* (KAPLAN, 2002). Entretanto, as duas espécies originalmente descritas como *Psyllophryne*, e popularmente conhecidas como sapos-pulga, apresentam tamanho do corpo menor em relação às demais espécies do gênero, padrão escuro de coloração, não possuem estruturas ossificadas no dorso e exibem habilidade de saltar em longas distâncias (IZECKSOHN, 1971; GIARETTA & SAWAYA, 1998). Também apresentam distribuição geográfica e altitudinal ampla, ocorrendo desde o nível do mar até aproximadamente 700 metros de altitude em diversas localidades nos estados do Paraná, São Paulo, Rio de Janeiro e Espírito Santo (IZECKSOHN, 1971; GIARETTA & SAWAYA, 1998; VERDADE et al. 2008; OLIVEIRA et al., 2011; OLIVEIRA et al., 2012). Apenas mais uma espécie próxima aos sapos-pulga foi descrita desde então, por Napoli e colaboradores (2011). Considerando a ampla distribuição geográfica, seu tamanho pequeno e coloração críptica é possível que, assim como para as demais espécies do gênero, a diversidade dos sapos-pulga esteja sendo subestimada.

A maior parte da diversidade conhecida atualmente para o gênero resulta da descrição recente de novas espécies, intensificada a partir do início do século XXI principalmente com as amostragens nos maciços serranos e áreas de altitude no Sul e Sudeste do Brasil. O gênero é endêmico da Mata Atlântica, composto atualmente por 21 espécies distribuídas em aproximadamente 1600 km, desde o estado da Bahia (15°23'S, 39°33'W; NAPOLI et al., 2011) até Santa Catarina (26° 47′ S, 48° 55′ W; PIE et al., 2013) (Figura 2).

Figura 2. Diversidade de espécies no gênero *Brachycephalus*. Da esquerda para a direita, início acima: *B. alipioi*, *B. brunneus*, *B. crispus*, *B. didactylus*, *B. ephippium*, *B. ferruginus*, *B. garbeanus*, *B. guarani*, *B. hermogenesi*, *B. margaritatus*, *B. nodoterga*, *B. pernix*, *B. pitanga*, *B. pulex*, *B. toby*, *B. tridactylus*, *B. vertebralis*, *B.* sp. 1, *B.* sp. 2, *B.* sp. 3, *B.* sp. 4, *B.* sp. 5, *B.* sp. 6, *B.* sp. 8, *B.* sp. 9, *B.* sp. 9, *B.* sp. 10 e *B.* sp. 11. Créditos das fotos: *B. alipioi* (J.L. Gasparini), *B. brunneus* e *B. garbeanus* (C.F.B. Haddad), *B. didactylus* e *B. pernix* (J.P. Pombal Jr.), *B. margaritatus* (I. Nunes), *B. tridactylus* (M.V. Garey), demais (T.H. Condez).

Em ordem cronológica, as espécies atualmente incluídas neste gênero são: *B. ephippium* (Spix, 1824), *B. atelopoide* Miranda-Ribeiro, 1920, *B. bufonoides* Miranda-Ribeiro, 1920, *B. garbeanus* Miranda-Ribeiro, 1920, *B. nodoterga* Miranda-Ribeiro, 1920, *B. didactylus* (Izecksohn, 1971), *B. pernix* Pombal, Wistuba & Bornschein, 1998, *B. hermogenesi* (Giaretta & Sawaya, 1998), *B. vertebralis* Pombal Jr., 2001, *B. brunneus* Ribeiro, Alves, Haddad & dos Reis, 2005, *B. izecksohni* Ribeiro, Alves, Haddad & dos Reis, 2005, *B. ferruginus* Alves, Ribeiro, Haddad & dos Reis, 2006, *B. pombali* Alves, Ribeiro, Haddad & dos Reis, 2006, *B. alipioi* Pombal & Gasparini, 2006, *B. pitanga* Alves, Sawaya, dos Reis & Haddad, 2009, *B. toby* Haddad, Alves, Clemente-Carvalho & dos Reis, 2010, *B. pulex* Napoli, Caramaschi, Cruz & Dias, 2011, *B. margaritatus* Pombal & Izecksohn 2011, *B. tridactylus* Garey, Lima, Hartmann & Haddad, 2012, *B. guarani* Clemente-Carvalho, Giaretta, Condez, Haddad & dos Reis, 2012 e *B. crispus* Condez, Clemente-Carvalho, Haddad & dos Reis, 2014. Estas espécies, juntamente com as espécies do gênero *Ischnochnema*, compõem a família Brachycephalidae (FROST, 2014). Essa família é atualmente composta por 54 espécies sendo caracterizada pelo compartilhamento de caracteres moleculares e morfológicos, incluindo o hábito terrestre associado à serapilheira e o desenvolvimento direto, em que ovos geralmente de tamanho grande eclodem diretamente em pequenos sapos, não passando pelo estágio de girino (HEDGES et al., 2008).

A maioria das espécies de *Brachycephalus* (18 espécies ou 85% da diversidade atual) ocorre em populações restritas a encostas e topos de morro na Mata Atlântica, em elevações de 600-1800 metros (POMBAL et al., 1998; CONDEZ et al., 2014). Apresentam tamanho do corpo reduzido, com comprimento rostro-cloacal geralmente inferior a 18 mm (CLEMENTE-CARVALHO et al., 2011). Com tamanho médio de 8.7 mm, *Brachycephalus didactylus* é considerado entre os dez menores vertebrados do mundo, ocupando a terceira posição depois dos microhylídeos de Nova Guiné *Paedophryne amauensis* e *Paedophryne swiftorum* (RIIMEYER et al., 2012). Considerando os processos evolutivos de miniaturização, espécies de *Brachycephalus* compartilham características morfológicas como a completa ausência ou redução de ossos e hiperossificação de alguns elementos do esqueleto (ALBERCH & GALE, 1985; HANKEN, 1993; CLEMENTE-CARVALHO et al., 2009). Em relação à morfologia, encontram-se disponíveis informações sobre os padrões de hiperossificação do crânio e coluna vertebral e morfologia da região auditiva de algumas espécies (CAMPOS, 2007, SILVA et al., 2007, CLEMENTE-CARVALHO et al., 2009). Outra importante característica deste gênero é a coloração conspícua que varia geralmente do amarelo até o alaranjado presente em várias espécies, associada a presença de tetrodotoxina e análogos na pele, fígado

e ovários (SEBBEN et al., 1986; HANIFIN, 2010; PIRES et al., 2002; 2005). Até o momento, pouco se sabe sobre a ocorrência destas toxinas e suas possíveis implicações comportamentais e ecológicas nas diferentes espécies do gênero.

A hipótese filogenética proposta para o gênero *Brachycephalus* indica três clados principais, coincidentes com a distribuição geográfica das espécies e o padrão de hiperossificação do esqueleto (CLEMENTE-CARVALHO et al., 2011). Embora estes agrupamentos principais tenham sido corroborados por estudos recentes (e.g. PADIAL et al., 2014), a relação entre as espécies ainda é pouco clara. A falta de resolução filogenética no relacionamento entre grupos ou espécies e, principalmente, a amostragem insuficiente da diversidade reforçam a necessidade de novos estudos com esta abordagem. Alguns aspectos de história natural e ecologia de *Brachycephalus* ainda são pouco conhecidos. O difícil acesso a maioria das localidades onde é prevista a ocorrência de espécies e sua possível atividade discreta sob a serapilheira resulta em uma grande dificuldade na coleta de dados (CONDEZ et al., 2014). Dados sobre comportamento reprodutivo são restritos a *B. ephippium* (POMBAL, 1999). Hábitos alimentares e outros aspectos ecológicos são conhecidos para *B. brunneus* (FONTOURA et al., 2011), *B. didactylus* (ALMEIDA-SANTOS et al., 2011), *B. ephippium* (LIMA et al., 2013), *B. garbeanus* (DORIGO et al., 2012) e *B. pitanga* (OLIVEIRA, 2013). Vocalizações estão descritas para *B. crispus* (CONDEZ et al., 2014), *B. ephippium* (POMBAL et al., 1994), *B. pernix* (WISTUBA, 1998), *B. hermogenesi* (VERDADE et al., 2008), *B. pitanga* (ARAÚJO et al., 2012; OLIVEIRA, 2013) e *B. tridactylus* (GAREY et al., 2012). A presença de endoparasitas é conhecida para *B. pernix* (RIBEIRO et al., 2014). As especificidades ambientais do gênero e sensibilidade a alterações climáticas globais para algumas espécies já foram avaliadas, indicando tratar-se de espécies microendêmicas cuja distribuição seria drasticamente reduzida com o aumento da temperatura e elevação da linha de neblina (HADDAD et al., 2008; PIE et al., 2013). Apesar de sua distribuição restrita e notável sensibilidade a alterações ambientais, a maioria das espécies de *Brachycephalus* ocorre em áreas legalmente protegidas, o que influencia na decisão de considera-las entre as espécies ameaçadas no território brasileiro. Atualmente, oito espécies encontram-se listadas como Deficiente em Dados (categoria DD) na lista internacional de espécies ameaçadas da IUCN, sendo cinco delas do estado do Paraná (*B. alipioi, B. brunneus, B. ferruginus, B. izecksohni, B. nodoterga, B. pernix, B. pombali* e *B. vertebralis*; IUCN, 2014).

Embora o conhecimento sobre a diversidade e a distribuição do gênero *Brachycephalus* tenha aumentado significativamente, sabemos ainda muito pouco sobre sua história evolutiva. Seu conhecimento atual evidencia a baixa tolerância das espécies a

alterações no ambiente, baixa capacidade de dispersão e, consequentemente, elevado isolamento geográfico entre populações ao longo das principais cadeias montanhosas na Mata Atlântica. Além disso, a extensa faixa latitudinal ocupada pelo gênero sugere a existência de espécies adaptadas a ambientes tropicais e subtropicais. A complexidade topográfica e a estratificação climática em áreas de altitude elevada podem favorecer o isolamento reprodutivo e aumentar a ocorrência de eventos de especiação alopátrica nestas áreas, em comparação com áreas de baixa altitude (GUARNIZO & CANATELLA, 2013). Devido principalmente a menor flutuação climática sazonal, ambientes tropicais apresentam pouca sobreposição de condições ambientais ao longo do gradiente altitudinal, o que favorece a ocorrência de organismos especializados em determinados tipos de ambiente e com tolerâncias fisiológicas muito menores em comparação aos organismos de ambientes temperados (JANZEN, 1967; GHALAMBOR et al., 2006). Desta forma, o isolamento entre populações de *Brachycephalus*, sua divergência e especiação podem ocorrer de maneira distinta em áreas baixas e de altitude elevada, bem como nas montanhas da porção tropical e subtropical do bioma.

O presente trabalho foi realizado com o objetivo principal de ampliar o conhecimento sobre o gênero *Brachycephalus* e estudar a variação de caracteres morfológicos, genéticos e ecológicos das populações ao longo de sua distribuição geográfica. Com estas informações conseguimos avaliar os fatores históricos e contemporâneos envolvidos na distribuição e diversificação destas espécies, contribuindo para a compreensão da historia evolutiva deste gênero e dos ambientes de altitude na Mata Atlântica. A tese encontra-se organizada em três capítulos. No primeiro capítulo descrevemos a variação morfométrica do gênero, com base em uma amostra bastante completa de sua diversidade e distribuição. Ainda avaliamos a relação entre a variação morfométrica e fatores climáticos e da paisagem, com a intenção de identificar a existência de gradientes altitudinais e latitudinais previstos na literatura. No segundo capítulo, apresentamos uma releitura das relações filogenéticas entre as espécies, mapeamos e reconstruímos os estados de caracteres ancestrais para coloração e tamanho do corpo, canto de anúncio e distribuição altitudinal e geográfica. Os objetivos principais deste capítulo consistiram em avaliar a hipótese taxonômica atual, descrever a diversidade de parâmetros morfológicos, ecológicos e de distribuição no gênero e discutir aspectos de sua evolução. No terceiro capítulo utilizamos uma amostragem mais completa e populacional para descrever os padrões de diversidade genética do gênero. Investigamos o tempo de divergência entre as linhagens e os principais mecanismos envolvidos na diversificação das mesmas. Neste capítulo testamos também a hipótese de que diferentes características climáticas e da

paisagem poderiam oferecer maior resistência ao fluxo gênico entre populações, ocasionando maior isolamento reprodutivo e oportunidades para especiação na porção tropical do bioma. Como resultado adicional decorrente do trabalho de campo realizado, apresentamos em anexo a descrição de uma nova espécie para o gênero.

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CAPÍTULO I

Spatial variation of morphometry in *Brachycephalus* (Anura: Brachycephalidae) along the altitudinal and latitudinal gradients in the Atlantic forest

Spatial variation of morphometry in *Brachycephalus* (Anura: Brachycephalidae) along the altitudinal and latitudinal gradients in the Atlantic forest

Abstract

The Brazilian Atlantic forest has an exceptional habitat diversity mainly related to climate and altitudinal variation. The genus *Brachycephalus* is endemic of Atlantic forest and currently comprises 21 species. These species exhibit extremely small adult body size and share morphological features due to evolutionary process of miniaturization. Most species present geographic distribution restricted to altitudinal areas, occurring in isolation from the other congeneric species in the Atlantic forest mountain ranges. Despite of the increasing diversity and knowledge of the genus, morphometric differences among several populations of *Brachycephalus* were never investigated. Here, we analyzed the geographical variation in morphometric traits among 106 populations of *Brachycephalus*. We identified two main groups of species based on body size and interorbital distance variation. These groups are congruent with variation in altitude and topographic complexity across the distribution in Atlantic forest. Also, temperature was the environmental variable that better explain the morphometric variation observed in the species of high elevation, probably accounting for the latitudinal pattern of body size variation in this group. We found that the little morphometric variation in populations of the flea-toads *B. hermogenesi*, *B. didactylus* and *B. pulex* is related to temperature fluctuations. In general, the body size in *Brachycephalus* does not follow altitudinal or latitudinal clines. Further investigations on species diversification at highlands and lowlands are supposed to help understanding the current geographic distribution and the evolution of this endemic miniaturized genus of toadlets in the Atlantic forest.

Introduction

Geographical variation of biological traits along spatial and environmental gradients is a central issue in biogeography (GASTON et al., 2008). The environmental conditions present several impacts on the evolution of life history traits by imposing strong selective pressure on morphology, physiology and genetic of organisms (GASTON & BLACKBURN, 2000; GASTON et al., 2008). The investigation of morphological variation across environmental heterogeneous conditions focus mainly on body size. Several ecogeographical hypotheses have been investigated since the Bergmann's rule, which predicts a decrease in body size with increasing temperature or latitude (BERGMANN, 1847; BLACKBURN et al., 1999; WATT et al., 2010; OLALLA-TÁRRAGA, 2011).

The presence of latitudinal and altitudinal Bergmann clines in amphibians is still controversial. Some anurans follow the rule (e.g. OLALLA-TÁRRAGA & RODRIGUEZ, 2007; BIDAU et al., 2011; GOUVEIA et al., 2013), others exhibit a converse pattern (e.g. CVEKTOVIC et al., 2008) and others simply do not exhibit any pattern (ADAMS & CHURCH, 2008). Despite of merely identify the presence of latitudinal and altitudinal clines, the unique life-history attributes of amphibians need to be accounted to access the evolutionary explanations for those patterns (ADAMS & CHURCH, 2008).

Regardless of the intense habitat loss and forest fragmentation the Brazilian Atlantic forest harbors exceptional diversity and endemism (MYERS et al., 2000). The Atlantic forest biota is probably the result of a complex evolutionary history involving vicariant models of diversification (DANTAS et al., 2011). The Pleistocene refuges (BROWN & AB'SABER, 1979; CARNAVAL et al., 2009), neotectonic activities and riverine barriers (e.g. GRAZZIOTIN et al., 2006; THOMÉ et al., 2010) may be plausible explanations to biological diversification in Atlantic forest. More than 530 species of amphibians are known to occur along the biome (HADDAD et al., 2013). Combined to the evolutionary history, the unique climate conditions and the large altitudinal range have favored the high diversity and endemism of amphibians in this region (HADDAD & PRADO, 2005; ROSSA-FERES et al., 2008).

With approximately 12% of the original cover remaining, the Atlantic forest is currently restricted to 15 million hectares distributed in small-size and isolated forest fragments with highly heterogeneous environmental conditions (RIBEIRO et al., 2009). The latitudinal extension of the biome is around 29° across tropical and subtropical climates (ALMEIDA & CARNEIRO, 1998). The longitudinal variation of rainfall regimes drives the differences between forests from the interior and those near to the coast (CÂMARA, 2003). Also, the forest remnants occur in a wide altitudinal range from sea level up to 2700 m of altitude. Above 700 m of altitude the sub-montane forests exhibit warmer average temperatures and narrower fluctuations in the environmental conditions than montane forests (OLIVEIRA-FILHO & FONTES, 2000). These latitudinal, longitudinal and altitudinal gradients created a unique diversity of landscapes, which is congruent with the extraordinary species diversity of the region (SILVA & CASTELETI, 2003).

Amphibian species richness is variable across the altitudinal gradient, generally declining in higher elevations (FU et al., 2006; ZANCOLLI et al., 2014). At the same time, the diversity of endemic, restricted range, rare and undescribed species highlights the importance of the high elevation sites to amphibian conservation (FU et al., 2006; SIQUEIRA

et al., 2013). Especially in the tropical forests, montane regions are considered hotspots (MYERS et al., 2000; RICKETTS et al., 2005). The mountainous regions of Atlantic forest are recognized to be more stable under the past climate shifts, possibly acting as forest refugia (BROWN & AB'SABER, 1979; CARNAVAL et al., 2009). The increasing number of herpetological surveys in high elevations of Atlantic forest is revealing high levels of endemism and diversity of anurans. From six new species described for the Atlantic forest since the last year, four species are restricted to high elevation areas (<1000 m of altitude; FROST, 2014).

The genus *Brachycephalus* is endemic to the Atlantic forest, with a range extension of approximately 1600 km, from the state of Bahia (15°23'S, 39°33'W; NAPOLI et al., 2011) to the state of Santa Catarina (26°47′S, 48°55′W; PIE et al., 2013). Most species of the genus has been described in the last decade as result of the increasing surveys in the Serra do Mar mountain range in the states of Paraná and São Paulo (ALVES et al., 2009; HADDAD et al., 2010; CLEMENTE-CARVALHO et al., 2012). Currently, 21 species are recognized within the genus (FROST, 2014). Eighteen species, representing 85% of them, are restricted to mountain slopes with 600-1800 m of altitude (POMBAL et al., 1998; CONDEZ et al., 2014).

Species of *Brachycephalus* are leaf-litter inhabitants and exhibit extremely small adult body size, with snout-vent length usually inferior to 18 mm (POMBAL, 1999; CLEMENTE-CARVALHO et al., 2011). Due to the evolutionary process of miniaturization these species share morphological characteristic features, including the complete absence, reduction and/or hyperossification of bones (ALBERCH & GALE, 1985; HANKEN, 1993; CLEMENTE-CARVALHO et al., 2009). *Brachycephalus* species also exhibit reduced fecundity, terrestrial breeding and direct development, in which large eggs hatch into toadlets thereby bypassing the tadpole stage (POMBAL, 1999; HEDGES et al., 2008; CONDEZ et al., 2014). Ecology of *Brachycephalus* species is poorly understood, especially because collecting data on these species is hampered by inaccessible study sites and possible activity under leaf litter (CONDEZ et al., 2014). More complete data on reproductive behavior is restricted to *B. ephippium* (POMBAL, 1999). Food habits and other ecological aspects are described for *B. brunneus* (FONTOURA et al., 2011), *B. didactylus* (ALMEIDA-SANTOS et al., 2011), *B. ephippium* (LIMA et al., 2013), *B. garbeanus* (DORIGO et al., 2012) and *B. pitanga* (OLIVEIRA, 2013). Advertisement calls are available for *B. crispus* (CONDEZ et al., 2014), *B. ephippium* (POMBAL et al., 1994), *B. pernix* (WISTUBA, 1998), *B. hermogenesi* (VERDADE et al., 2008), *B. pitanga* (ARAÚJO et al., 2012; OLIVEIRA, 2013) and *B. tridactylus* (GAREY et al., 2012). Niche modeling studies revealed that *Brachycephalus*

species are microendemic and sensitive to global climate changes (HADDAD et al., 2008; PIE et al., 2013). The recently proposed phylogenetic hypothesis for the genus has three main clades coincident with the geographic distribution and the skeleton hyperossification pattern (CLEMENTE-CARVALHO et al., 2011). However, the incongruence between the gene trees and the poor resolution in the species tree maintain the phylogenetic relationships within *Brachycephalus* species still unclear.

The highly restricted geographical range of most species of *Brachycephalus* suggests narrow environmental tolerance, reduced dispersal capacity and, consequently, geographic isolation in high elevation areas along the Atlantic forest domain. The complex mountain topography and climate variation in high elevation localities could provide more genetic isolation among populations and higher allopatric speciation rates compared to low altitudes (WOLLEMBERG et al., 2011; GUARNIZO & CANATELLA, 2013). Exceptionally, three *Brachycephalus* species have wide altitudinal ranges, occurring from the sea level up to the highlands (IZECKSOHN, 1971; GIARETTA & SAWAYA, 1998; NAPOLI et al., 2011). In anurans, widely distributed species are supposed to have climatically distinct niches than the microendemic sister species (e.g., WOLLEMBERG et al., 2011). Even so, phenotypic and genetic differences among populations of *Brachycephalus* were never accurately investigated. Further studies on species diversification at highlands and lowlands are supposed to help understanding the mechanisms underlying the biogeographical history and evolution of this endemic miniaturized genus of toadlets in the Atlantic forest.

In this study we analyzed the geographical variation in morphometric traits among 106 *Brachycephalus* populations across the genus distribution. Our main goals were: (1) to describe morphometric groups and the environmental conditions associated to their occurrence, (2) to identify altitudinal and latitudinal patterns of morphometric variation across the geographic distribution and (3) to infer which climate and landscape variables better explain the morphometric variation observed.

Material and Methods

Morphometric variation

We examined 1008 *Brachycephalus* specimens from 106 populations distributed in 100 localities along the coastal Brazilian Atlantic forest (Figure 1). The specimens analyzed are deposited in CFBH (Célio F. B. Haddad Collection, Universidade Estadual Paulista, Campus de Rio Claro, São Paulo, Brazil), MZUSP (Herpetology Collection of Museu de

Zoologia, Universidade de São Paulo, São Paulo, Brazil) and MNRJ (Herpetology Collection of Museu Nacional do Rio de Janeiro, Rio de Janeiro, Brazil) (Appendix 1).

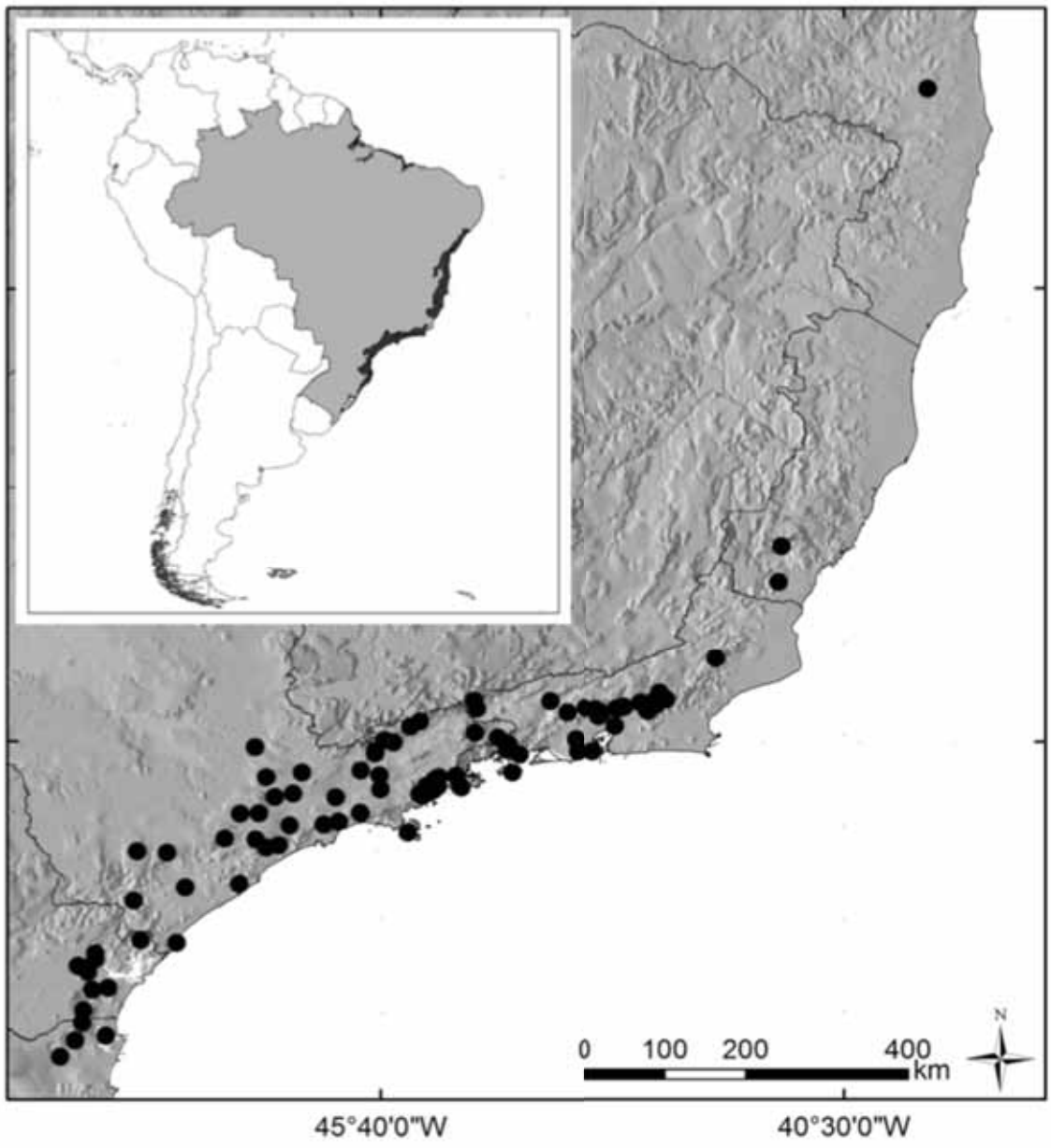

Figure 1. Populations of *Brachycephalus* used for morphometric variation analysis.

Fourteen metric traits were measured (in mm) with a micrometric ocular fitted to the stereomicroscope (Figure 2). The abbreviations used were: SVL (snout-vent length), HL (head length; from tip of snout to angle of jaw), HW (head width; greatest width of head located between angle of jaw), ND (nostril diameter), IND (inter-nostril distance; between inner margins of nostrils), ED (eye diameter), IOD (interorbital distance; between anterior corners of eyes), END (eye-nostril distance; from anterior corner of the eye to posterior

margin of nostril), THL (thigh length), TBL (tibia length), FL (foot length; between the centrale region to the tip of the longest toe), AL (arm length), FAL (forearm length) and HAL (hand length; between the centrale region to the tip of the longest finger). Arms and limbs measurements (THL, TBL, FL, AL, FAL and HAL) and HL were taken preferably on the right side. The same person performed all measurements.

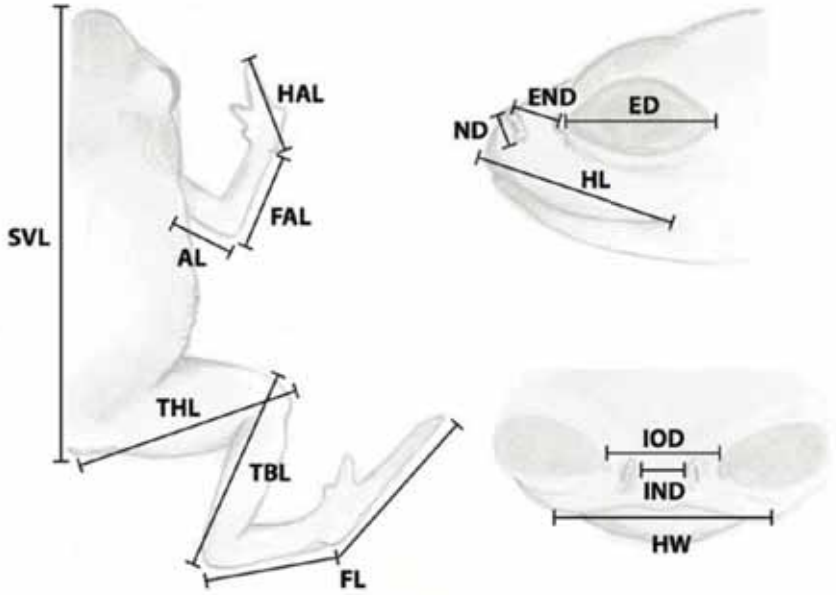

Figure 2. Fourteen morphometric measurements taken in *Brachycephalus*: SVL (snout-vent length); HL (head length); HW (head width); ND (nostril diameter); IND (inter-nostril distance); ED (eye diameter); IOD (interorbital distance); END (eye-nostril distance); THL (thigh length); TBL (tibia length); FL (foot length); AL (arm length); FAL (forearm length) and HAL (hand length).

Only adult specimens were included in our morphometric dataset. We considered juveniles the individuals smaller than 10.0 mm SVL for *Brachycephalus alipioi, B. bufonoides, B. ephippium*, *B. garbeanus, B. margaritatus*, *B. nodoterga*, *B. pitanga*, *B. toby* and *B. vertebralis*; smaller than 8.0 mm SVL for *B. brunneus*, *B. ferruginus*, *B. izecksohni*, *B. pernix*, *B. pombali* and *B. tridactylus;* and smaller than 7.0 mm SVL for *B. didactylus, B. hermogenesi* and *B. pulex*. Sexual dimorphism of adults is mainly evidenced by SVL, the most distinctive dimorphic external character in *Brachycephalus*. Females are generally larger than males but sexual precise identification can only be confirmed with observation of gonads and sometimes by the mature oocytes visible by the translucent body walls in females. Considering the inaccuracy of SVL sex determination in *Brachycephalus*, sexual size dimorphism was not considered in our analysis.

Morphometric data were transformed into z-scores values for standardization (GOTELLI & ELLISON, 2004). We use all morphometric variables for size comparisons and a size-free dataset for shape comparisons. The size-free dataset was obtained through residuals of linear regressions of each morphometric measurement on SVL. To access the patterns of geographical variation in morphometry we performed Canonical Variate Analysis with both datasets (CVA; MANLY, 2004). The axes that accounted for the majority of variance were extracted for each set of variables and used in posterior analysis. To access the mean distance to centroid of each group of populations discriminated by CVA we use a Permutational Analysis of Multivariate Dispersion (ANDERSON, 2006). We account for differences in sample sizes subsampling larger groups 100 times. Pairwise comparisons of group mean dispersions of subsampled data were made using a T-test combined with permutations and a TukeyHSD test to access significant differences between groups.

Spatial analysis

Geographic coordinates of each population were checked individually and adjusted to UTM WGS 1984 datum to minimize projection errors. We used correlograms based on Moran's I index (LEGENDRE & LEGENDRE, 1998; PEREZ et al., 2010) to inspect the spatial autocorrelation in population morphometric data. The Moran's I index was calculated for 14 distance classes (10, 20, 50, 100, 200, 300, 400, 500, 600, 700, 800, 900, 1000 and 1500 km) and the values ranged from 1 (positive spatial dependence) to -1 (negative spatial dependence).

To characterize population environment we overlapped geographic coordinates to six environmental layers (~800 m of resolution) using ArcMap 10.0 (ESRI, 2010). To calculate the surrounding climate and landscape features we delimited circular regions of 1 km radius buffering each sampled point. The environmental values were extracted by multiple points inside these circular regions and the average environmental value was used to describe each population. For climate variables we used layers of annual mean temperature, annual temperature range and precipitation (BIO1, BIO7 and BIO12 respectively, HIJMANS et al., 2005). For landscape variables we considered altitude (JARVIS et al., 2009) and we also calculated topographic complexity using the raster calculator feature in ArcMap 10.0 (ESRI, 2010), where for each elevation pixel was assigned the variance of the neighbor pixels (HUAXING, 2008). Once is related to relief roughness this metric provides significant information on habitat heterogeneity and microclimate turnover. We also used vegetation density (USGS & FAO, 2000) as an important measure of current landscape changes.

As environmental variables do not match the assumptions of normality and homogeneity of variances we use the Welch's T-test to compare landscape and climate variables among morphometric groups previously identified (RUXTON, 2006).

Environmental predictors of morphometric variation

Since we found significant spatial autocorrelation in morphometric data at short distances (Figure 3), we fit generalized linear mixed models to test the effects of environmental variables on morphometric variation considering latitude as a random effect (GLMM; ZUUR et al., 2009). We constructed 20 explanatory models assuming *Poisson* error distribution, log-link function and Gauss-Hermite Quadrature technique for parameter estimations (BOLKER et al., 2009). We used the corrected Akaike's Information Criterion (AIC*c*) to rank the best models and calculate the relative AIC*c* weights (BURNHAM & ANDERSON, 2002). The AIC*c* weight expresses the weight of evidence in favor of each model in contrast of all those in the model set (BURNHAM & ANDERSON, 2002). We used these weights to select the model that best explains the morphometric variation in all *Brachycephalus* populations and in the previous morphometric groups evidenced by the CVA.

Analyses were performed in R environment (R CORE TEAM, 2013; FRIENDLY & FOX, 2013; BIVAND, 2013; BOSTRÖM, 2013; BARTON, 2013; OKSANEN et al., 2013).

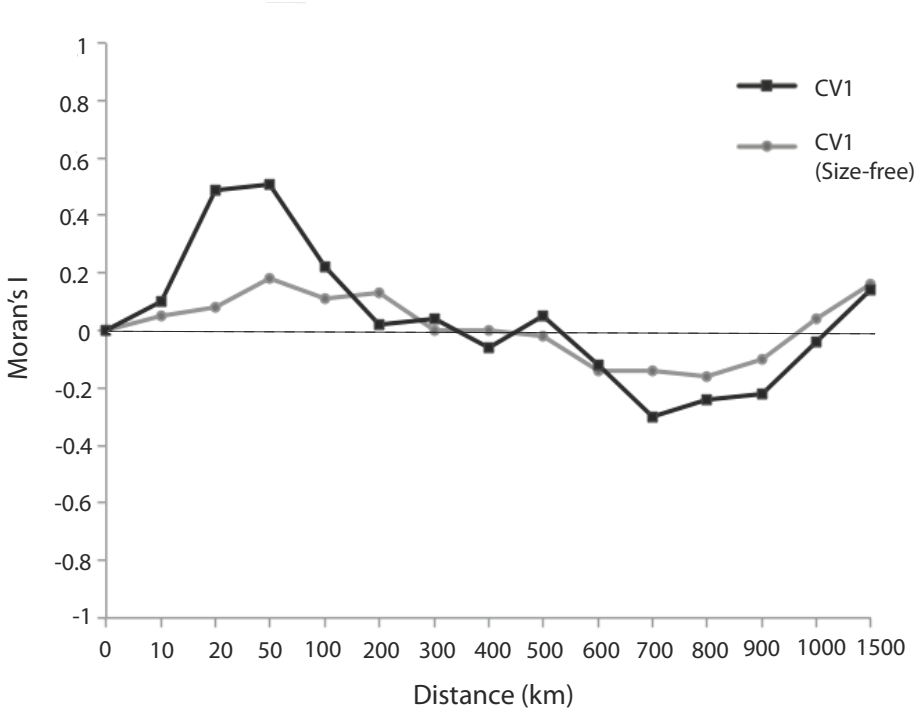

Figure 3. Correlograms based on Moran's I indices for spatial autocorrelation of all the morphometric data (CV1) and size-free data (CV1 size-free) in *Brachycephalus.*

Results

Morphometric variation

Canonical Variate Analysis of size dataset showed that 72% of morphometric variations among populations of *Brachycephalus* were explained by two canonical functions (Table 1). The first canonical function (CV1) is mainly correlated with negative variation in IOD, and positive variation in SVL. The second canonical function (CV2) is strongly correlated with a negative variation in HW (Table 1). Thus, populations with positive CV1 eigenvalues exhibit smaller body size accompanied by narrower interorbital distance. Populations with positive CV2 eigenvalues also exhibit narrower interorbital distance than those associated with negative values. According to this variation, two main groups of populations are distinguished within *Brachycephalus* (Figure 4A).

Table 1. Results of Canonical Variate Analysis (CVA) of size and size-free dataset of *Brachycephalus* morphometric measurements. Bold values are the most representative variables in the correspondent axis.

	Size dataset		**Size-free dataset**	
Variables	CV1	CV2	CV1	CV2
SVL	**0.53**	-0.20	-	-
HL	0.10	0.17	-0.15	-0.32
HW	0.29	**-1.13**	**1.16**	-0.66
ND	-0.06	0.33	-0.37	-0.24
IND	-0.41	0.01	-0.07	0.57
ED	-0.29	0.18	0.19	0.16
IOD	**-0.57**	-0.11	0.06	**0.73**
END	0.01	-0.19	0.28	-0.52
THL	-0.16	0.17	-0.21	0.40
TBL	-0.25	0.54	-0.61	-0.36
FL	0.02	0.55	-0.64	-0.57
AL	-0.09	-0.02	0.04	-0.18
FAL	-0.10	0.09	-0.13	0.27
HAL	-0.08	-0.16	0.22	-0.06
Eigenvalues	15.92	3.28	3.28	2.50
% Variance	59.72	12.30	26.77	20.38
% Cumulative	-	72.03	-	47.16

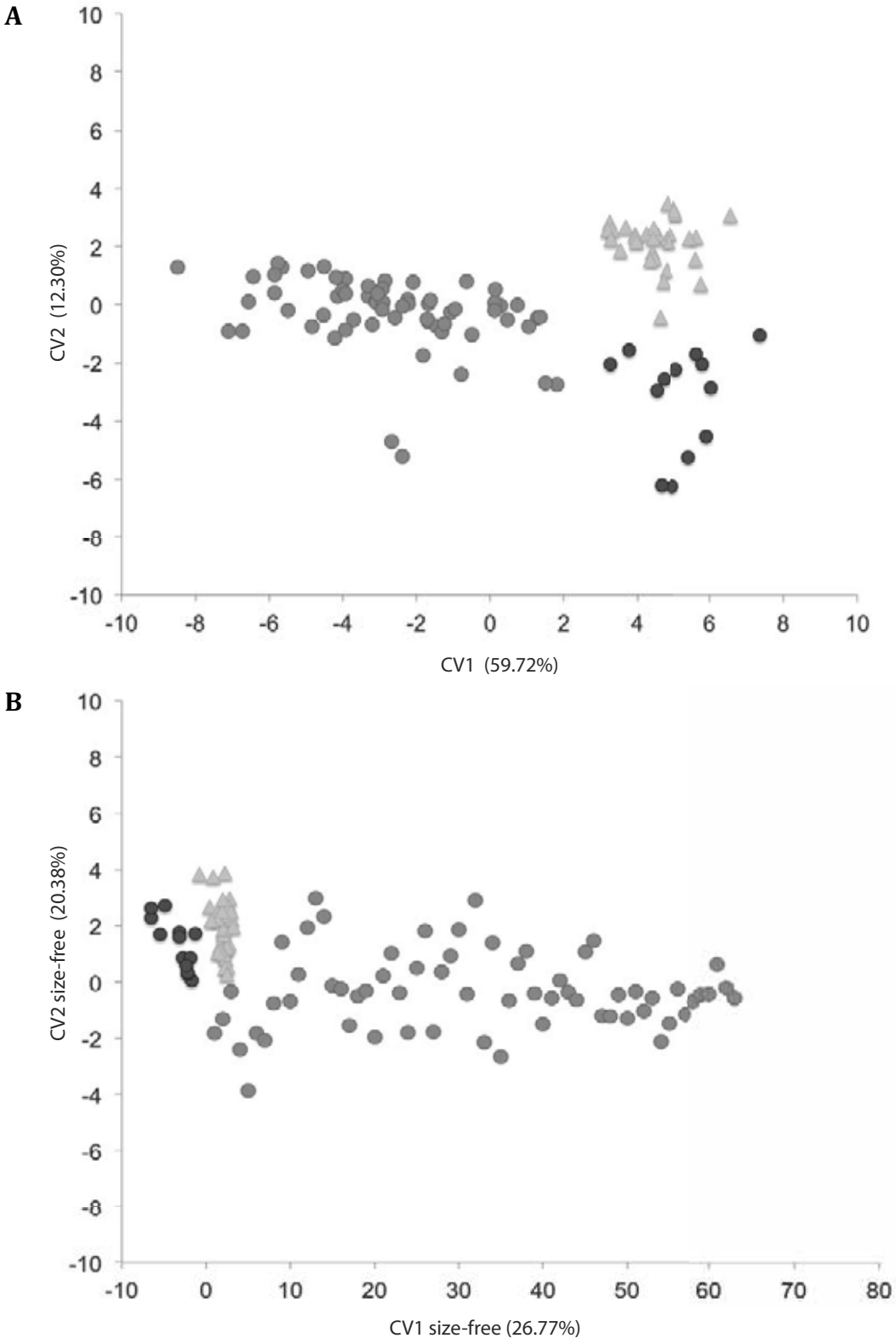

Figure 4. Results of the Canonical Variate Analysis of the size (A) and size-corrected (B) datasets of morphometric measurements in *Brachycephalus*. Symbols correspond to groups of populations identified as: light and dark circles (aposematic coloration species, generally associated at high elevation sites, group A) and triangles (dull colored species, associated with wide altitudinal range, group B). Dark circles correspond to populations slightly differentiated in the group A, see text for details.

The group A is composed by 74 populations with SVL ranged from 9.36 to 18.18 mm ($\bar{x}$ = 13.30 ± 1.94), IOD ranged from 1.65 to 4.15 mm ($\bar{x}$ = 3.07 ± 0.57) and HW ranged from 2.21 to 6.13 mm ($\bar{x}$ = 4.26 ± 0.71). This group includes populations of *B. alipioi*, *B. brunneus*, *B. bufonoides*, *B. crispus*, *B. ephippium*, *B. ferruginus*, *B. garbeanus*, *B. guarani*, *B. izecksohni*, *B. margaritatus*, *B. nodoterga*, *B. pernix*, *B. pitanga*, *B. pombali*, *B. toby*, *B. tridactylus*, *B. vertebralis* and ten undescribed species. These species are generally associated with high elevation sites and frequently exhibit aposematic color (Figure 5). Inside this group we identified a latitudinal gradient of morphometric variation (Figure 6), clearly distinguishing populations with smaller body size and narrower interorbital distance in the southern portion of geographic distribution (represented in Figure 6 by the dark triangles). These populations are represented by the species *B. brunneus*, *B. ferruginus*, *B. izecksohni*, *B. pernix*, *B. pombali* and other six undescribed species. The pattern of increasing body size as latitude decrease revealed that this group of species within *Brachycephalus* exhibits a converse response to Bergmann's rule (Figure 7A).

The group B is composed by 32 populations with SVL ranged from 7.58 to 10.43 mm ($\bar{x}$ = 8.88 ± 0.63), IOD ranged from 1.57 to 2.13 mm ($\bar{x}$ = 1.89 ± 0.13) and HW ranged from 2.03 to 3.83 mm ($\bar{x}$ = 2.52 ± 0.34). This group includes populations of *B. didactylus*, *B. hermogenesi*, *B. pulex* and three undescribed species. These species are generally dull colored and widespread distributed, occurring from sea level up to 1000 m of altitude (Figure 5). We found no geographic pattern of morphometric variation inside this group (Figures 7B and 8).

In order to avoid differences in sample sizes (74 populations from group A compared to 32 from group B), we subsampled for 100 times 32 populations from the group A. For each subsample we calculated the average distance to centroid and then compared the point dispersion between groups. The average distance to centroid was significantly different between groups A and B in 56% of subsampled cases (TukeyHSD test $p < 0.01$). The multivariate dispersion was larger in the group A than in the group B (mean distances to centroid: group A = 1.90, group B = 0.82), suggesting more morphometric variation within group A than within group B (Figure 4A).

The CVA of size-free dataset showed that 47.16% of morphometric variation among populations was explained by the two first canonical functions (Table 1). The first canonical function (CV1 size-free) is mainly correlated with a positive variation in HW. The second canonical function (CV2 size-free) is strongly correlated with a positive variation in IOD and a negative variation in HW (Table 1). In this case, populations with positive CV1 eigenvalues exhibit larger head width and population with positive CV2 eigenvalues exhibit narrower

snout than those associated with negative values. Groups A and B are not clearly distinguished in this analysis (Figure 4B).

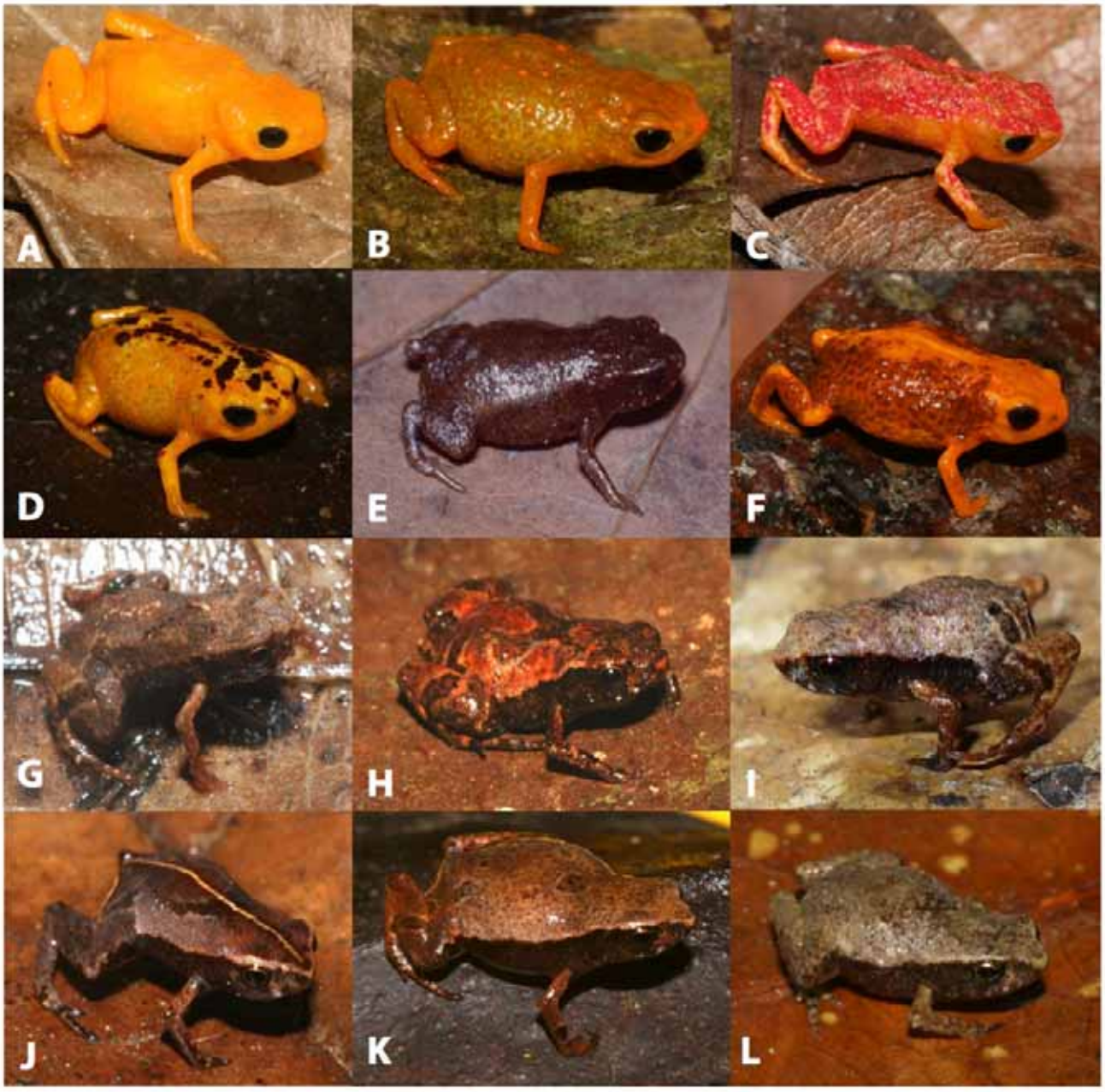

Figure 5. Some *Brachycephalus* species associated to each morphometric group of populations identified in this study. Species from group A, generally aposematic and associated with high elevation sites: (A) *B. ephippium*, (B) *B. nodoterga*, (C) *B. pitanga*, (D) *B. ferruginus*, (E) *B. brunneus* and (F) *B.* sp. Species from group B, generally dull colored and widespread distributed, occurring from sea level up to 1000 m: (G) *B. didactylus*, (H) *B. hermogenesi*, (I) *B. hermogenesi*, (J) *B. pulex*, (K) *B.* sp., (L) *B.* sp. Photo credits (E) C. F. B. Haddad and (G) J. P. Pombal Jr.

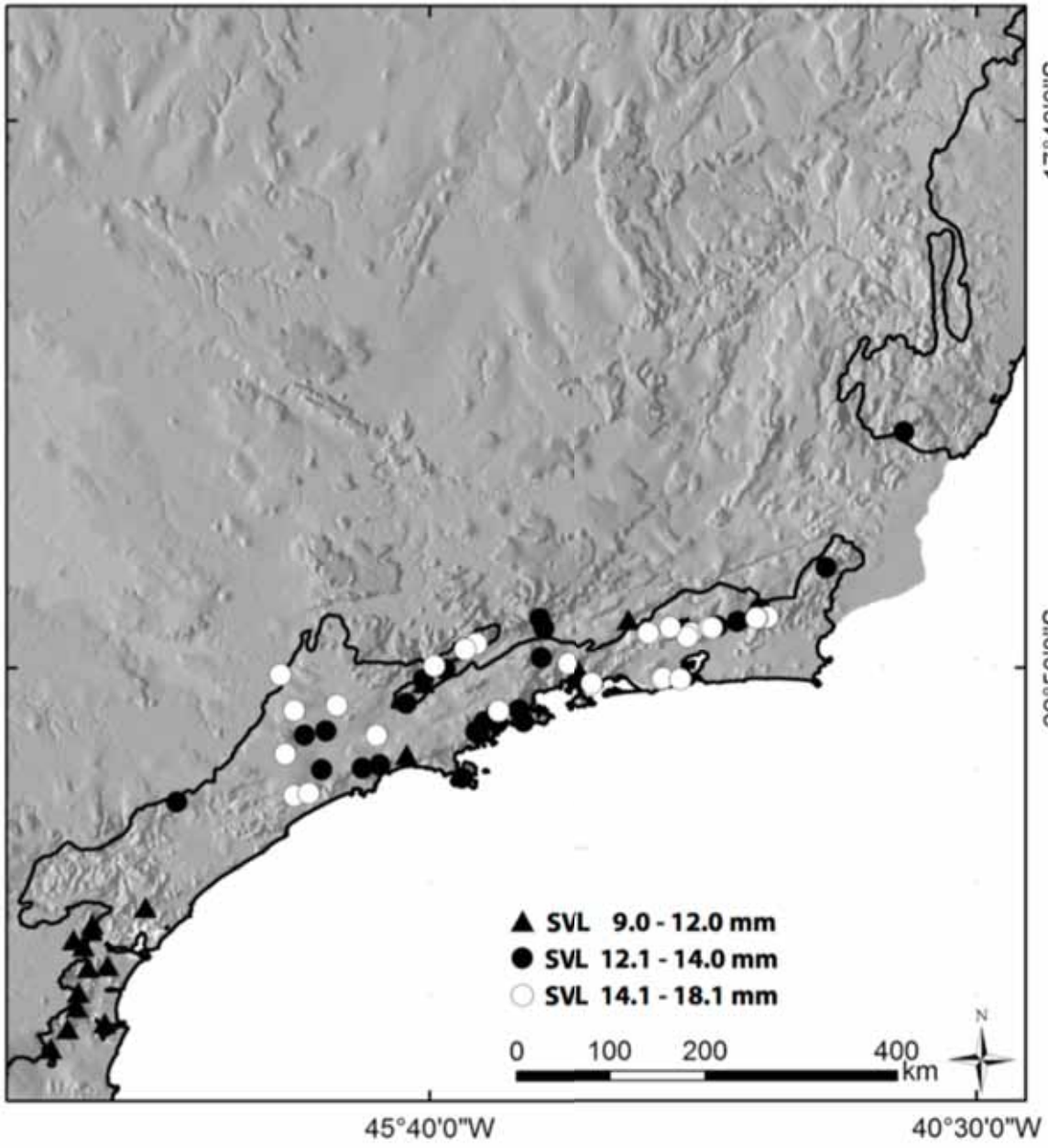

Figure 6. Latitudinal pattern of body size variation in populations associated to the morphometric group A. Ombrophilous Atlantic forest is delimited in black. SVL: snout-vent length.

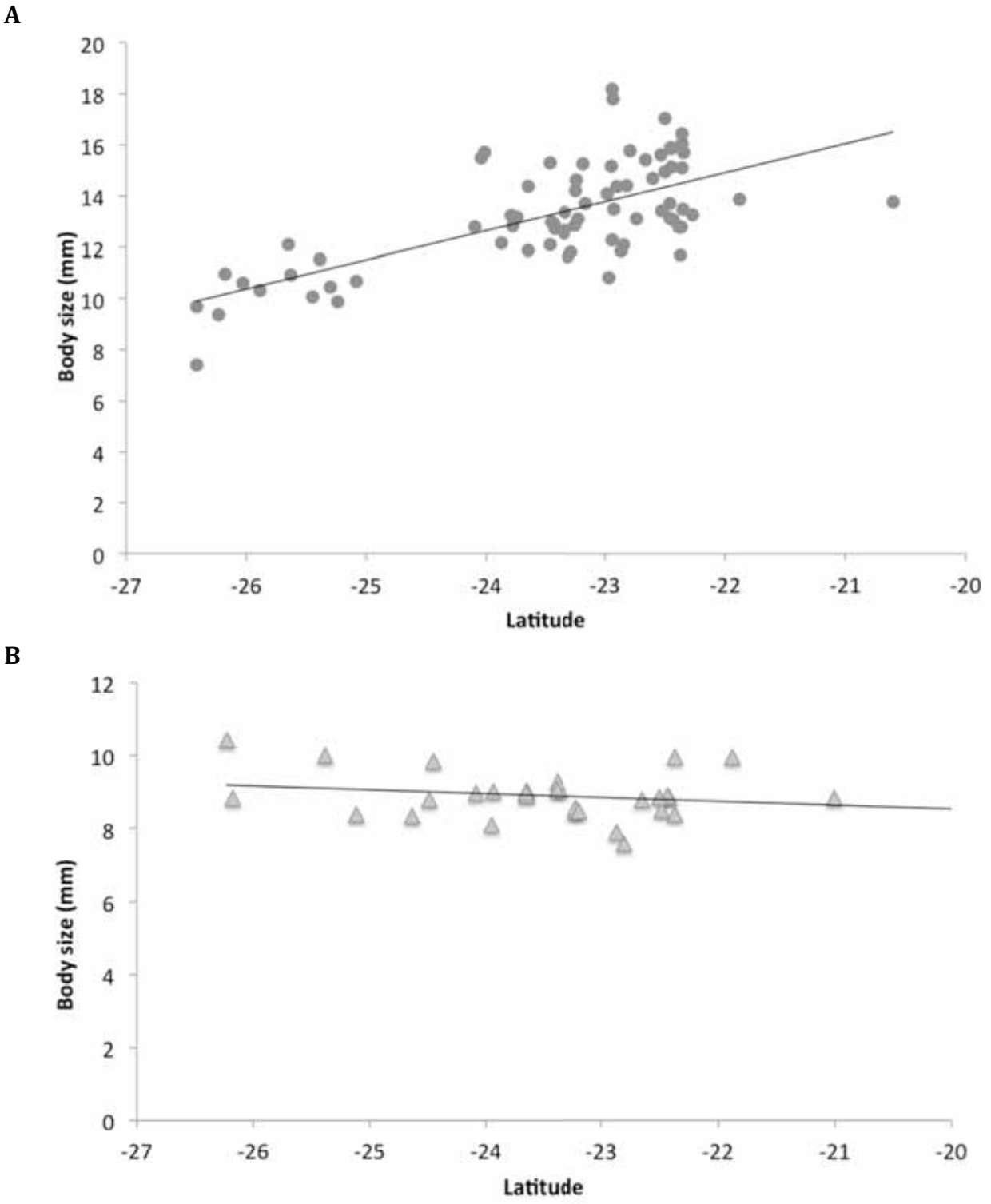

Figure 7. Body size variation in *Brachycephalus* populations with latitude. (A) Species from group A, generally aposematic and associated with high elevation sites and (B) species from group B, generally dull colored and widespread distributed, occurring from sea level up to 1000m.

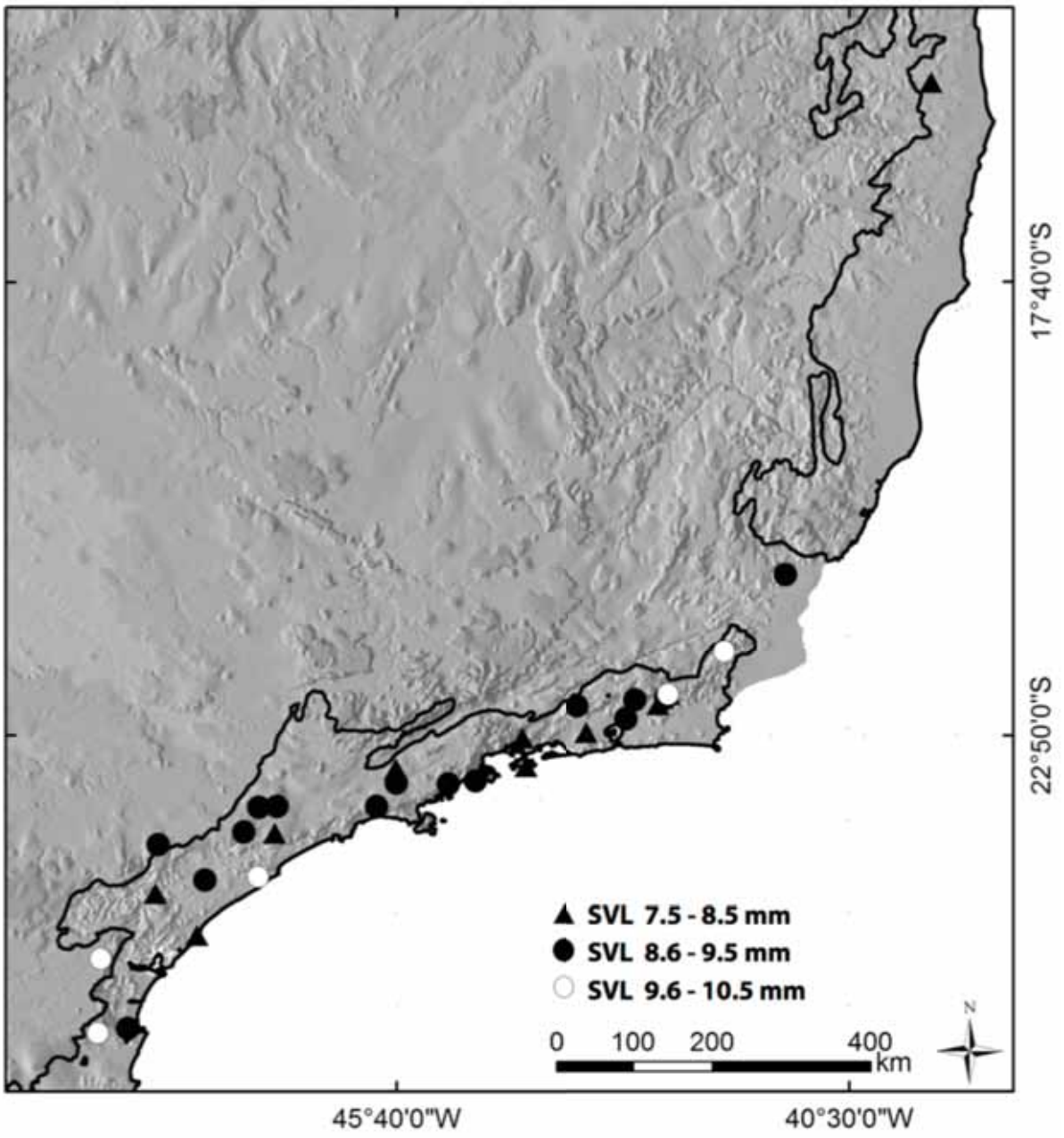

Figure 8. Absence of latitudinal pattern of body size variation in populations associated to the morphometric group B. Ombrophilous Atlantic Forest is delimited in black. SVL: snout-vent length.

Environmental predictors of morphometric variation

Species of *Brachycephalus* are in general restricted to ombrophilous phytophysiognomy of Atlantic forest (Figures 6 and 8). The current altitudinal range of *Brachycephalus* is 17 to 2164 m above sea level (Table 2). The topographic conditions surrounding localities are generally complex with valleys and mountaintops over short geographic distances. Annual mean temperature at occurrence sites ranged from 9.75-20.1°C

and annual precipitation ranged from 1100-3252 mm (Table 2). Probably due to the proximity to mountain top rock formations, localities have around 12% of vegetation density (Table 2).

Table 2. Climate and landscape descriptors for all sampled populations of *Brachycephalus* and the groups evidenced by the morphometric analysis.

	Group A (N = 74)		**Group B** (N = 32)		**All populations** (N = 106)	
Variables	$\bar{x}$ ± SD	Min-Max	$\bar{x}$ ± SD	Min-Max	$\bar{x}$ ± SD	Min-Max
Annual mean temperature (°C)	14.3 ± 1.6	9.7-20.1	16.1 ± 1.9	13.0-19.8	14.9 ± 1.9	9.75-20.1
Annual temperature range (°C)	14.4 ± 0.8	13.0-16.5	14.0 ± 1.0	10.1-16.0	14.3 ± 0.5	10.1-16.5
Annual precipitation (mm)	1759 ± 339	1100-3252	1642 ± 327	1135-2566	1725 ± 340	1100-3252
Elevation (m)	862 ± 385	206-2164	620 ± 374	17-1354	791 ± 397	17-2164
Topographic complexity	52065 ± 47265	186-237297	29232 ± 31657	82-130327	45325 ± 44483	82-237297
Vegetation Density (%)	10 ± 9	2-32	16 ± 12	2-32	12 ± 10	2-32

Populations from group A occur in relative mild temperatures compared to group B (Welch's test $p < 0.01$, $df = 50.33$, $t = -4.12$; Figure 9A). The localities occupied by group A have higher altitudes and are topographically more complex than localities from group B (Welch's test elevation: $p < 0.01$, $df = 57.28$, $t = 2.96$; topographic complexity $p < 0.01$, $df = 81.89$, $t = 2.85$; Figure 9B and 9C). Vegetation density is also different between groups A and B (Welch's test $p = 0.05$, $df = 46.01$, $t = -1.96$; Figure 9D).

The most important environmental variables for explaining morphometric variation in *Brachycephalus* were annual mean temperature, annual temperature range and topographic complexity. The model that best predicted the morphometric variation of all populations was related to topographic complexity (Table 3). The populations from group A are especially associated with topographically more complex localities than populations from group B (Figures 9C and 10). Also, due to their discrete morphometric variation and their widespread altitudinal occurrence, populations from group B did not exhibit altitudinal pattern of body size variation (Figure 10). Considering only group A, the model that best predicted morphometric variation was annual mean temperature (Table 3). So, temperature is driving

the latitudinal gradient of morphometric variation inside this group of populations (Figures 6 and 7A). For the group B, the model that best predicted morphometric variation was annual temperature range (Table 3).

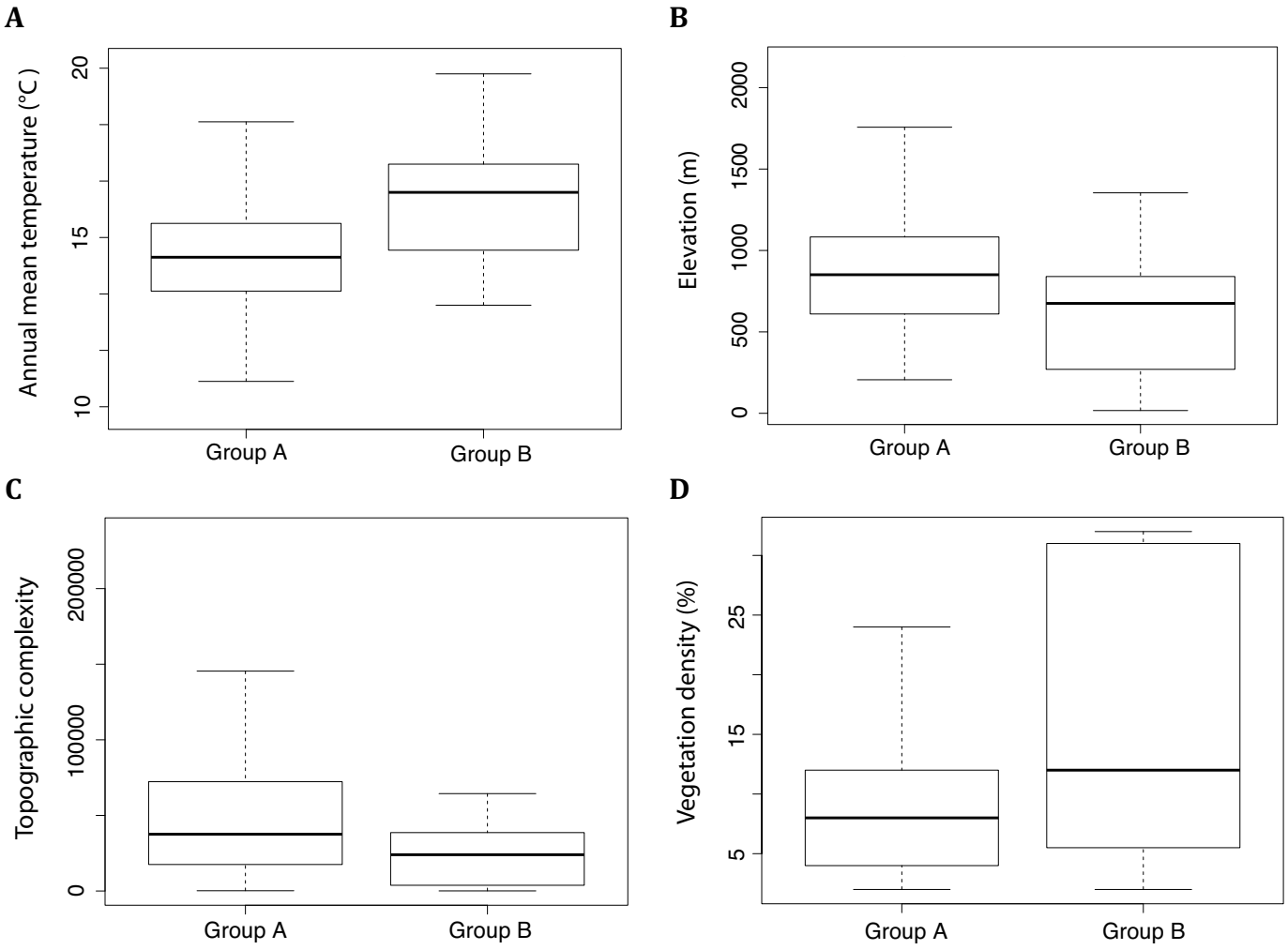

Figure 9. Climate and landscape differences between localities of the morphometric groups identified in this study. (A) Annual mean temperature; (B) elevation; (C) topographic complexity; (D) vegetation density.

Discussion

Morphometric variation in *Brachycephalus* is essentially related to body size. We identified two clearly distinct groups of species within *Brachycephalus* based on body size, head width and interorbital distance variation. Although there is no evidence for this separation in the molecular phylogeny (CLEMENTE-CARVALHO et al., 2011) the informal distinction of these two groups of species has already been suggested in literature (VERDADE et al., 2008; NAPOLI et al., 2011). Our study confirmed that these groups are consistently differentiated by morphometric traits.

Table 3. Multi-model inference results of the explanatory climate and landscape effects on the morphometric variation (first canonical axis, CV1) of *Brachycephalus*. *k* = number of parameters, *df* = degrees of freedom, AIC*c* = corrected Akaike's Information Criterion, Δ*i* = difference between the criterion values of the best-ranked model and model *i, w*AIC*c* = Akaike's weights. [a] Best model for the group A, [b] best model for the group B and [c] best model for all populations (see text for details).

			Group A (n=74 populations)				Group B (n=32 populations)				All populations (n=106)			
Type	Model	*k*	*df*	AIC*c*	Δ*i*	*w*AIC*c*	*df*	AIC*c*	Δ*i*	*w*AIC*c*	*df*	AIC*c*	Δ*i*	*w*AIC*c*
Climate/ Landscape	Temperature +Temperature range + Precipitation + Elevation +Topographic complexity +Vegetation density	7	66	114.51	9.07	0.003	24	29.01	14.81	0.000	98	185.95	6.70	0.008
Climate/ Landscape	Temperature * Elevation + Topographic complexity	5	68	112.25	6.81	0.009	26	22.82	8.62	0.002	100	181.18	1.93	0.082
Climate/ Landscape	Temperature range * Elevation + Topographic complexity	5	68	113.41	7.97	0.005	26	22.31	8.11	0.003	100	185.46	6.21	0.010
Climate/ Landscape	Precipitation * Elevation + Topographic complexity	5	68	112.98	7.54	0.006	26	23.18	8.98	0.002	100	183.55	4.30	0.025
Climate/ Landscape	Temperature*Elevation	4	69	109.96	4.52	0.027	27	19.82	5.62	0.011	101	181.86	2.61	0.058
Climate	Temperature + Temperature range + Precipitation	4	69	107.96	2.52	0.074	27	19.48	5.28	0.013	101	184.53	5.28	0.015
Climate/ Landscape	Temperature range * Elevation	4	69	111.09	5.65	0.015	27	19.31	5.11	0.014	101	185.85	6.60	0.008
Climate/ Landscape	Precipitation * Elevation	4	69	110.68	5.24	0.019	27	20.13	5.93	0.009	101	185.02	5.77	0.012
Landscape	Elevation + Topographic complexity + Vegetation density	4	69	111.06	5.62	0.016	27	20.12	5.92	0.009	101	182.75	3.50	0.037
Landscape	Elevation + Topographic complexity	3	70	108.94	3.50	0.045	26	15.86	1.66	0.077	102	182.75	3.50	0.037
Landscape	Elevation + Vegetation density	3	70	108.82	3.38	0.048	28	17.33	3.13	0.037	102	183.52	4.27	0.025
Landscape	Topographic complexity + Vegetation density	3	70	112.30	6.86	0.008	28	17.35	3.15	0.037	102	180.59	1.34	0.110
Landscape	Elevation	2	71	106.75	1.31	0.135	29	14.76	0.56	0.134	103	181.85	2.60	0.059
Landscape	**[c] Topographic complexity**	**2**	**71**	**110.11**	**4.67**	**0.025**	**29**	**29.05**	**14.85**	**0.000**	**103**	**179.25**	**0.00**	**0.215**
Landscape	Vegetation density	2	71	110.40	4.96	0.022	29	14.79	0.59	0.132	103	181.59	2.34	0.067
Climate	Temperature + Precipitation	3	70	105.74	0.30	0.224	28	17.41	3.21	0.036	102	183.77	4.52	0.022
Climate	Temperature range + Precipitation	3	70	112.12	6.68	0.009	28	16.83	2.63	0.048	102	183.03	3.78	0.032
Climate	**[a] Temperature**	**2**	**71**	**105.44**	**0.00**	**0.260**	**29**	**14.85**	**0.65**	**0.128**	**103**	**181.69**	**2.44**	**0.063**
Climate	**[b] Temperature range**	**2**	**71**	**110.40**	**4.96**	**0.022**	**29**	**14.20**	**0.00**	**0.177**	**103**	**181.9**	**2.65**	**0.057**
Climate	Precipitation	2	71	109.93	4.49	0.028	29	14.79	0.59	0.132	103	181.89	2.64	0.057

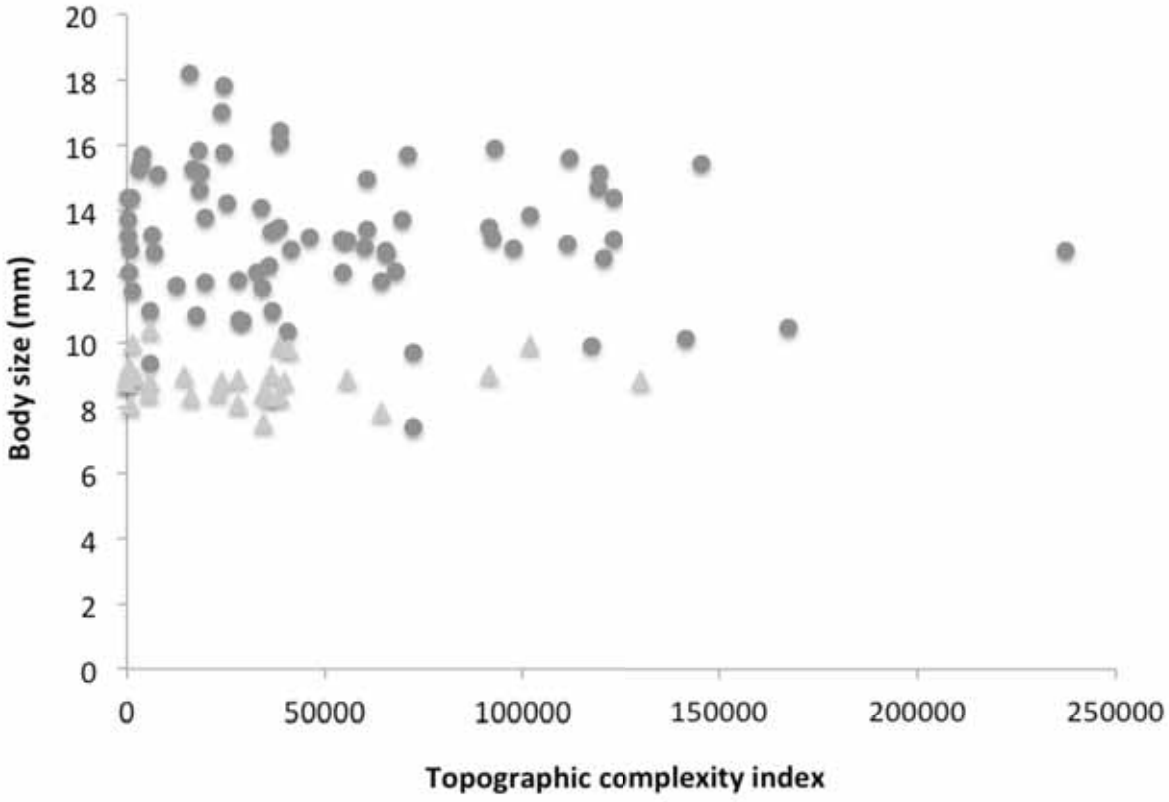

Figure 10. Body size variation in *Brachycephalus* populations with topographic complexity. Circles represent the populations from group A, generally aposematic and associated with high elevation sites, and triangles represent populations from group B, generally dull colored and widespread distributed, occurring from sea level up to 1000 m.

Seventeen species of *Brachycephalus* exhibit a general "bufoniform body", namely by presenting length of legs proportional to overall body size (MIRANDA-RIBEIRO, 1920). Furthermore, these species present the larger SVL within the genus, head as wide as long, and commonly exhibit a conspicuous orange coloration which has been associated to tetrodotoxin toxicity (SEBBEN et al., 1986; HANIFIN, 2010; PIRES et al., 2002, 2005). This group of species presents high phenotypic diversity, comprising minute body size species (e.g. *B. brunneus*, average SVL = 9.8 mm) and also the largest species within the genus (e.g. *B. ephippium*, *B. margaritatus* and *B. garbeanus*, average SVL = 14.9 mm). Herein, we identified this group of species as group A.

In the group A we can also observe a gradient in the degree of mineralization of the skull and post-cranial skeleton, since the condition of complete lack of hyperossification (e.g., *B. brunneus*) to a completely hyperossified condition, including a dorsal shield (e.g., *B. garbeanus*; CLEMENTE-CARVALHO et al., 2009). Some species have also osteoderms (e.g. *B. crispus, B. margaritatus* and *B. nodoterga*; CONDEZ et al., 2014).

Body color is another variable character among members of this group of species. Most have the background color conspicuous orange, covered or not by colored spots (e.g. reddish dorsal pattern of *B. pitanga*). As an exception, *B. brunneus* presents general body color brownish (RIBEIRO et al., 2005).

Most of species within group A exhibit restricted geographic range, represented by one or two populations known only from the type locality and its surroundings. *Brachycephalus brunneus*, *B. ephippium*, *B. margaritatus*, *B. garbeanus* and *B. nodoterga* are exceptions because of their occurrence in more than three localities. Considering the altitudinal preferences, species from this group are generally associated with mountain slopes along the main Atlantic rain forest mountain ranges (POMBAL et al., 1998; POMBAL, 2010; RIBEIRO et al., 2005; ALVES et al., 2006, ALVES et al., 2009). In general, these montane species occur in elevations around 800 m above sea level. Exceptionally, we found one population at 206 m and the higher limit of occurrence is attributed to 2164 m above sea level.

Differently, the group B includes *Brachycephalus didactylus*, *B. hermogenesi* and *B. pulex*, which exhibits a general "leptodactyliform body" (MIRANDA-RIBEIRO, 1920). These three species are dull colored with skills to long-distances jumps (VERDADE et al., 2008; NAPOLI et al., 2011). The skull and skeleton of these species lack hyperossification (CLEMENTE-CARVALHO et al., 2009). It comprises only minute body size species (maximum SVL = 10.4 mm) including *B. didactylus* and *B. hermogenesi*, the Brazilian toadlets considered among the ten smallest vertebrates in the world (RITTMEYER et al., 2012). *Brachycephalus didactylus* (SVL = 8.7 mm in average) occupies the third position in the smallest vertebrate rank, being only larger than two microhylids from New Guinea, *Paedophryne amauensis* and *Paedophryne swiftorum* (RITTMEYER et al., 2012). Despite of being not phenotypically diverse as the group A, mentioned before, the small body size and cryptic coloration of species from group B might contribute to their under estimated diversity.

Brachycephalus didactylus and *B. hermogenesi* are widespread distributed, presenting a wide altitudinal range of occurrence, from the sea level up to 1000 m of altitude (IZECKSOHN, 1971; GIARETTA & SAWAYA, 1998; PIMENTA et al., 2007; VERDADE et al., 2008). The exception here is *Brachycephalus pulex*, which is only known from type locality at 840 m of altitude (NAPOLI et al., 2011). In general, populations from this group occur in elevations around 600 m above sea level (*pers. obs.*). *Brachycephalus didactylus* and *B. hermogenesi* were until recently allocated into the no longer recognized genus *Psyllophryne* Izecksohn, 1971. *Brachycephalus* and *Psyllophryne* were considered distinct genera based on the presence of *omosternum* in the pectoral girdle of *Psyllophryne* and

absence in *Brachycephalus*. Currently, *Psyllophryne* is a junior synonym of *Brachycephalus* based on the presence of a reduced omosternum in *Brachycephalus ephippium* (KAPLAN, 2002). Although *B. pulex* has been described originally as *Brachycephalus*, authors evidenced the species similarities with *B. didactylus* and *B. hermogenesi* and attributed to this group of species the popular name of flea-toads, in allusion to the inconspicuous body color and jumping skills of the individuals (NAPOLI et al., 2011).

Our results revealed that higher elevations in Atlantic forest are usually associated with topographically more complex localities. The relief roughness and variability expressed by the topographic complexity index was indicated as the most important variable for determining morphometric variation in *Brachycephalus* species. Topographically complex localities are generally heterogeneous and exhibit an altitudinal gradient of environmental conditions, such as variation in temperature and humidity due to the relief irregularities. With some altitudinal overlap, the flea-toads populations are mainly associated with lower elevations and topographically less complex localities compared to populations of the other species that are larger bodied and associated to higher elevations. Even so, larger species of *Brachycephalus* were not restricted to high elevation localities and the expected pattern of increasing body size with altitude and topographic complexity, as predicted by the Bergmann's rule, was not found in these species.

Temperature was the best explanatory variable for morphometric variation within the high elevation species of *Brachycephalus*. The relationship of environmental temperature and body size is largely explored in tetrapods, especially in the light of latitudinal variation (ANGILETTA et al., 2004; ASHTON, 2004). Our findings suggest the presence of a latitudinal pattern of body size variation within this group; species from the southern portion of geographic distribution, with higher latitudes and mild temperatures, present smaller body size compared to species from the northern portion of the distribution. This body size variation pattern confirms that these species exhibits a converse Bergmann-type response to latitude. This pattern is common in salamanders (ASHTON, 2002; OLALLA-TÁRRAGA & RODRIGUEZ, 2007; ADAMS & CHURCH, 2008) and some anurans (e.g. in the majority of species within the genus *Bufo*; CVETKOVIK et al., 2008). In this case, species do not follow the expected larger adult size in colder environments as other ectothermic do (ATKINSON & SIBLY, 1997). Generally, amphibians of high latitudes and altitudes face cooler average temperatures and higher climatic fluctuations, dealing with thermoregulatory problems (WELLS, 2007). Probably due to the small body size, species of *Brachycephalus* would have body temperature closely fluctuating with environmental conditions and so, tend to minimize

body size in colder regions to reduce heating time (OLALLA-TÁRRAGA et al., 2006; OLALLA-TÁRRAGA, 2011). To corroborate this physiological trait, most *Brachycephalus* species are diurnal (POMBAL et al., 1994; LIMA et al., 2014) and probably exhibit extremely reduced dispersal capacity (e.g. PABIJAN et al., 2012; GASTON & BLACKBURN, 1996).

The recognition of two main groups of species within high elevation *Brachycephalus* is phylogenetic supported and was confirmed by the body size variation. The species from southern portion of Atlantic forest with small body size, *Brachycephalus brunneus*, *B. ferruginus*, *B. izecksohni*, *B. pernix* and *B. pombali* are, in almost all gene trees, clustered in a supported clade, sister to all other species within the genus (CLEMENTE-CARVALHO et al., 2011). These species are also geographically separated from the others by the Guapiara lineament, a physical barrier that coincides with phylogeographic distribution breaks for several vertebrate species in the Atlantic forest (e.g. GRAZZIOTIN et al., 2006; THOMÉ et al., 2010; D'HORTA et al., 2011; AMARO et al., 2012; RAPOSO et al., 2013). In addition to being influenced by temperature, the phylogenetic signal might contribute to the body size similarities in closely related species of *Brachycephalus*.

We did not find significant morphometric variation in populations of the flea-toads *Brachycephalus didactylus*, *B. hermogenesi* and *B. pulex* across the latitudinal gradient. The little body size variation found in this group of species is related to temperature fluctuation, calculated by the difference between the maximum temperature of the warmest month and the minimum temperature of the coldest month. Considering that these three species of flea-toads are widespread distributed and exhibits a wide altitudinal range of occurrence (IZECKSOHN, 1971; GIARETTA & SAWAYA, 1998; PIMENTA et al., 2007; VERDADE et al., 2008), distinct populations of the same species are exposed to completely different environmental conditions. *Brachycephalus didactylus* can be found in ombrophilous sub-montane and montane forests across its distribution (OLIVEIRA et al., 2012), in which the local climate conditions seem to be very distinct. Moreover, due to the extremely degraded state of the Atlantic forest, the remnants are usually small and susceptible to microclimate and vegetation structure changes related to edge effects (RIBEIRO et al., 2009). Populations of *B. hermogenesi* are in the same way distributed in pristine areas at Serra do Mar mountain range and in secondary forest remnants in the Atlantic Plateau of São Paulo state (VERDADE et al., 2008). Compared to mountainous areas of the Serra do Mar, the flatter relief of the Atlantic Plateau favors deforestation practices and forest fragmentation effects can be intense in these localities.

The first attempt to access the relationship of environmental variables and *Brachycephalus* occurrence was made by Pie et al. (2013). Although these authors recognized the importance of accounting for phylogenetic structure in modeling climatic preferences, they identified within *Brachycephalus* three morphometric and phylogenetic unsupported clusters of species based purely on predicted climatic niches. Our results suggested that the environmental conditions are very distinct among localities of occurrence of the flea-toads and the species of *Brachycephalus* associated to high elevations, except in those from where species of both groups are occurring in sympatry. There are no records of sympatry among high elevation species of *Brachycephalus*, mainly due to their habitat specialization and narrow range distribution. Although flea-toads have a widespread distribution and wider ecological tolerances, no sympatry was recorded among species of flea-toads. Pairs of sympatric species are for example *B. hermogenesi* and *B. nodoterga* (from Boracéia, Salesópolis, São Paulo), *B. hermogenesi* and *B. ephippium* (from Morro Grande Reserve, Cotia, São Paulo), *B. didactylus* and *B. margaritatus* (from Engenheiro Paulo de Frontin, Sacra Família do Tinguá, Rio de Janeiro) and *B. didactylus* and *B. garbeanus* (from Teodoro de Oliveira, Rio de Janeiro). The wider environmental tolerances of *B. hermogenesi* and *B. didactylus* are reinforced by their occurrence in sympatry with other species in high elevation localities and also in less topographically complex localities, which also are associated with high annual temperatures and high vegetation densities.

Our results corroborated the expected differences in morphometric traits and the environmental conditions associated to *Brachycephalus* occurrence at highlands and lowlands in the Atlantic forest. Topographic complexity was the most important variable to explain the morphometric variation between species from lowlands and highlands. Despite of this, we did not find altitudinal or latitudinal patterns of variation in body size, except when analyzing only the high elevation species. In this case, species associated to lower temperatures and high latitudes exhibit smaller body size than those from higher temperatures. The higher morphometric variation within the high elevation species of *Brachycephalus* corroborates the phenotypic diversity of this group when compared to the flea-toads. Further investigations on *Brachycephalus* diversification at highlands and lowlands, including genetic and natural history aspects, may provide valuable information to the knowledge of these species and the evolutionary processes related to diversification in montane regions of the Atlantic forest.

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Appendix 1. Specimens examined. Abbreviations of Brazilian states are: BA = Bahia, ES = Espírito Santo, PR = Paraná, RJ = Rio de Janeiro, SC = Santa Catarina, and SP = São Paulo.

B. alipioi, Santa Teresa, ES (MNRJ 25405, 25406, 25407); *B. alipioi*, Vargem Alta, ES (CFBH 25437-38, 24663-67; MNRJ 26042, 26044, 26046-50, 26052-55); *B. brunneus*, Campina Grande do Sul, PR (CFBH 7880, 7893-94; MNRJ 40289-91); *B. bufonoides*, Macaé, RJ (MZUSP 1458-59); *B. crispus*, Cunha, SP (CFBH 10698-99, 10701-03, 10705-08, 23894-903, 27857-68, 29659-61, 29822-27, 29829-30, 29832-40; MNRJ 17443-44); *B. didactylus*, Atílio Vivacqua, ES (CFBH 32636-40; MNRJ 58974, 66733, 66735-37, 66746, 66749, 66755, 66759, 66766); *B. didactylus*, Ilha Grande, RJ (MNRJ 31201, 49294-96, 49298, 55475, 59361-62, 70585-86); *B. didactylus*, Magé, RJ (MNRJ 56156-57, 60980, 62841-42); *B. didactylus*, Lídice Rio Claro, RJ (MNRJ 71334), *B. didactylus*, Parque Estadual dos Três Picos, Cachoeiras de Macacu, RJ (MNRJ 54402-03, 69868, 69875, 69877, 69880, 69884, 69891-93); *B. didactylus*, Reserva Ecológica de Guapiaçu, Cachoeiras de Macacu, RJ (MNRJ 53231-32, 55473-74, 57736, 68819-20, 72358-59); *B. didactylus*, Vale da Revolta, Teresópolis, RJ (MNRJ 70581, 39096); *B. didactylus*, Sacra Família do Tinguá, RJ (MNRJ 4062-73; MZUSP 58319-20, 64810-12, 94620-21); *B. didactylus*, Santa Maria Madalena, RJ (CFBH 28125-27, 27343); *B. didactylus*, Teodoro de Oliveira, RJ (MNRJ 56514-15); *B. ephippium*, Angra dos Reis, RJ (MNRJ 17458-59); *B. ephippium*, Atibaia, SP (MZUSP 104203-04; CFBH 16807-11, 16813, 16815, 16817-18, 16820-21, 16824, 16826); *B. ephippium*, Alto do Soberbo, Teresópolis, RJ (MZUSP 49991, 49996, 50008-09, 50011, 50014, 50022-23, 50028, 50032, 50039, 50043, 50051, 50054, 50055); *B. ephippium*, Cotia, SP (MZUSP 104149-58, 132267); *B. ephippium*, Guaratinguetá, SP (CFBH 31981-83); *B. ephippium*, Itanhaém, SP (CFBH 19348, 25809, 25816); *B. ephippium*, Joaquim Egydío, Campinas, SP (CFBH 994, 996, 1343, 2565-66, 2568-69, 4259-60; MNRJ 25347-52); *B. ephippium*, Juquitiba, SP (CFBH 32311); *B. ephippium*, Lídice, Rio Claro, RJ (MNRJ 25353-54, 66619-20, 71337, 76000, 81016); *B. ephippium*, Mangaratiba, RJ (MNRJ 2542, 11574-83, 13264-66, 13269); *B. ephippium*, Monteiro Lobato, SP (MNRJ 70620-22); *B. ephippium*, Mogi das Cruzes, SP (CFBH 28658-59, 28663-64, 28667-68, 28670; MZUSP 104208-10, 136758, 136762, 136764, 136787, 137319, 138775, 138776); *B. ephippium*, Parque Estadual Carlos Botelho, São Miguel Arcanjo, SP (MZUSP 136670); *B. ephippium*, Parque Nacional do Itatiaia, Itatiaia, RJ (MZUSP 4134, 7755, 104081, 104101-02; MNRJ 23583-86, 52428-29, 52439); *B. ephippium*, Parque Nacional da Serra dos Órgaos, Teresópolis, RJ (CFBH 22013-15, 24763-64, 32569-70; MZUSP 11205, 34646, 71707-08; MNRJ 17438-39, 17450, 51580-83); *B. ephippium*, Pedra Branca, Jacarepaguá, RJ (MNRJ 3327, 13818-19, 27577-79); *B. ephippium*, Pedra Branca, Paraty, RJ (ALMN 894, 896, 897-99; MNRJ 632, 2435, 10271); *B. ephippium*, Pindamonhangaba, SP (MNRJ 70616-18); *B. ephippium*, Piquete, SP (MZUSP 534, 851, 861, 864-66, 871, 3821-24, 3827-28, 3831-32, 3835, 3837-38, 3840, 3844-45, 4387, 54387); *B. ephippium*, Represa Billings, São Paulo, SP (CFBH 11623); *B. ephippium*, Resende, RJ (MNRJ 73096-99, 78465); *B. ephippium*, Reserva Ecológica do Guapiaçu, Cachoeiras de Macacu,

RJ (MNRJ 38104, 56517-18, 59946); *B. ephippium*, Vale da Revolta, Teresópolis, RJ (MNRJ 50260-61, 70580); *B. ephippium*, Represa Rio Grande, RJ (MZUSP 9031, 100277, 100281, 100283, 100285-86, 100290, 100292, 100295, 100298-99, 100303-05, 100307, 100312, 100317, 100342-44, 100346, 100349, 100356, 100436, 100580, 100718); *B. ephippium*, Rio de Janeiro, RJ (MZUSP 100278-80, 103809, 103813, 103817, 103820, 103823, 103828, 103831, 103835, 103839, 103841, 103847, 103850, 103856, 103864, 103869); *B. ephippium*, Rócio, Petrópolis, RJ (CFBH 13946); *B. ephippium*, São Franscisco Xavier, SP (CFBH 16827-34; MNRJ 70619, 70623-25; MZUSP 2761-62); *B. ephippium*, Santo Antônio do Pinhal, SP (MNRJ 70628); *B. ephippium*, Serra do Japi, Jundiaí, SP (CFBH 9431, 16837, 16839, 16857, 16859, 16864, 16870, 30834-35, 30842; MZUSP 507; AP 1, 2, 3, TC 98, 99); *B. ephippium*, Taubaté, SP (MNRJ 70627); *B. ephippium*, Valença, RJ (MNRJ 73395); *B. ferruginus*, Morretes, PR (CFBH 8028, 8030, 8032; TC 142-44); *B. garbeanus*, Alto Caledonia, RJ (MNRJ 39585, 39602, 67498); *B. garbeanus*, Baixo Caledonia, RJ (MNRJ 57124, 57293); *B. garbeanus*, Nova Friburgo, RJ (MNRJ 17433, 27008-09; MZUSP 811, 1460-61); *B. garbeanus*, Alto de Macaé, RJ (CFBH 23564-67, 23569-71, 23574, 23576-77; MNRJ 17440-41, 25391, 25398, 39617); *B. garbeanus*, Serra Nevada, RJ (MNRJ 39342-43); *B. garbeanus*, Teodoro de Oliveira (MNRJ 51472-73); *B. guarani*, Prumirim, Ubatuba, SP (CFBH 27231-35, 27237); *B. hermogenesi*, Salesópolis, Boracéia, SP (CFBH 20125, 20128; MZUSP 138347; MNRJ 47932-34; TC 127, 129, 133, 135); *B. hermogenesi*, Capão Bonito, SP (A-RG 1-7); *B. hermogenesi*, Fazenda Capricórnio, Ubatuba, SP (MNRJ 18624-25, 18662-63); *B. hermogenesi*, Ibiúna, SP (MZUSP 142568); *B. hermogenesi*, Jambeiro, SP (CA 60, 64-65, 195-97, 208-10, 213-14, 221-27, 240, 248, 273, 280); *B. hermogenesi*, Juquitiba, SP (MZUSP 134806-07); *B. hermogenesi*, Cotia, SP (MZUSP 132257-62, 134377); *B. hermogenesi*, Paraibuna, SP (AAG-UFU 175-82); *B. hermogenesi*, Peruíbe, SP (CFBH 24086); *B. hermogenesi*, Picinguaba, Ubatuba, SP (CFBH 29793-95, 29797; MNRJ 18907; MZUSP 137219); *B. hermogenesi*, Registro, SP (MZUSP 138279); B. hermogenesi, Tapiraí, SP (A-TC 1-13, 15-16); *B. izecksohni*, Campina Grande do Sul, PR (CFBH 7375, 7384, 7386-87; MZUSP 132892-93); *B. margaritatus*, Castelo Country Club, Petrópolis, RJ (MNRJ 73700-02); *B. margaritatus*, Castelo Monte Belo, Petrópolis, RJ (MNRJ 70428-29); *B. margaritatus*, Paty de Alferes, RJ (MNRJ 76100-01); *B. margaritatus*, Petrópolis, RJ (MNRJ 60869-70, 60891, 62976-84); *B. margaritatus*, Quitandinha, Petrópolis, RJ (MNRJ 75174); *B. margaritatus*, Sacra Família do Tinguá, RJ (MNRJ 21675, 25373-76, 25385, 25387-89, 25401, 39312); *B. nodoterga*, Salesópolis, Boracéia, SP (MZUSP 30625-26, 30653; TC 128, 130-32, 136-38); *B. nodoterga*, Serra da Cantareira, Mairiporã, SP (CFBH 28413; MZUSP 809, 975, 112785-91; MNRJ 73674-75; TC 140); *B. nodoterga*, Ilhabela, SP (CFBH 7030, 7032-33, 7035, 7037-42, 7045-48; MNRJ 23633-36; 23638-40; TC 105-111); *B. nodoterga*, Santana de Parnaíba, SP (CFBH 9606; MZUSP A147711-22); *B. pernix*, Quatro Barras, PR (CFBH 28888-91; MNRJ 17343); *B. pitanga*, São Luis do Paraitinga, SP (CFBH 15758, 16746-47, 16750, 16755-56, 16760-66, 24299); *B. pitanga*, Natividade da Serra, SP (TC 115-121); *B. pombali*, Guaratuba, PR (CFBH 8048, 8051); *B. pulex*, Camacan, BA (MNRJ 69646; TC 180-184, 186-187,

189-194); *B. toby*, Corcovado, Ubatuba, SP (CFBH 12902-03, 20581-91, 20593-99, 23004-06; TC 113-114); *B. tridactylus*, Salto Morato, Guaraqueçaba, PR (MNRJ 67312-13; MZUSP 137952-57); *B. vertebralis*, Pedra Branca, Paraty, RJ (CFBH 3416-17; MNRJ 2053, 2338, 10599, 11095-96, 11098, 11100-07, 11111, 11113-15, 11117-18, 11120-21, 11124, 11127, 11132); *B. vertebralis*, Picinguaba, Ubatuba, SP (CFBH 3953-54, 3964, 7907-08, 7910, 8361-63, 29789-91); *B.* sp. (aff. *ephippium*), Bananal, SP (MNRJ 132166-81, 138647-56; TC 122-126); *B.* sp. (aff. *ephippium*), Cunha, SP (CFBH 27852-54, 27856, 27879, 29810-21, 29841; TC 158-162); *B.* sp. (aff. *ephippium*), Parque das Neblinas, Mogi das Cruzes, SP (MZUSP 135753, 136870-79; PNG 4); *B.* sp. (aff. *ephippium*), Paranapiacaba, Santo André, SP (CFBH 27187-90, 28168, 28206-10; MNRJ 25367, 42873; MZUSP 93350-51); B. sp. (aff. *vertebralis*), Cunha/Ubatuba, SP (CFBH 29843-55; TC 145-151); *B.* sp. (aff. *hermogenesi*), Parque Estadual do Jacupiranga, Barra do Turvo, SP (MZUSP 138640-43); B. sp. (aff. *hermogenesi*), São Francisco do Sul, SC (TC 251-252, 263-264); B. sp. (aff. *hermogenesi*), Joinville, SC (TC 234, 244-245); *B.* sp. (aff. *hermogenesi*), Quatro Barras, PR (TC 141); *B.* sp. (aff. *hermogenesi*), Ilha do Cardoso, Cananéia, SP (TC 267-268); *B.* sp., Santa Maria Madalena, RJ (CFBH 27342, 27344-45, 28048-49, 28124, 28128-38; MNRJ 42408-10, 52718-21); *B.* sp., Mendanha, RJ (MNRJ 53167-68, 57008-09); *B.* sp., Teodoro Oliveira, RJ (MNRJ 51470-71, 56504-11, 56513, 56516, 66543); *B.* sp., Joinville, SC (TC 235-243); *B.* sp., Corupá, SC (TC 246-248); *B.* sp., Garuva, SC (MZUSP 132902, 132904-07, 132910, 132912, 132914, 132916, 132918, 132920, 132921, 132925-29; TC 202, 205, 208, 209, 213, 215, 216, 218, 220, 221, 225, 227-231); *B.* sp., Serra do Ibitiraquire, Campina Grande do Sul, PR (MZUSP 132894-97, 132900); *B.* sp., Morro dos Perdidos, Guaratuba, PR (CFBH 20940, 20941, 20943-55); *B.* sp., São Francisco do Sul, SC (TC 232, 233, 253-262).

CAPÍTULO II

Phylogenetic relationships and the evolution of morphological and ecological traits within the miniaturized species of *Brachycephalus* (Anura: Brachycephalidae)

Phylogenetic relationships and the evolution of morphological and ecological traits within the miniaturized species of *Brachycephalus* (Anura: Brachycephalidae)

Abstract

The genus *Brachycephalus* is endemic to the Atlantic forest of Brazil and currently includes 21 species. These species exhibit extremely small adult body size and share morphological features due to evolutionary process of miniaturization. Morphological and ecological features have been used to support the informal distinction of the flea-toads (*B. didactylus*, *B. hermogenesi*, and *B. pulex*) and other species within *Brachycephalus*. Herein, we present a molecular phylogenetic hypothesis based on mitochondrial DNA sequences in attempt to examine these species relationships. Additionally, we mapped and reconstructed ancestral states of body color, hyperossification pattern, body size, advertisement call, altitudinal range, and geographic distribution with the main purpose of describe the extant diversity and discuss the evolution of these traits. The topology confirmed that the genus *Brachycephalus* is monophyletic and we found no molecular evidence of distinction for the flea-toads among *Brachycephalus* species. Nine well-supported clades were reconstructed and *B. pulex* was the single species not assigned to any of these clades. The basal clade comprises populations of *B. hermogenesi* and the relationship between this and all other sister species of *Brachycephalus* are well supported. The extant species are supposed to be evolved from a dull colored ancestor, without hyperossification in skull and postcranial skeleton, with wide altitudinal range, and short advertisement call composed by one to four high frequency notes with 1-3 pulses. Distinct events are supposed to drive the genus evolution in southern and northern portions of Atlantic forest and further studies are necessary to address those divergences.

Introduction

The terrestrial breeding and direct-developing frogs included in Terrarana (HEDGES et al., 2008; PYRON & WIENS, 2011) exhibit several cases of miniaturization, the evolutionary process leading to an extremely small adult body size (HANKEN & WAKE, 1993). At least six genera from distinct Terraranan families (e.g. *Adelophryne*, *Brachycephalus*, *Eleutherodactylus, Euparkerella, Phyzelaphryne*, and *Noblella*) include examples of miniaturized species (RITTMEYER et al., 2012; FROST, 2014; FOUQUET et al., 2012; FUSINATTO et al., 2013).

Terrestrialization and miniaturization are recurring evolutionary trends in amphibians (ZIMKUS et al., 2012). The evolution of non-aquatic lifestyle enable species to occupy new ecological niches far from water bodies, but in the same way directs the species requirements to very specific habitat conditions, as the high humidity microhabitats inside forests (DUELLMAN & TRUEB, 1994). At the same time, due to the small body size and the predicted low dispersal ability, miniaturized species are mostly microendemic, with very restricted geographic distribution (WOLLEMBERG et al., 2011; PIE et al., 2013). So, these species are predicted to present increased speciation rates and are considered excellent models to diversification studies (WOLLEMBERG et al., 2011; ZIMKUS et al., 2012).

The genus *Brachycephalus* is endemic to the Atlantic forest of southern, southeastern and northeastern Brazil, and currently comprises 21 species (RIBEIRO et al., 2005; NAPOLI et al., 2011; FROST, 2014). Species of *Brachycephalus* are leaf-litter inhabitants and exhibit extremely small adult body size, with snout-vent length usually inferior to 18 mm (POMBAL, 1999; CLEMENTE-CARVALHO et al., 2011). *B. didactylus* and *B. hermogenesi* are considered among the ten smallest vertebrates in the world, being smaller than 10 mm (RITTMEYER et al., 2012). Due to the miniaturization, *Brachycephalus* species share morphological characteristics, as the loss of phalanges in the manus and pes, a reduced number of functional toes, the loss of bones such as the neopalatine and columellae in the skull, and the complete ossification of epicoracoid cartilages with clavicle and scapula (IZECKSOHN, 1971; POMBAL et al., 1998; YEH, 2002). Another remarkable phenotypic feature among species of *Brachycephalus* is a gradient in the degree of mineralization of the skull and post-cranial skeleton, including a hyperossified dorsal shield in some species (CLEMENTE-CARVALHO et al., 2009).

Most *Brachycephalus* species exhibits restricted distribution along the main Atlantic forest mountain ranges, occurring from 600 to 1800 m elevation (CLEMENTE-CARVALHO et al., 2011; CONDEZ et al., 2014). These species share generalized bufoniform morphology and exhibit the highest snout-vent-length values for the genus (CONDEZ et al., unpublished data). These species have conspicuous orange coloration and, in some cases, the skin contains toxic tetrodotoxin-like substances (SEBBEN et al., 1986; PIRES et al., 2002, 2005; HANIFIN, 2010). Other three species within the genus are popularly known as flea-frogs (*B. didactylus, B. hermogenesi*, and *B. pulex*; NAPOLI et al, 2011). These species have a wide altitudinal range, occurring from sea level up to 950 m (IZECKSOHN, 1971; GIARETTA & SAWAYA, 1998; NAPOLI et al., 2011) and exhibit relatively smaller body sizes compared to the most species of *Brachycephalus*, leptodactyliform morphology, dull inconspicuous

coloration, and the ability to jump longer distances (VERDADE et al., 2008; NAPOLI et al., 2011). These characteristics have been used to support informal distinction of the flea-toads and all the other species within *Brachycephalus* (VERDADE et al., 2008; NAPOLI et al., 2011).

The existing phylogenetic hypothesis for the genus is based on molecular evidence; the phylogenetic groups are mainly associated to geographic distribution and the different conditions of hyperossification of the skull and skeleton (CLEMENTE-CARVALHO et al., 2011). Despite of this, the basal relationships and the relationship between the flea-toads and other species within *Brachycephalus* were not clearly resolved (CLEMENTE-CARVALHO et al. 2011). Natural history and ecological traits of *Brachycephalus* species are poorly understood, especially because of the possible species activity under leaf litter and the difficulties associated to access the study sites (CONDEZ et al., 2014). However, these data might reflect species relationships and provide valuable information for comparative and evolutionary studies (e.g. COCROFT & RYAN, 1995; PADIAL et al., 2010; GEHARA et al. 2013). The recent description of new species (CLEMENTE-CARVALHO et al., 2012; GAREY et al., 2012; CONDEZ et al., 2014) and the increasing information on ecology and natural history (FONTOURA et al., 2011; ALMEIDA-SANTOS et al., 2011; ARAÚJO et al., 2012; DORIGO et al., 2012) are currently revealing an extraordinary diversity and evidencing that species of *Brachycephalus* are particularly interesting models to study evolutionary trends.

Herein, we present a molecular phylogenetic hypothesis based on mitochondrial DNA sequences in attempt to examine the relationships within *Brachycephalus*. Our specific goals were: (1) to test the monophyly of *Brachycephalus*, (2) to evaluate molecular evidence for distinction between the flea-toads and other *Brachycephalus* species, and (3) to identify the main clades and correlate this data with available geographical, morphological, and ecological information. Additionally, we mapped and reconstructed ancestral states of six morphological and ecological traits with the main purpose of describing extant diversity and discuss the evolution of these traits.

Material and Methods

Molecular Data

We obtained molecular data for 65 individuals, from 18 formally named and 11 undescribed *Brachycephalus* species (Appendix 1). Our ingroup sampling represents

approximately 85% of the known diversity of *Brachycephalus* (21 species; FROST, 2014). These data include representatives distributed in all regions and elevational ranges in which these anurans are known (Figure 1).

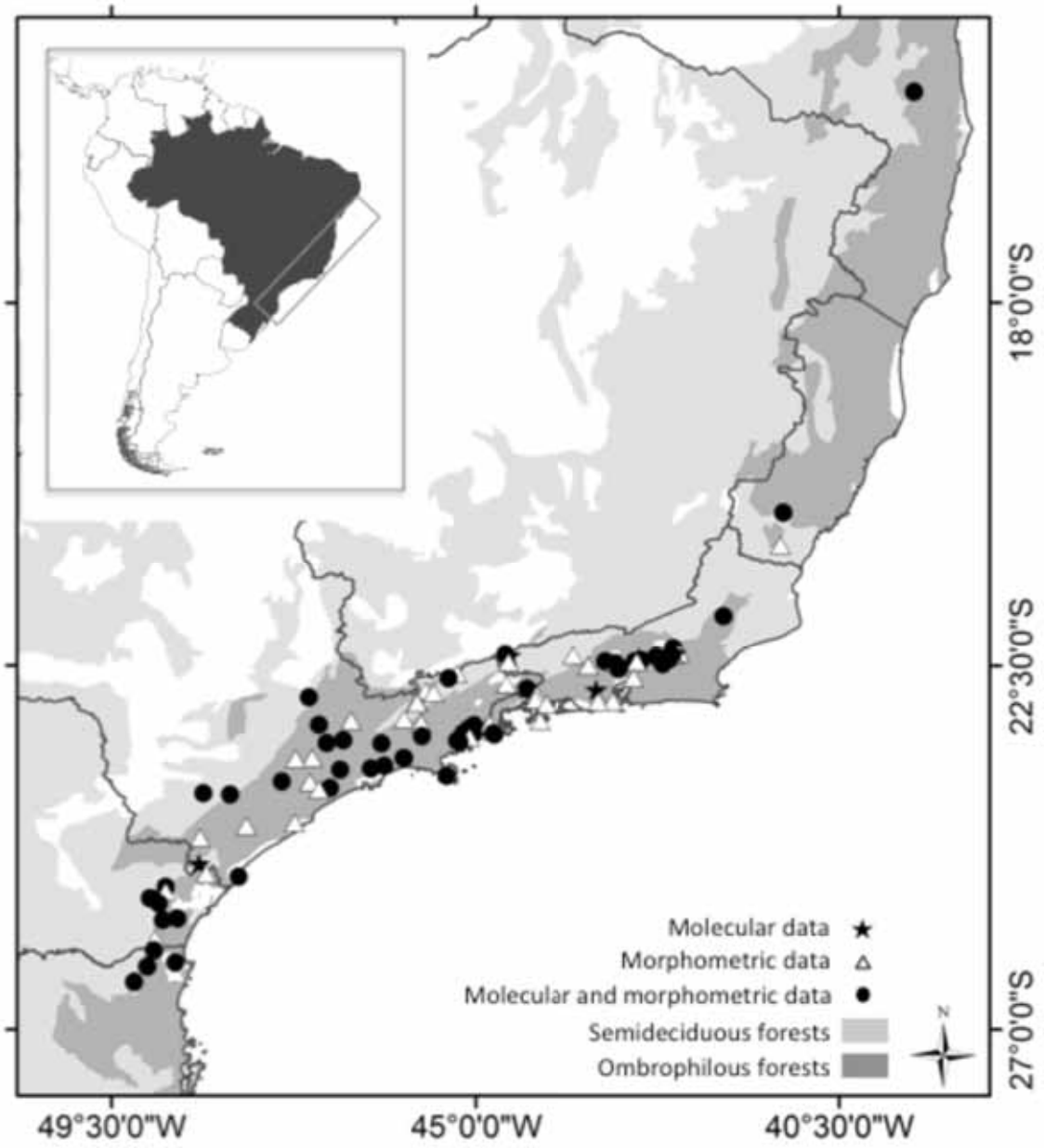


Figure 1. Populations of *Brachycephalus* analyzed in this study. Black dots represent populations used in molecular and morphometric analyses, white triangles represent populations used only in morphometric analysis, and black stars represent populations used only in molecular analysis.

Molecular studies have established *Ischnocnema* as the sister group of *Brachycephalus* (HEDGES et al., 2008; PYRON & WIENS, 2011; CANEDO & HADDAD, 2012). The species *Haddadus binotatus* and *Ischnocnema parva* were used as outgroups and the former species was used to root the phylogeny. The analyzed tissue samples were deposited in

CFBHT (Célio F. B. Haddad Tissue Collection, Departamento de Zoologia, I.B., Universidade Estadual Paulista, Campus de Rio Claro, São Paulo, Brazil), CTMZ (Tissue collection at Museu de Zoologia, Universidade de São Paulo, Brazil), and MNRJ (Tissue Collection of Museu Nacional, Rio de Janeiro, Rio de Janeiro, Brazil).

The selected genes were the mitochondrial fragments 12S rRNA (12S; 814 bp), 16S rRNA (16S; 864 bp), and Cytochrome b (Cytb; 694 bp). Total DNA was extracted from ethanol-preserved muscle or liver tissues using DNeasy Tissue Kits (Qiagen Inc., Valencia, CA). The amplification and sequence strategy of mitochondrial fragments followed similar procedures as in a previous study (CLEMENTE-CARVALHO et al., 2011). We performed Polymerase Chain Reaction (PCR) for the amplification of the selected fragments using specific primers (Table 1) and PCR Master Mix (Taq Polymerase 0.05 u/ul, PCR reaction buffer with 4 mM $MgCl_2$, 0.4 mM of each dNTP) or PCR Grade Nucleotide Mix (Roche Diagnostics GmbH, Mannheim, Germany), according to the manufacturer's instructions. Fragments of the mitochondrial 12S gene were amplified using primer pair 12SJL and 12SKH (GOEBEL et al., 1999). Thermocycling conditions for dsDNA amplification began with a 5-min denaturation at 94^{o}C followed by 35 cycles each of which had a 1-min denaturation at 94^{o}C, a 1-min annealing at 50-52^{o}C, and a 1-min extension at 72^{o}C. A 5-min extention at 72^{o}C followed the final cycle. A section of the mitochondrial 16S gene was amplified using primer pair 12SM and 16SA (DARST & CANNATELLA, 2004). Thermocycling conditions were similar to those for 12S except that annealing temperatures were 60-61^{o}C. Partial sequences of Cytb gene were obtained using five primers. Primers MVZ15-L and CytbAR-H were previously published (MORITZ et al., 1992; GOEBEL et al., 1999) and primers BrachyCytb-F2, BrachyCytb-F3, and BrachyCytb-R1 were designed for this study and amplified a fragment just below 700 bp. Thermocycling conditions were similar to those for 12SrDNA except that annealing temperatures were 60-61^{o}C for primer pair BrachyCytb-F2 and BrachyCytb-R1, 55^{o}C for primer pair BrachyCytb-F3 and BrachyCytb-R1, and 50-55^{o}C for primer pair MVZ15-L and CytbAR-H. PCR products were visualized in 1% agarose gels. PCR products were purified using Exonuclease and Shrimp Alkaline Phosphatase (Affimetrix/USB, Cleveland, OH), according to the manufacturer's instructions. Cycle sequencing reactions were completed with Applied Biosystems BigDye Terminator chemistry (Applied Biosystems). Sequencing was performed by Macrogen Inc. on an Applied Biosystems automated 3730 DNA analyzer or at Cornell University under the same conditions. Sequences were obtained in both directions with the same primers used for PCR amplification and subjected to BLAST searches in GenBank to verify amplification of desired

sequences. Chromatograms were fully inspected, embedded primer sequences were deleted, and forward and reverse strands were compared before assembling consensus sequences with CodonCode Aligner 3.5 (Codon Code Corporation). Data also include sequences downloaded from GenBank, NCBI (Appendix 1). Sequences were aligned using CLUSTAL W (THOMPSON et al., 1994) in BioEdit software (HALL, 1999).

Table 1. Primers used in this study.

Primer		Gene	Sequence	Reference
12SJL	F	12S rRNA	AAAGRTTTGGTCCTRRSCTT	Goebel et al., 1999
12SKH	R	12S rRNA	TCCRGTAYRCTTACCDTGTTACGA	Goebel et al., 1999
12SM	F	16S rRNA	GGCAAGTCGTAACATGGTAAG	Darst & Cannatella, 2004
16SA	R	16S rRNA	ATGTTTTTGGTAAACAGGCG	Darst & Cannatella, 2004
MVZ15-L	F	Cytb	GAACTAATGGCCCACACWWTACGNAA	Moritz et al., 1992
CytbAR-H	R	Cytb	TAWAAGGGTCTTCTACTGGTTG	Goebel et al., 1999
BrachyCytb-F2	F	Cytb	AAGCAAATAAGAAGTATCAYTCTGG	this study
BrachyCytb-F3	F	Cytb	CCGGACTCTTCCTAGCYATA	this study
BrachyCytb-R1	R	Cytb	CCATCACCGGACTCTTCCTAGC	this study

Phylogenetic analysis

We used the concatenated mtDNA dataset (2372 bp) based on one individual per locality to infer the phylogenetic relationship within *Brachycephalus*. We used maximum parsimony (MP), maximum likelihood (ML), and Bayesian inference (BI) for phylogenetic reconstructions. We performed MP analysis in TNT 1.1, Willi Hennig Society Edition (GOLOBOFF et al., 2008) using New Technology Search at initial level 50, including sectorial searches, ratched, drift, and tree fusing; requested directed search to hit 15 times the best length; random addition sequence 1000; random seed 1; replace existing trees, auto-constrain; we also performed a total of 1000 jacknife replicates with the same specifications. We performed ML analysis in RAxMLGUI 0.9.3 (STAMATAKIS, 2006) using the GTR+I+G model of nucleotide substitution. We performed a total of 100 runs and assessed node support via 1000 bootstrap replicates (ML + thorough bootstrap; n. threads 10). We partitioned the dataset by gene (12S rRNA, 16S rRNA) and codon position in protein-coding genes (Cytb). Saturation analysis of transitions and transversions against genetic distances were obtained in Dambe 4.1.19 (XIA & XIE, 2001). Because we did not detect any kind of

saturation all substitutions of these three genes were used for phylogenetic reconstructions. We performed BI analyses in MrBayes 3.1 (RONQUIST & HUELSENBECK, 2003) based on the best substitution models selected for each locus according to the Akaike Information Criterion (AIC) in PartitionFinder 1.0 (LANFEAR et al., 2012). The models of nucleotide evolution used in BI for the five partitions were: 12S (GTR+I+G), 16S (GTR+I+G), Cytb 1st codon (TVMef+I+G), Cytb 2nd codon (TVM+G) and Cytb 3rd codon (GTR+G). We performed analyses with 10 million generations, consisted of two independent runs, four Markov chains with default heating values and default priors. Trees were sampled every 1000 generations and the first 25% of generated trees were discarded as burn-in. Output files were examined visually in Tracer 1.5 (RAMBAUT & DRUMMOND, 2007) and trees were visualized and edited using FigTree 1.1.2 program, available at http://tree.bio.ed.ac.uk/software/figtree/.

Character coding and evolution

We categorically coded and reconstructed the evolution of six traits within *Brachycephalus*: (1) body color, (2) hyperossification pattern, (3) body size, (4) advertisement call, (5) altitudinal range, and (6) geographic distribution (Appendix 2). The characters were plotted in the terminals and reconstructed to ancestral states using Maximum Likelihood (PAGEL, 1999; CUNNINGHAM, 1999) in Mesquite 2.74 (MADDISON & MADDISON, 2010). Ancestral states were reconstructed on a 50% major rule consensus tree, based on a set of 7.500 post burn-in trees obtained from Bayesian inference, by tracing characters over trees. Ancestral state reconstructions were completed under a single-rate Mk likelihood model (mk1) with the likelihood decision threshold set to default value of 1.0. Reconstructions of internal nodes equivocal or absent in more then 50% of the examined trees were assigned to equivocal state.

Body color and body size

For body color and body size coding we examined 1008 *Brachycephalus* specimens from 106 populations distributed in 100 localities along the coastal Brazilian Atlantic forest, representing 99% of known diversity of *Brachycephalus* (Figure 1). The specimens analyzed are deposited in CFBH (Célio F. B. Haddad Collection, Departamento de Zoologia, I.B., Universidade Estadual Paulista, Campus de Rio Claro, São Paulo, Brazil), MZUSP (Herpetology Collection of Museu de Zoologia, Universidade de São Paulo, São Paulo,

Brazil) and MNRJ (Herpetology Collection of Museu Nacional, Rio de Janeiro, Rio de Janeiro, Brazil) (Appendix 3).

Body size is represented by the average snout-vent length (SVL) in millimeters, resulting from several adults measured in the same population. We considered juveniles the individuals smaller than 10.0 mm SVL for *Brachycephalus alipioi, B. bufonoides, B. ephippium*, *B. garbeanus, B. margaritatus*, *B. nodoterga*, *B. pitanga*, *B. toby*, and *B. vertebralis*; smaller than 8.0 mm SVL for *B. brunneus*, *B. ferruginus*, *B. izecksohni*, *B. pernix*, *B. pombali*, and *B. tridactylus;* and smaller than 7.0 mm SVL for *B. didactylus, B. hermogenesi*, and *B. pulex*. SVL was taken with a digital caliper with precision of 0.01 mm or a micrometric ocular fitted to the stereomicroscope. Sexual size dimorphism was not considered in our analysis. Body sizes of *Haddadus binotatus* and *Ischnocnema parva* were obtained from literature (HEYER et al., 1990; DIAS et al., 2012). Based on these data continuous snout-vent length values have been allocated among five categories: (0) SVL 8.0–10.0 mm; (1) SVL 10.1–12.0 mm; (2) SVL 12.1–14.0 mm; (3) SVL 14.1-18.0 mm, and (4) SVL > 30.0 mm.

Body color was preferentially evaluated in live animals or photographs taken in life. General body color was coded as a binary character: (0) dull colored, as dark brown or another cryptic coloration similar to the leaf-litter habitat, and (1) conspicuous, generally with the entire body color orange or yellow and clearly distinct from the leaf-litter habitat.

Hyperossification pattern

The hyperossification pattern is related to the degree of mineralization of the skin elements, skull, and postcranial skeleton in *Brachycephalus*. Bony projections are associated with an intricate network between bone tissue and collagen fibers, resulting in three distinct conditions of skeletal mineralization (CLEMENTE-CARVALHO et al., 2009). In the condition of absence of hyperossification species do not have mineralized elements associated to the skin, skull, and postcranial skeleton (Figure 2A). In the intermediate condition of hyperossification the spinous processes, presacral vertebrae, and some portions of the skull are mineralized (Figure 2B). In the extreme condition of hyperossification the skull and postcranial skeleton are strongly mineralized including a dorsal bone-shield (Figure 2D). These conditions, except for the one with absence of hyperossification, can be also combined with the presence of dermal ossifications (Figure 2C). The hiperossification pattern was described for the majority of species within *Brachycephalus* by cleared and double-stained, microradiography, or electronic microscopy prepared material (e.g. CLEMENTE-

CARVALHO et al., 2009; CLEMENTE-CARVALHO et al., 2012; CONDEZ et al., 2014). For the species that the condition was not described, we prepared the material or followed species descriptions to attribute one of these conditions.

Hyperossification pattern was classified among five categories: (0) hyperossification absent (no mineralized elements associated to the skin, skull, and postcranial skeleton); (1) intermediate condition of hyperossification (skull and vertebrae mineralized); (2) intermediate condition of hyperossification + osteoderms (skull and vertebrae mineralized and variable amount of osteoderms); (3) extreme hyperossification (skull and postcranial skeleton mineralized including a dorsal bone-shield); (4) extreme hyperossification + osteoderms (skull and postcranial skeleton mineralized, dorsal bone-shield, and variable amount of osteoderms).

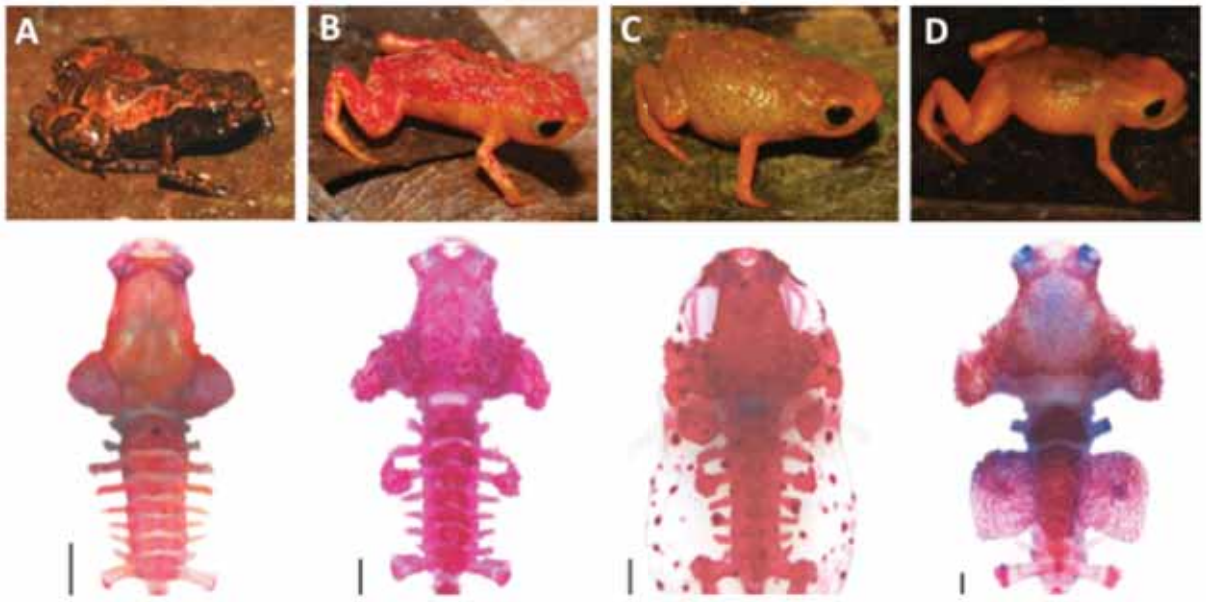


Figure 2. Live specimens and cleared and double-stained skull and vertebral column representative of the hyperossification pattern in *Brachycephalus*. (A) hyperossification absent represented by *B. hermogenesi*; (B) intermediate condition of hyperossification represented by *B. pitanga*; (C) intermediate condition of hyperossification and osteoderms represented by *B. nodoterga*; and (D) extreme hyperossification represented by *B. ephippium*. Scale bar 1 mm.

Acoustic data

We analyzed 73 advertisement calls from eight formally named and six undescribed species of *Brachycephalus* (Appendix 4). One to six individual were analyzed from each population. Calling males were recorded with a Marantz PMD-660 digital recorder coupled to an external unidirectional Sennheiser ME-66 microphone. We analyzed 10-20 calls per

individual using a sampling frequency rate of 44.1 kHz and 16-bit resolution in the mono pattern. Bioacoustic analyses were performed using the program Raven Pro 1.4 (BIOACOUSTICS RESEARCH PROGRAM, 2011). Audiospectrograms were produced with a fast Fourier transform of 256 points, overlap 50%, and hamming window type. Ten advertisement call parameters were quantified: number of notes per call; note duration (s); internote duration (s); call repetition rate (call/s); minimum, maximum, and dominant frequency (Hz); number of pulses per note; pulse duration (ms); and pulse rate (pulses/s). Oscillograms and spectrograms were used to illustrate sound features. Information on the advertisement calls of *Haddadus binotatus* and *Ischnocnema parva* were obtained from literature (CARVALHO & MARTINS, 2012; DIAS et al., 2012; HEYER et al., 1990).

According to the variation of sound parameters the advertisement calls were classified in six categories, four of them occurring in *Brachycephalus* (Figure 3): (0) advertisement call I (long, composed by the regular repetition of one low frequency note with 8-20 pulses); (1) advertisement call II (long, composed by five or six high frequency notes, each one composed by 6-13 pulses, in a set regularly repeated); (2) advertisement call III (short, composed by one to three low frequency notes with 1-3 pulses in each note); (3) advertisement call IV (short, composed by one to four high frequency notes with 1-3 pulses); (4) advertisement call V (short, composed by 20-25 low frequency notes with 2 pulses in each note), and (5) advertisement call VI (short, composed by one single low frequency note with 3-10 pulses).

Altitudinal and geographic distribution

To characterize altitudinal and geographic distribution of species we used all georeferenced records available for the genus in literature and scientific collections. The geographic coordinates were checked individually and adjusted to UTM (WGS 1984 Datum) to minimize projection errors. We overlapped geographic coordinates to the layers of altitude (JARVIS et al., 2009) and Brazilian relief units (IBGE, 2010) using ArcMap 10.0 (ESRI, 2010).

Information provided by the collectors was combined to the information extracted from the altitude layer to investigate the altitudinal range of the species. Continuous elevation values have been allocated in two categories: (0) species occurrence in lowlands and highlands (widespread altitudinal distribution from sea level to 700 m above sea level); (1) species occurrence restricted to highlands (restricted to elevations > 700 m above sea level).

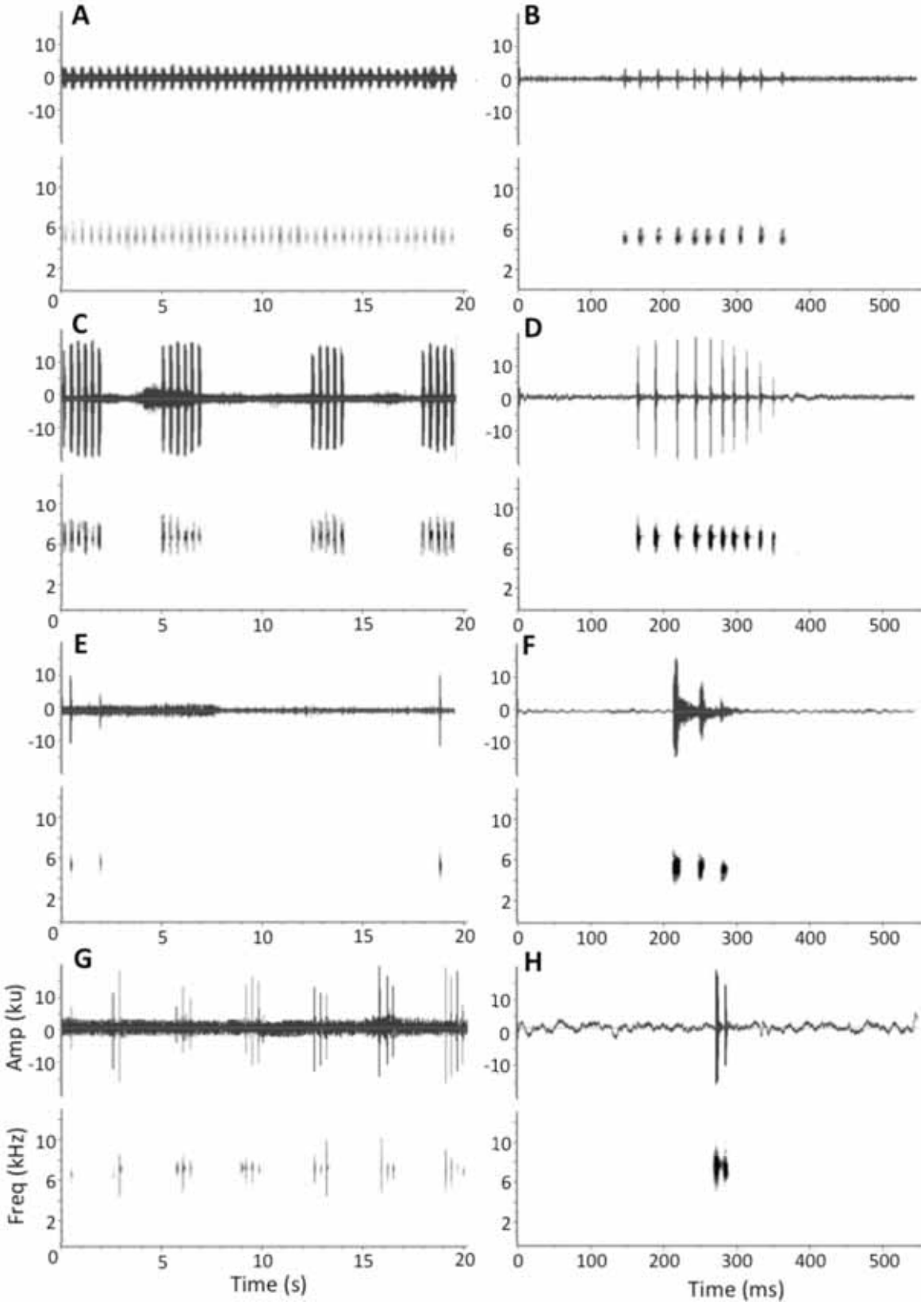


Figure 3. Spectrograms and oscillograms of the advertisement call in the genus *Brachycephalus*. (A) advertisement call and (B) detail of the note of *B. pitanga*, classified as type I; (C) advertisement call and (D) detail of the note of *B.* sp. *2*, classified as type II; (E) advertisement call and (F) detail of the note of *B. pombali*, classified as type III; and (G) advertisement call and (H) detail of the note of *B. hermogenesi*, classified as type IV.

Geographic distribution was investigated considering the relief units delimited by Brazilian Institute of Geography and Statistics (Figure 4). Due to the latitudinal extension of Serra do Mar, we additionally considered the subdivision of this relief unit into two smaller units (northern and southern) corresponding to the tropical and subtropical climates occurring along the Atlantic forest (ALMEIDA & CARNEIRO, 1998). We subdivided northern and southern units considering the narrower width of the Serra do Mar mountain range, at Ribeira de Iguape river, southern São Paulo state.

Thus, we coded geographic distribution in six categories: (0) restricted to northern Serra do Mar; (1) restricted to southern Serra do Mar; (2) restricted to Serra da Mantiqueira; (3) restricted to Paranapiacaba Plateau; (4) restricted to riverine, marine and coastal plains, including islands; (5) restricted to Rio das Contas mountains in Bahia State, and (6) widespread distributed in more than one region.

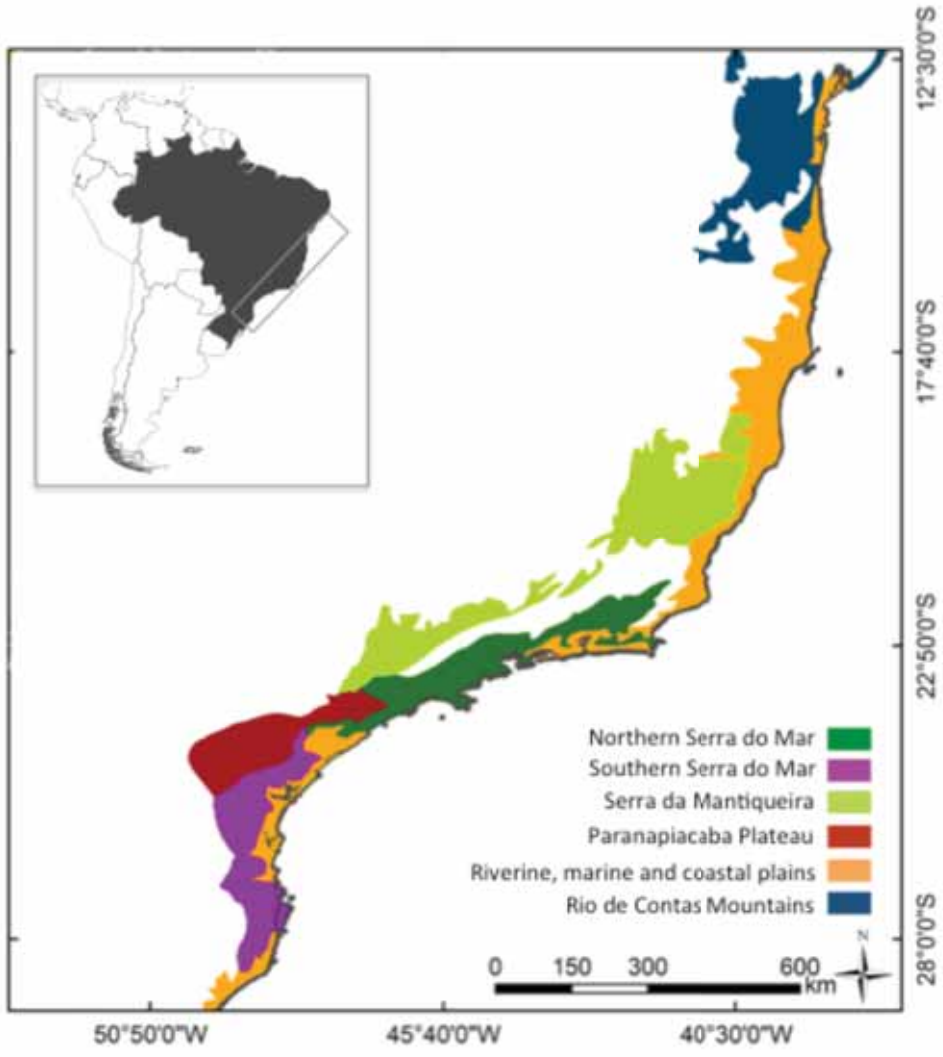


Figure 4. Map of the coastal Atlantic forest showing the relief units considered in this study. Limits are obtained from Brazilian Institute of Geography and Statistics (IBGE, 2010).

Results

Phylogenetic relationships within *Brachycephalus*

Phylogenetic reconstructions based on concatenated mtDNA sequences inferred from the different methods were almost identical. The conflicts among BI, ML, and MP were restricted to weakly supported clades. We present the node support values for the three methods in the 50% majority rule consensus tree obtained from the Bayesian inference analysis (Figure 5).

The topology confirmed that the genus *Brachycephalus* is monophyletic and *Ischnocnema* is the closest taxon. We found no evidence of distinction for the flea-toads, until recently allocated into the genus *Psyllophryne*. Species of flea-toads were widely distributed among the other species within the genus, which corroborate the synonymy proposed by Kaplan (2002). The topology also revealed nine well-supported clades within *Brachycephalus* and *B. pulex*, a species not assigned to any of these clades. The basal clade A comprises populations of *B. hermogenesi* and the relationship between this and all other sister species of *Brachycephalus* are well supported. Other two main groups comprise the clades B, C, and D and clades E, F, G, H, and I, respectively. Both groups had internal nodes well supported but the relationships between them remain unresolved.

In the first group, the clade B comprises two species of flea-toads *B.* sp. 1 and *B. didactylus*, from northern Serra do Mar. The sister clades C and D are distributed exclusively in the southern Serra do Mar of São Paulo, Paraná, and Santa Catarina states. The Clade C comprises five populations of flea-toads from *B.* sp. 2 while the clade D comprises *B. ferruginus*, *B. pernix*, *B. pombali*, *B. izecksohni*, *B. brunneus*, and the undescribed species *B.* sp. 3, *B.* sp. 4, *B.* sp. 5, and *B.* sp. 6. All species within this group share the condition of absence of hyperossification of skull and postcranial skeleton.

In the other group, the clade E comprises distinct populations of the flea-toads *B. didactylus* from Rio de Janeiro. The clade F comprises the flea-toads of *B.* sp. 7 also from Rio de Janeiro. As the other flea-toads, *B. didactylus* and *B.* sp. 7 exhibit also absence of hyperossification of skull and postcranial skeleton. The clade G comprises *B. alipioi*, *B. crispus*, *B. guarani*, *B. nodoterga*, *B. pitanga*, *B. toby*, *B. vertebralis*, and the undescribed species *B.* sp. 8 and *B.* sp. 9. Species within this clade share the intermediate condition of hyperossification of skeleton. The clade H comprises *B. ephippium*, *B. margaritatus*, *B.* sp. 10, and B. sp. 11, species with extreme hyperossification of skull and postcranial skeleton.

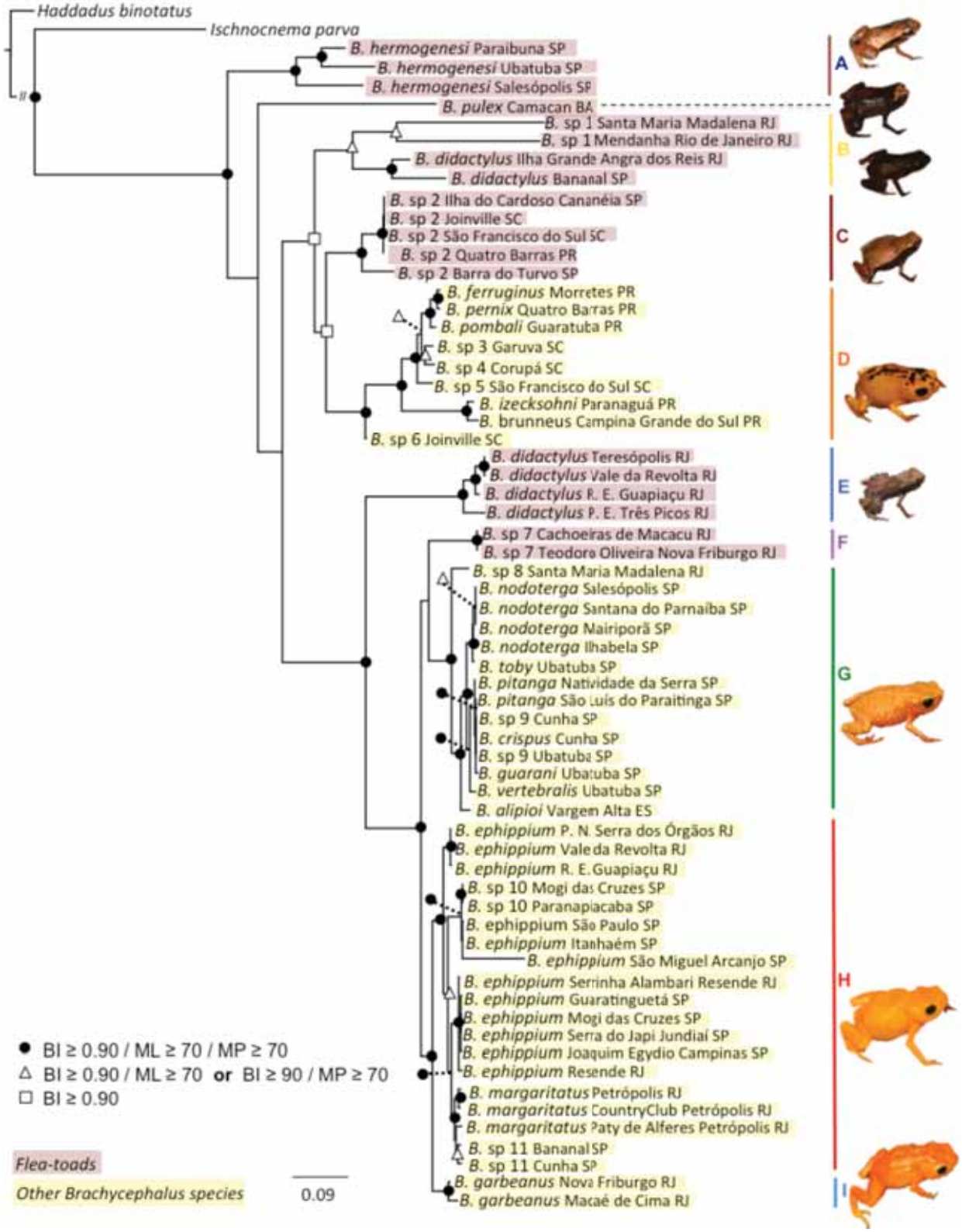


Figure 5. The 50% majority rule consensus tree from Bayesian inference analysis of concatenated mitochondrial fragments (12S, 16S and Cytb). Black dots indicate nodes supported by Bayesian posterior probabilities (BI ≥ 0.90); Maximum likelihood bootstrap support (ML ≥ 70%) and Maximum Parsimony Jacknife support (MP ≥ 70%). Triangles indicate nodes supported by Bayesian posterior probabilities (BI ≥ 0.90) and one of the other methods (ML ≥ 70% or MP ≥ 70%). Squares indicate nodes supported only by Bayesian posterior probabilities (BI ≥ 0.90). Nodes without symbols are poorly supported (BI ≤ 0.90, MP ≤ 70% and MP ≤ 70%). Terminals in in red were popularly known as flea-toads. Main clades were identified with letters in the right side of the tree. See text for details.

The clade I comprises distinct populations of *B. garbeanus,* which has the same extreme hyperossification condition of clade H. All of the species within this group are distributed in northern Atlantic forest and the condition of hyperossification of skull and postcranial skeleton are variable.

Evolution of morphological and ecological traits

Body color – Most *Brachycephalus* species (14 currently known and eight undescribed species) exhibits conspicuous coloration, which has been associated to tetrodotoxin toxicity. This condition was not exhibited in the most basal clade, which is composed by a dull colored flea-toad species. The conspicuous coloration has evolved independently at least two times (Figure 6). The clades D, G, H, and I comprise predominantly bright-colored species. According to ancestral reconstructions, the clade F and *B. brunneus* (in clade D) represent special cases of reversion to ancestral state.

Hyperossification pattern – Ancestral state reconstructions reveal a trend towards increasing skull and skeleton mineralization in *Brachycephalus* (Figure 6). The most basal clade A and all taxa contained in clades B, C, D, E, and F exhibit the condition of absence of hyperossification of skull and postcranial skeleton. The evolution of hyperossification has occurred two times, in clade G as the intermediate condition and in clades H and I as the extreme condition. In both cases some terminal taxa presented the dermal ossifications in addition to different levels of hyperossification of skeleton and skull, suggesting a subsequent modification. The absence of hyperossification of the skull and postcranial skeleton is exhibited primarily by the flea-toads and also by species from the southern Atlantic forest. Species from the northern Atlantic forest can exhibit both the ancestral and derived conditions.

Body size – The miniaturization is a remarkably feature within *Brachycephalus* species, which usually exhibit snout-vent-length smaller than 18 mm and share morphological novelties related to this evolutionary process. Although most of ancestral states reconstructions were equivocal, the results reveal a trend towards increasing body size (Figure 7). The flea-toads and the species from clade D (from southern Atlantic forest) are the smallest within the genus, exhibiting SVL usually inferior to 12 mm. The clades G, H, and I with intermediate and extreme conditions of hyperossification present the larger body size values within the genus. These species are distributed in the northern Atlantic forest, and the larger body sizes can be associated to the extreme condition of hyperossification of skull and postcranial skeleton.

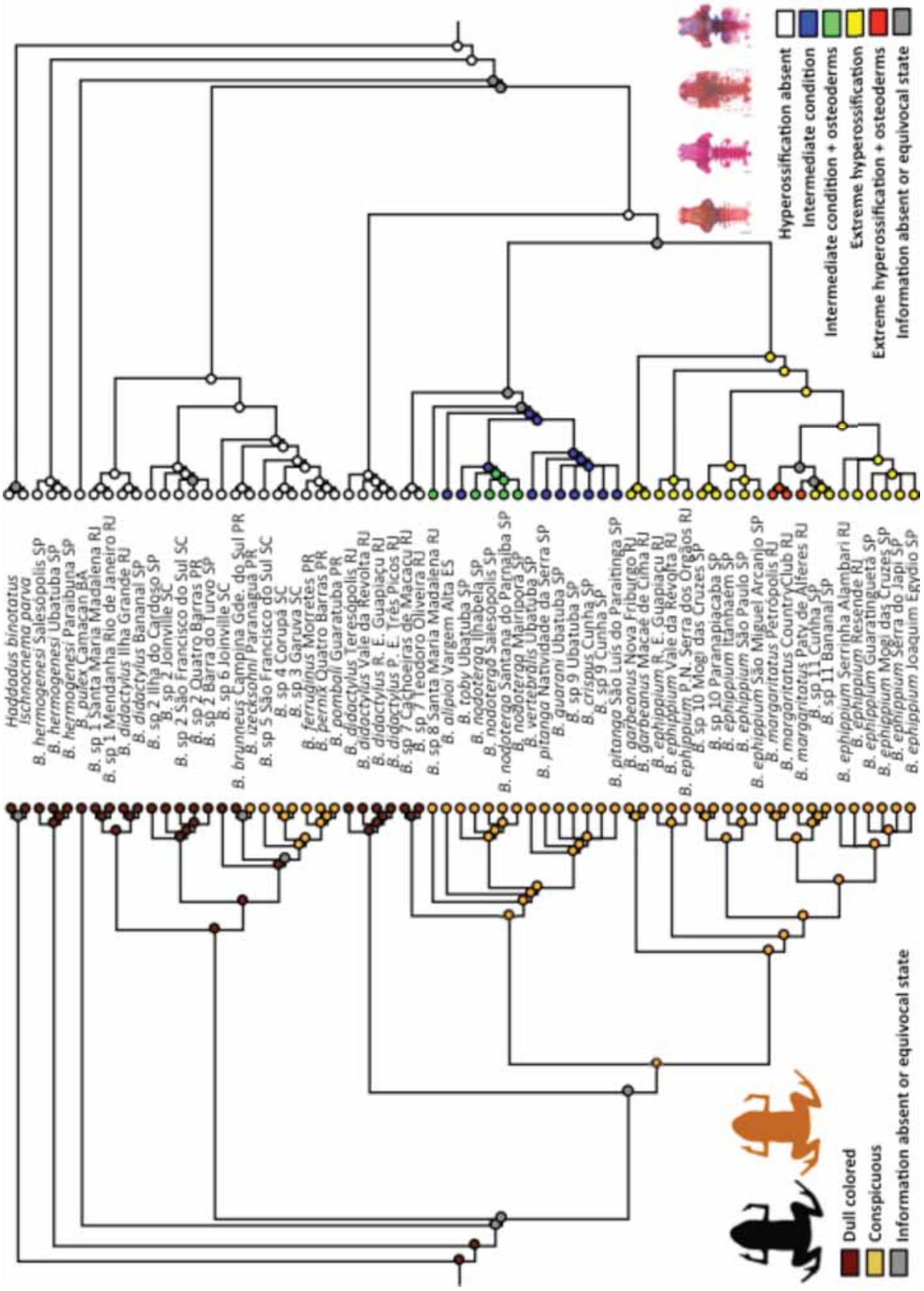
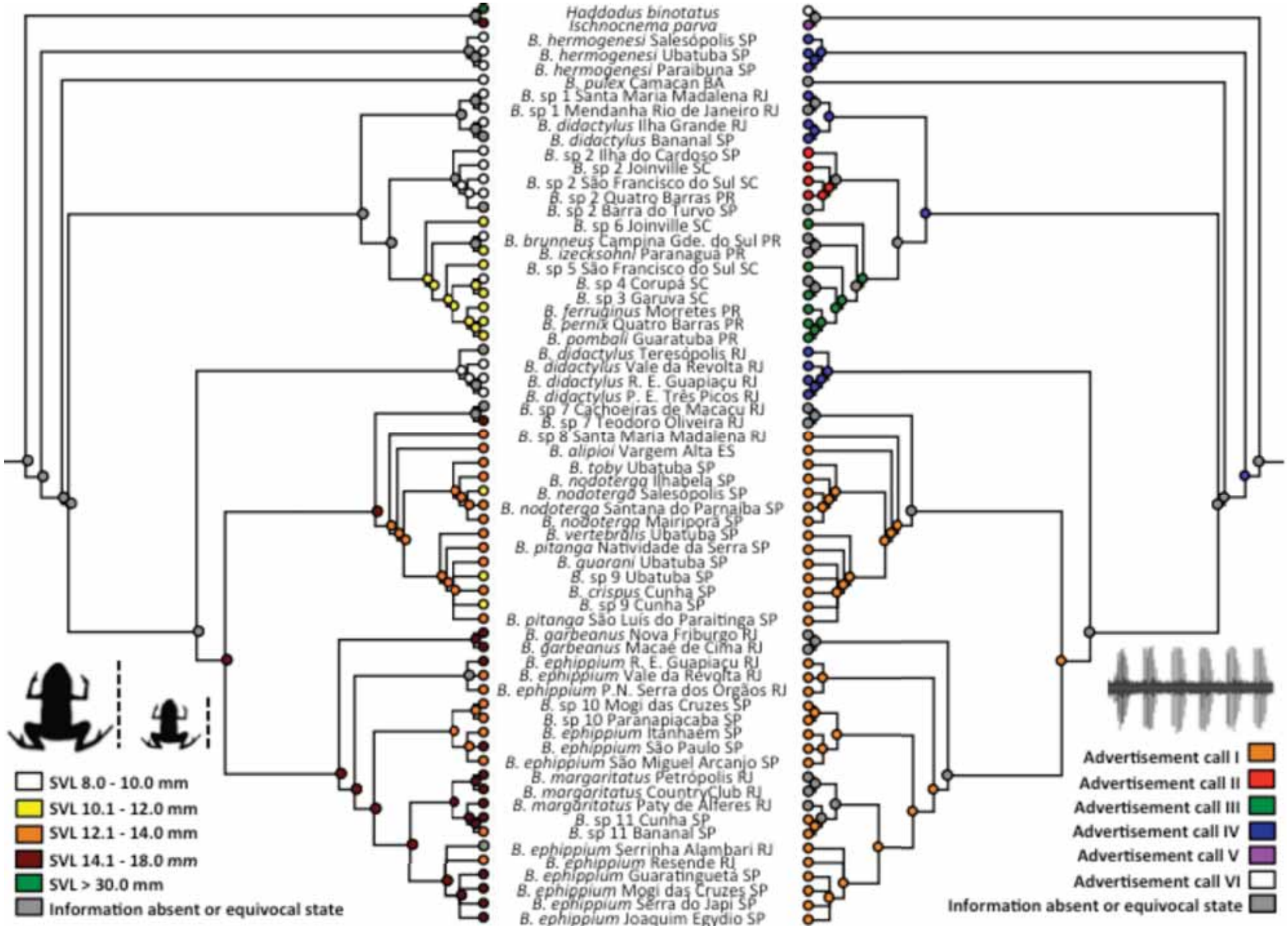

Hyperossification absent
Intermediate condition
Intermediate condition + osteoderms
Extreme hyperossification
Extreme hyperossification + osteoderms
Information absent or equivocal state
Haddadus binotatus
Ischnocnema parva
B. hermogenesi Salesópolis SP
B. hermogenesi Ubatuba SP
B. hermogenesi Paraibuna SP
B. pulex Camacan BA
B. sp 1 Santa Maria Madalena RJ
B. sp 1 Mendanha Rio de Janeiro RJ
B. didactylus Ilha Grande RJ
B. didactylus Bananal SP
B. sp 2 Ilha do Cardoso SP
B. sp 2 Joinville SC
B. sp 2 São Francisco do Sul SC
B. sp 2 Quatro Barras PR
B. sp 2 Barra do Turvo SP
B. sp 6 Joinville SC
B. brunneus Campina Gde. do Sul PR
B. izecksohni Paranaguá PR
B. sp 5 São Francisco do Sul SC
B. sp 4 Corupá SC
B. sp 3 Garuva SC
B. ferruginus Morretes PR
B. pernix Quatro Barras PR
B. pomboli Guaratuba PR
B. didactylus Teresópolis RJ
B. didactylus Vale da Revolta RJ
B. didactylus R. E. Guapiaçu RJ
B. didactylus P. E. Três Picos RJ
B. sp 7 Cachoeiras de Macacu RJ
B. sp 7 Teodoro Oliveira RJ
B. sp 8 Santa Maria Madalena RJ
B. alipioi Vargem Alta ES
B. toby Ubatuba SP
B. nodoterga Ilhabela SP
B. nodoterga Salesópolis SP
B. nodoterga Santana do Parnaíba SP
B. nodoterga Mairiporã SP
B. vertebralis Ubatuba SP
B. pitanga Natividade da Serra SP
B. guarani Ubatuba SP
B. sp 9 Ubatuba SP
B. crispus Cunha SP
B. sp 9 Cunha SP
B. pitanga São Luis do Paraitinga SP
B. garbeanus Nova Friburgo RJ
B. garbeanus Macaé de Cima RJ
B. ephippium R. E. Guapiaçu RJ
B. ephippium Vale da Revolta RJ
B. ephippium P. N. Serra dos Órgãos RJ
B. sp 10 Mogi das Cruzes SP
B. sp 10 Paranapiacaba SP
B. ephippium Itanhaém SP
B. ephippium São Paulo SP
B. ephippium São Miguel Arcanjo SP
B. margaritatus Petrópolis RJ
B. margaritatus CountryClub RJ
B. margaritatus Paty de Alferes RJ
B. sp 11 Cunha SP
B. sp 11 Bananal SP
B. ephippium Serrinha Alambari RJ
B. ephippium Resende RJ
B. ephippium Guaratinguetá SP
B. ephippium Mogi das Cruzes SP
B. ephippium Serra do Japi SP
B. ephippium Joaquim Egydio SP
Dull colored
Conspicuous
Information absent or equivocal state

Figure 6. Evolution of general body color and hyperossification pattern of the skull and skeleton in *Brachycephalus*. The states of general body color (adult dorsal view) and hyperossification pattern (degree of mineralization of the skin elements, skull, and skeleton) were mapped in the terminals and reconstructed to each ancestral node of the consensus tree using Maximum Likelihood. General body color was classified into (0) dull colored (dark brown or another cryptic coloration similar to the leaf-litter habitat) and (1) conspicuous (generally orange or yellow, clearly distinct from the leaf-litter habitat). Hyperossification pattern was classified among five categories: (0) hyperossification absent (no mineralized elements associated to the skin, skull and skeleton); (1) intermediate condition of hyperossification (skull and vertebrae mineralized); (2) intermediate condition of hyperossification + osteoderms (skull and vertebrae mineralized and variable amount of osteoderms); (3) extreme hyperossification (skull and skeleton mineralized including a dorsal bone-shield); (4) extreme hyperossification + osteoderms (skull and skeleton mineralized, dorsal bone-shield and variable amount of osteoderms). Gray circles denote either terminals with information absent or internal nodes for which reconstructions were not possible due to equivocal state in more then 50% of the trees.


Haddadus binotatus
Ischnocnema parva
B. hermogenesi Salesópolis SP
B. hermogenesi Ubatuba SP
B. hermogenesi Paraibuna SP
B. pulex Camacan BA
B. sp 1 Santa Maria Madalena RJ
B. sp 1 Mendanha Rio de Janeiro RJ
B. didactylus Ilha Grande RJ
B. didactylus Bananal SP
B. sp 2 Ilha do Cardoso SP
B. sp 2 Joinville SC
B. sp 2 São Francisco do Sul SC
B. sp 2 Quatro Barras PR
B. sp 2 Barra do Turvo SP
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B. brunneus Campina Gde. do Sul PR
B. izecksohni Paranaguá PR
B. sp 5 São Francisco do Sul SC
B. sp 4 Corupá SC
B. sp 3 Garuva SC
B. ferruginus Morretes PR
B. pernix Quatro Barras PR
B. pomboli Guaratuba PR
B. didactylus Teresópolis RJ
B. didactylus Vale da Revolta RJ
B. didactylus R. E. Guapiaçu RJ
B. didactylus P. E. Três Picos RJ
B. sp 7 Cachoeiras de Macacu RJ
B. sp 7 Teodoro Oliveira RJ
B. sp 8 Santa Maria Madalena RJ
B. alipioi Vargem Alta ES
B. toby Ubatuba SP
B. nodoterga Ilhabela SP
B. nodoterga Salesópolis SP
B. nodoterga Santana do Parnaíba SP
B. nodoterga Mairiporã SP
B. vertebralis Ubatuba SP
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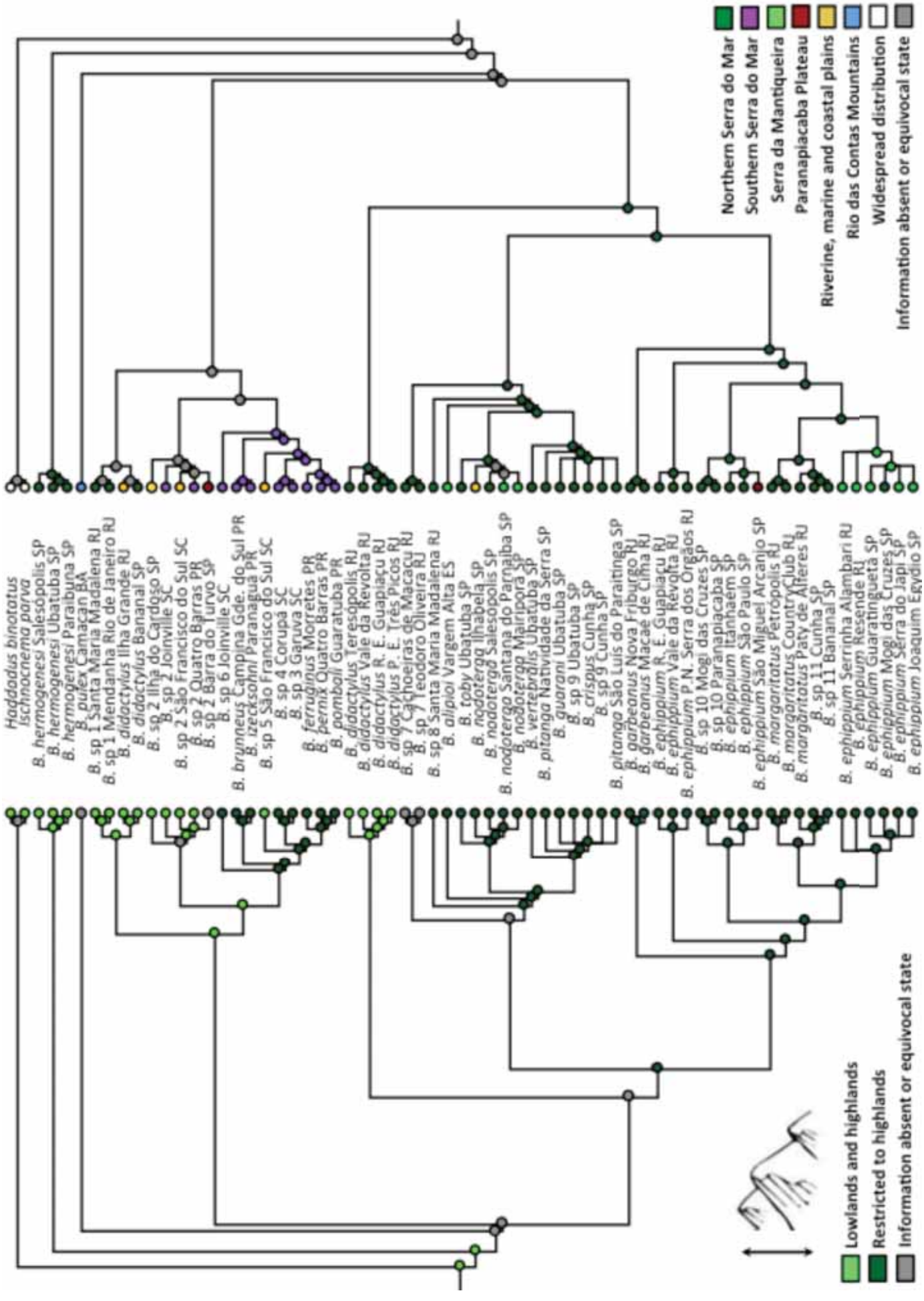
B. ephippium P.N. Serra dos Órgãos RJ
B. sp 10 Mogi das Cruzes SP
B. sp 10 Paranapiacaba SP
B. ephippium Itanhaém SP
B. ephippium São Paulo SP
B. ephippium São Miguel Arcanjo SP
B. margaritatus Petrópolis RJ
B. margaritatus CountryClub RJ
B. margaritatus Paty de Alferes RJ
B. sp 11 Cunha SP
B. sp 11 Bananal SP
B. ephippium Serrinha Alambari RJ
B. ephippium Resende RJ
B. ephippium Guaratinguetá SP
B. ephippium Mogi das Cruzes SP
B. ephippium Serra do Japi SP
B. ephippium Joaquim Egydio SP
SVL 8.0 - 10.0 mm
SVL 10.1 - 12.0 mm
SVL 12.1 - 14.0 mm
SVL 14.1 - 18.0 mm
SVL > 30.0 mm
Information absent or equivocal state
Advertisement call I
Advertisement call II
Advertisement call III
Advertisement call IV
Advertisement call V
Advertisement call VI
Information absent or equivocal state

Figure 7. Evolution of body size and advertisement call in *Brachycephalus*. The states of body size (adult average snout-vent length) and advertisement call (classified in this study) were mapped in the terminals and reconstructed to each ancestral node of the consensus tree using Maximum Likelihood. Continuous snout-vent length values have been allocated among five categories: (0) SVL 8.0–10.0 mm; (1) SVL 10.1–12.0 mm; (2) SVL 12.1–14.0 mm; (3) SVL 14.1-18.0 mm; (4) SVL > 30.0 mm. Advertisement calls were classified among six categories: (0) advertisement call I (long, composed by the regular repetition of one low frequency note with 8-20 pulses); (1) advertisement call II (long, composed by five or six high frequency notes with 6-13 pulses, in a set regularly repeated); (2) advertisement call III (short, composed by one to three low frequency notes with 1-3 pulses); (3) advertisement call IV (short, composed by one to four high frequency notes with 1-3 pulses); (4) advertisement call V (short, composed by 20-25 low frequency notes with 2 pulses, according to Heyer et al. (1990)); (5) advertisement call VI (short, composed by one single low frequency note with 3-10 pulses, according to Carvalho & Martins (2012) and Dias et al. (2012)). Gray circles denote either terminals with information absent or internal nodes for which reconstructions were not possible due to equivocal state in more then 50% of the trees.


Northern Serra do Mar
Southern Serra do Mar
Serra da Mantiqueira
Paranapiacaba Plateau
Riverine, marine and coastal plains
Rio das Contas Mountains
Widespread distribution
Information absent or equivocal state
Haddadus binotatus
Ischnocnema parva
B. hermogenesi Salesópolis SP
B. hermogenesi Ubatuba SP
B. hermogenesi Paraibuna SP
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B. sp 4 Corupá SC
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B. ferruginus Morretes PR
B. pernix Quatro Barras PR
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B. didactylus R. E. Guapiaçu RJ
B. didactylus P. E. Três Picos RJ
B. sp 7 Cachoeiras de Macacu RJ
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B. ephippium Resende RJ
B. ephippium Guaratinguetá SP
B. ephippium Mogi das Cruzes SP
B. ephippium Serra do Japi SP
B. ephippium Joaquim Egydio SP
Lowlands and highlands
Restricted to highlands
Information absent or equivocal state

Figure 8. Evolution of altitudinal range and geographic distribution in *Brachycephalus*. The states of altitudinal range (elevation in meters) and geographic distribution in the relief units proposed by IBGE (2010) were mapped in the terminals and reconstructed to each ancestral node of the consensus tree using Maximum Likelihood. Continuous elevation values have been allocated in two categories: (0) species occurrence in lowlands and highlands (widespread altitudinal distribution); (1) species occurrence restricted to highlands (restricted to elevations > 700 meters). Geographic distribution was classified in seven categories: (0) restricted to northern Serra do Mar; (1) restricted to southern Serra do Mar; (2) restricted to Serra da Mantiqueira; (3) restricted to Paranapiacaba Plateau; (4) restricted to riverine, marine and coastal plains, including islands; (5) restricted to Rio das Contas mountains in Bahia State; (6) widespread distributed in more than one region. Gray circles denote either terminals with information absent or internal nodes for which reconstructions were not possible due to equivocal state in more then 50% of the trees.

Advertisement call –The flea-toads of the most basal clade exhibit the advertisement call IV, composed by one to four high frequency notes with 1-3 pulses (Figures 6 and 2). The ancestral reconstructions suggest the evolution of advertisement call II (long, composed by five or six high frequency notes with 6-13 pulses, in a set regularly repeated) in clade C and the evolution of advertisement call III (short, composed by one to three low frequency notes with 1-3 pulses) in clade D. The clades G, H, and I exhibit the advertisement call I, the longest advertisement call, composed by the regular repetition of one low frequency note with 8-20 pulses. So, the flea-toads of northern distribution present the advertisement call IV while those of the southern distribution present the advertisement call II. And for the other *Brachycephalus* species we observed the advertisement call I for the northern distribution and the advertisement call III for the southern distribution.

Altitudinal range – The most basal clade, as the majority of other flea-toads species (clades B, C, E, and F), presents a wide altitudinal range of occurrence, from the sea level up to 1000 m of altitude (Figure 8). As an exception, *B. pulex* is only known from its type locality at 840 m of altitude, and further studies are necessary to infer the altitudinal range for this species. The other *Brachycephalus* species present restricted geographic distribution in mountain slopes, occurring in elevations superior to 700 m above sea level. Exceptionally, *B.* sp. 5 is known only in lowlands. The ancestral reconstructions suggest that the evolution of groups restricted to highlands has occurred at least two times. This restricted altitudinal range has evolved in clade D, with a case of reversion to ancestral state occurring in *B.* sp. 5; and also in the group of clades F, G, H, and I.

Geographic distribution – Most *Brachycephalus* species occurs in Serra do Mar mountain range at Rio de Janeiro, São Paulo, Paraná, and Santa Catarina states (Figure 8). Four species were distributed in riverine, marine, and coastal plains: *B. didactylus* (Ilha Grande, RJ), *B. nodoterga* (Ilhabela, SP), *B.* sp. 2 (Ilha do Cardoso, SP), and *B.* sp. 5 (Ilha de São Francisco do Sul, SC). These are the only species within the genus associated to continental islands. Among them, only populations from Ilha Grande, RJ and Ilha de São Francisco do Sul, SC occur in lowlands. Populations from Ilhabela, SP e Ilha do Cardoso, SP are associated to mountains with more than 500 m of altitude. The Serra da Mantiqueira region harbors three species: *B. alipioi*, *B. nodoterga* and *B. ephippium*. The Paranapiacaba Plateau harbors two species, *B. ephippium* and *B.* sp. 2. Only *B. pulex* is found in Rio das Contas Mountains, Bahia state, constituting the further north occurrence for the genus. The most basal clade is known to occur in the northern portion of Serra do Mar. Ancestral reconstructions suggests a common ancestor living in northern Serra do Mar for the extant

taxa from clades E, F, G, H, and I. The geographic distribution of the ancestral of clades B, C, and D was not predicted.

Discussion

Phylogenetic relationships within *Brachycephalus*

The Bayesian topology based on three concatenated mtDNA genes did not recover the distinction of the flea-toads as a monophyletic group within *Brachycephalus*. *Brachycephalus* and *Psyllophryne* (which contained the species popularly known as flea-toads) were considered distinct genera based on the presence of omosternum in the pectoral girdle of *Psyllophryne* and absence in *Brachycephalus*. Currently, *Psyllophryne* is considered a junior synonym of *Brachycephalus* based on the presence of a reduced *omosternum* in *B. ephippium* (KAPLAN, 2002). Despite of the morphological and ecological features that clearly distinguish the flea-toads from the other *Brachycephalus* species, the molecular data corroborate the decision of synonymy.

Another remarkable result was the definition of *Brachycephalus hermogenesi* as a sister taxon of all other species within the genus. This basal position was not defined in the previous published phylogenetic hypothesis (CLEMENTE-CARVALHO et al., 2011). Despite of this, some clades reconstructed in this topology (D, E, G, H, and I) are completely concordant with the results presented by Clemente-Carvalho et al. (2011). The *Brachycephalus* species, other than the flea-toads, are clearly divided into two well-supported clades (D and a major clade composed by G, H, and I), corresponding to subtropical and tropical geographic regions of the Atlantic forest, respectively. In addiction to reflect the geographic distribution, the distinction of these two groups of species is also correlated with the hyperossification pattern (CLEMENTE-CARVALHO et al., 2011).

The inclusion of taxa and sequences from more than one population per species evidenced the increasing resolution in understanding the *Brachycephalus* species relationships, compared to the results of Clemente-Carvalho et al. (2011). The shallower nodes of the topology are completely resolved, with the exception of a small group of species from northern Serra do Mar, at São Paulo state, occurring in the clade G. The relationships among *B. crispus*, *B. guarani*, *B. pitanga*, and *B.* sp. 9 were not resolved. Due to the very recent history of these closely related species, another molecular markers may be useful to access their relationships.

The node of the relationship between *Brachycephalus pulex* and the other species of the genus, as well as the node of the relationship between the two major groups within the

genus (clades B, C, and D) and (clades E, F, G, H, and I) has shown lower support values for the three applied methods (node support values 55/30/79 for BI, ML, and MP, respectively). Considering the recent discovery of new species and populations of *Brachycephalus* (e.g. CLEMENTE-CARVALHO et al., 2012; GAREY et al., 2012; CONDEZ et al., 2014) is quite conceivable that additional sampling could help to improve the understanding of the phylogenetic relationships within this genus. Our sampling did not include, for example, sequence data from *B. atelopoide, B. bufonoides*, and *B. tridactylus*, known only from type material fixed in formalin. The two former species were recently removed from the synonymy of *B. ephippium* by the detailed exam of the descriptions provided by Miranda-Ribeiro (1920) and the old and scarce material available in the scientific collections (POMBAL, 2010). Field surveys are necessary to access the occurrence of these species and precisely evaluate their identity and the relationships with other *Brachycephalus* species.

Evolution of morphological and ecological traits

The conspicuous orange or yellow coloration exhibited from *Brachycephalus* species has been associated to tetrodotoxin present in the skin and some organs of *B. ephippium* (SEBBEN et al., 1986; PIRES et al., 2002). This potent neurotoxin is widely distributed in marine taxa while in terrestrial vertebrates its occurrence is limited to amphibians (HANIFIN, 2010). Tetrodotoxin or analogs have been identified in a total of four anuran families: Brachycephalidae, Bufonidae, Dendrobatidae, and Rhacophoridae (HANIFIN, 2010). The physiological effects of tetrodotoxin include muscle paralysis or death by direct action in the sodium channels of cell membranes, indicating that its presence primarily serves as an antipredator defense (DUELLMAN & TRUEB, 1994; HANIFIN, 2010).

Information on occurrence of tetrodotoxin in *Brachycephalus* is restricted to studies with *B. ephippium*, *B. nodoterga*, and *B. pernix* (PIRES et al., 2005). Even though, more than 66% of the species of *Brachycephalus* exhibits conspicuous body color. The conspicuousness should indicate an aposematic species, when the visual warning color is truly related to toxicity, or a mimetic species, when advertisement color is not assigned to toxicity (DUELLMAN & TRUEB, 1994). Our results suggested that the conspicuous coloration has evolved independently at least two times in the phylogeny and for each clade at least one species can be considered aposematic by presenting tetrodotoxin (e.g. *B. pernix* in clade D and *B. ephippium* in clade H). The multiple evolution of chromatic specializations and toxicity is a recurrent pattern in anurans. In the same way as reported here for *Brachycephalus*, the aposematic taxa do not constitute a monophyletic group in the

Neotropical poison frogs of the family Dendrobatidae (VENCES et al., 2003). For the Dendrobatids, multiple and parallel evolution of body color are supposed to explain some brightly colored species within cryptic clades (VENCES et al., 2003). Considering this, due to the *Brachycephalus* disjunctive geographic distribution in mountain slopes, the absence of simpatry among conspicuous species and the low frequency of mimicry among anurans (DUELLMAN & TRUEB, 1994), it is quite possible that all conspicuous colored species within *Brachycephalus* exhibit toxins. Further investigations could provide useful information to the study of the defensive behavior of these species.

Available information on natural history of *Brachycephalus* suggests that the behavior of living hidden under the leaf-litter or exposed is not exclusive from dull colored or conspicuous species, respectively (VERDADE et al., 2008). Even when exposed the flea-toads are supposed to be cryptically hidden, mainly due to its minute size and dull coloration. Depending on the optimal climatic conditions, both the flea-toads and other *Brachycephalus* species can be found exposed (POMBAL et al., 1994; VERDADE et al., 2008). Except for *B. garbeanus*, most of the conspicuous species of *Brachycephalus* are diurnal (DORIGO et al., 2012). In some species, males perform visual signaling to hold territories in the reproductive season, which clearly indicates the importance of visual communication in *Brachycephalus* (e.g. POMBAL et al., 1994; CONDEZ et al., 2014). Besides of providing visual warning related to toxicity, conspicuous body color in *Brachycephalus* species could also be related to intra-specific communication (HÖDL & AMÉZQUITA, 2001).

The extremely small adult body size, the reduced number of functional fingers and toes and the complete absence of some bones, suggest that *Brachycephalus* have evolved through miniaturization (ALBERCH & GALE, 1985; HANKEN, 1993; HANKEN & WAKE, 1993; YEH, 2002). The miniaturized phenotype can represent a complex combination of ancestral and derived traits, including structural simplification by morphological reduction or loss of bones, increased morphological variability, and novel structural configurations (HANKEN & WAKE, 1993). Thus, the remarkable pattern of skull and postcranial skeleton hyperossification within *Brachycephalus* may be evolved as a byproduct of miniaturization (CLEMENTE-CARVALHO et al., 2009).

Brachycephalus species present reduction in body size relatively to its most recent common ancestors, genera *Ischnocnema* and *Haddadus*. The most basal *Brachycephalus* species is among the flea-toads, group of species that comprises the smallest body size within the genus (CONDEZ et al., unpublished data). These species also share the absence of mineralization in the skull and skeleton (CLEMENTE-CARVALHO et al., 2009). Despite of

this, at shallower divergence levels *Brachycephalus* species exhibit a slight increase in body size and mineralization of bones. The increase in body size in these species seems to be related to a physical constraint associated to the support for a heavier skeleton.

Hyperossification of skull and postcranial skeleton appears to have evolved independently two times in *Brachycephalus*, both in the northern Atlantic forest. Except for the flea-toads, northern species of *Brachycephalus* are clearly distinguished in two major clades. One, composed by species with intermediate condition of hyperossification (*B. alipioi*, *B. crispus*, *B. guarani*, *B. nodoterga*, *B. pitanga*, *B. toby*, *B. vertebralis*, and *B.* sp. 9), and other clade composed by species with extreme condition of hyperossification (*B. ephippium*, *B. garbeanus*, *B. margaritatus*, *B.* sp. 10, and *B.* sp. 11). In both clades the hyperossification can be accompanied by osteoderms (e.g. *B. nodoterga* and *B. crispus* with intermediate hyperossification and B. *margaritatus* with extreme hyperossification).

Clemente-Carvalho et al. (2009) suggested that hyperossification might be a response to differences in specific microhabitat conditions, being more related to water economy than defense against predators. The functional significance of these structures is not clear for *Brachycephalus*, but might be also related to the decreasing rates of water evaporation by the general isolation of the species integument from the external environment. This function has been observed in the cranial-ossification of the casque-headed tree frogs, *Aparasphenodon brunoi* and *Corythomantis greeningi* (SEIBERT et al., 1974; ANDRADE & ABE, 1997; JARED et al., 1999; NAVAS et al., 2002). Despite of living in moist habitats of Atlantic forest, *Brachycephalus* species are predominantly diurnal and are more susceptible to evaporative water loss than nocturnal species.

Distinct advertisement calls found in *Brachycephalus* species are derived from pulsed notes, varying in structural and temporal parameters. A detailed description of these parameters and a comparative analysis considering distinct species are necessary to accurately evaluate the differences. For instance, the call parameters were used simply to group species into the four main types of advertisement calls (Figure 3; Appendix 4). One expected result is that the flea-toads presented higher dominant frequency ($\bar{x}$ = 7.0 Hz, range = 6.4-8.3 Hz) compared to other *Brachycephalus* species ($\bar{x}$ = 5.3 Hz, range = 3.6-6.9 Hz) possibly due to their small body size (DUELLMAN & PYLES, 1983; GERHARDT & HUBER, 2002). Additionally, the higher dominant frequency in the flea-toads could be explained in cases of sympatry with another *Brachycephalus* species. In these cases, if the frequencies of acoustic signals are similar in both species, they are supposed to overlap and result in interspecific interference (GERHARDT & HUBER, 2002).

We add to the current knowledge of *Brachycephalus* by analyzing advertisement calls of 14 species. Until recently the acoustic information was restricted to six species, *B. ephippium* (POMBAL et al., 1994), *B. pernix* (WISTUBA, 1998), *B. hermogenesi* (VERDADE et al., 2008), *B. pitanga* (ARAÚJO et al., 2012; OLIVEIRA, 2013), and *B. tridactylus* (GAREY et al., 2012), which represent only 23% of known diversity of the genus. Due to the lack of information, all flea-toads are supposed to call like *B. hermogenesi* (advertisement call IV), first described by Verdade et al. (2008). We found among the flea-toads of southern distribution a distinct advertisement call (advertisement call II), initially supposed to be a derivative of the advertisement call I exhibited by *B. ephippium* (POMBAL et al., 1994). Except for the flea-toads, the northern distributed species of *Brachycephalus* are supposed to exhibit the advertisement call I (POMBAL et al., 1994; ARAÚJO et al., 2012; OLIVEIRA, 2013), while those species from southern Atlantic forest are supposed to exhibit the advertisement call III (WISTUBA, 1998; GAREY et al., 2012).

Although the structural sound features were not coded and reconstructed in the phylogeny (e.g. COCROFT & RYAN, 1995; ROBILLARD et al., 2006), the ancestral reconstructions of the four major types of advertisement calls provided very interesting results. The most basal advertisement call (advertisement call IV) is composed by one to four high frequency notes with 1-3 pulses. Two different advertisement calls are supposed to be derived from this condition in the southern species. The evolution should have occurred independently, once to the advertisement call II (long, composed by five or six high frequency notes with 6-13 pulses, in a set regularly repeated) and another time to advertisement call III (short, composed by one to three low frequency notes with 1-3 pulses). In the northern Atlantic forest the ancestral condition might be evolved just one time for the advertisement call I (long, composed by the regular repetition of one low frequency note with 8-20 pulses), exhibited for the majority of species within this distribution.

The ancestral altitudinal range reconstruction suggests the evolution of the restricted highland distribution has occurred at least two times in *Brachycephalus*. This condition might have evolved once in southern and another in the northern distributed species. The flea-toads are supposed to be widespread distributed, presenting a wide altitudinal range of occurrence, from the sea level up to 1000 m of altitude (IZECKSOHN, 1971; GIARETTA & SAWAYA, 1998; PIMENTA et al., 2007; VERDADE et al., 2008; NAPOLI et al., 2011). The remaining species are generally associated with mountain slopes along the main Atlantic rain forest mountain ranges, occurring in elevations above 700 m (POMBAL et al., 1998; POMBAL, 2010; RIBEIRO et al., 2005; ALVES et al., 2006, ALVES et al., 2009). An exception to this

pattern is *B.* sp. 5 that is found at 206 m above sea level but belongs to the highland species group. This finding reinforces the idea of insufficient knowledge about this group of species and encourages further studies in the Atlantic forest, even though this is one of the best-studied biomes in Brazil.

Most *Brachycephalus* species are distributed along the ombrophilous phytophysionomy of Atlantic forest (*sensu* OLIVEIRA-FILHO & FONTES, 2000). The phylogenetic relationships within *Brachycephalus* lead us to interpret the northern Serra do Mar as the ancestral geographic distribution. Since 47 populations are known to occur in Serra do Mar mountain range, it is surely the most diverse region for *Brachycephalus*.

Conclusion

A combination of approaches and evidences in addition to mitochondrial DNA sequences will probably help to improve the taxonomy of *Brachycephalus*. The increasing information about diversity, natural history, and distribution are surely adding valuable information for a better comprehension of the evolution in the genus *Brachycephalus*. We confirmed the *Brachycephalus* monophyly and recognized nine well-supported clades, with emphasis on the basal position of *B. hermogenesi.* Thus, the extant species of *Brachycephalus* are supposed to be evolved from a dull colored ancestor, without hyperossification in skull and postcranial skeleton, with wide altitudinal range, and short advertisement call composed by one to four high frequency notes with 1-3 pulses. Distinct events are supposed to drive the genus evolution in southern and northern portions of Atlantic forest. For the flea-toads, advertisement call distinguishes species from northern and southern distributions. For other species, body size, hyperossification pattern, and advertisement call constitute divergences between north and south species. Further studies should address the evolutionary history of the genus, considering divergence times and historical explanations for these molecular, morphological, and ecological divergences.

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Appendix 1. Specimens examined in molecular analysis. Abbreviations of Brazilian states are: BA = Bahia, ES = Espírito Santo, PR = Paraná, RJ = Rio de Janeiro, SC = Santa Catarina, and SP = São Paulo.

B. alipioi, Vargem Alta, ES (CFBH 11759); *B. brunneus*, Pico Caratuva, PR (CFBH 7905, GenBank 12S 332672734, 16S 332672748 and Cytb 333102159); *B. didactylus*, Bananal, RJ (CTMZ 01522); *B. didactylus*, Parque Estadual dos Três Picos, RJ (MNRJ 54402); *B. didactylus*, Reserva Ecológica de Guapiaçu, RJ (MNRJ 68818); *B. didactylus*, Santa Maria Madalena, RJ (CFBH 13722); *B. didactylus*, Teresópolis, RJ (MNRJ 37327); *B. didactylus*, Vale da Revolta, RJ (CFBH 11506); *B. didactylus*, Ilha Grande, RJ (CFBH 12907, GenBank 12S 332672735, 16S 332672749, Cytb 333102161); *B. ephippium*, Guaratinguetá, SP (CFBH 31982); *B. ephippium*, Itanhaém, SP (CFBHT 7926); *B. ephippium*, Joaquim Egydío, Campinas, SP (CFBHT 53); *B. ephippium*, Mogi das Cruzes, SP (TC 07); *B. ephippium*, Parque Estadual Carlos Botelho, São Miguel Arcanjo, SP (CTMZ 02585); *B. ephippium*, Parque Nacional da Serra dos Órgaos, RJ (CFBH 12355); *B. ephippium*, Represa Billings, SP (CFBH 2978); *B. ephippium*, Resende, RJ (MNRJ 73097); *B. ephippium*, Reserva Ecológica do Guapiaçu, RJ (MNRJ 56517); *B. ephippium*, Serra do Alambari, Resende, RJ (MNRJ 78465); *B. ephippium*, Serra do Japi, SP (TC 103); *B. ephippium*, Vale da Revolta, RJ (MNRJ 50260); *B. ferruginus*, Pico Marumbi, PR (CFBH 8025, GenBank 12S 332672738, 16S 332672752, Cytb 333102167); *B. garbeanus*, Nova Friburgo, RJ (MNRJ57293); *B. garbeanus*, Alto de Macaé, RJ (CFBH 12688); *B. guarani*, Prumirim, Ubatuba, SP (CFBH 12997); *B. hermogenesi*, Boracéia, SP (TC 129); *B. hermogenesi*, Paraibuna, SP (ARI623); *B. hermogenesi*, Picinguaba, Ubatuba, SP (TC 47); *B. izecksohni*, Serra da Prata, PR (CFBH 7374, GenBank 12S 332672740, 16S 332672753, Cytb 333102171); *B. margaritatus*, Castelo Country Club, Petrópolis, RJ (MNRJ 73701); *B. margaritatus*, Paty de Alferes, RJ (MNRJ 76100); *B. margaritatus*, Petrópolis, RJ (CFBH 2420); *B. nodoterga*, Boracéia, SP (TC 106); *B. nodoterga*, Serra da Cantareira, SP (TC 140); *B. nodoterga*, Ilhabela, SP (TC 108); *B. nodoterga*, Santana de Parnaíba, SP (CFBH 2226); *B. pernix*, Anhangava, Quatro Barrras, PR (CFBH 2597, GenBank 12S 332672742, 16S 332672755, Cytb 333102175); *B. pitanga*, São Luis do Paraitinga, SP (CFBH 14868); *B. pitanga*, Natividade da Serra, SP (TC 115); *B. pombali*, Pico da Igreja, PR (CFBH 8043, GenBank 12S 332672744, 16S 332672757, Cytb 333102179); *B. pulex*, Camacan, BA (TC 184); *B. toby*, Corcovado, Ubatuba, SP (TC 113); *B. vertebralis*, Picinguaba, Ubatuba, SP (CFBH 1389); *B.* sp. 1, Mendanha, RJ (MNRJ 58178); *B*. sp. 2, São Francisco do Sul, SC (TC 251); *B.* sp. 2, Joinville, SC (TC 245); *B*. sp. 2, Parque Estadual do Jacupiranga, Barra do Turvo, SP (CTMZ 02970); *B.* sp. 2, Quatro Barras, PR (TC 141); *B.* sp. 2, Ilha do Cardoso, SP (TC 267); *B.* sp. 3, Garuva, SC (TC 202); *B.* sp. 4, Corupá, SC (TC 246); *B.* sp. 5, São Francisco do Sul, SC (TC 232); *B.* sp. 6, Joinville, SC (TC 236); *B.* sp. 7, Divisa Cachoeiras de Macacu e Nova Friburgo, RJ (MNRJ 56504); *B.* sp. 7, Teodoro Oliveira, RJ (56516); *B.* sp. 8, Santa Maria Madalena, RJ (CFBH 13724); B. sp. 9, Cunha, SP (TC 146); B. sp. 9, Ubatuba, SP (TC 156); *B.* sp. 10, Parque das Neblinas,

SP (CFBH 14576); *B.* sp. 10, Paranapiacaba, SP (CFBH 14586); *B.* sp. 11, Bananal, SP (TC 125); *B.* sp. 11, Cunha, SP (TC 158). Outgroups: *Haddadus binotatus* (CAB33) and *Ischnocnema parva* (MNRJ 51356, GenBank 12S 399568180).

Appendix 2. Characters assigned to each taxa used in ancestral reconstruction analysis. Code for body color: (0) dull colored or (1) aposematic. Code for hyperossification pattern: (0) hyperossification absent, (1) intermediate condition of hyperossification, (2) intermediate condition of hyperossification + osteoderms, (3) extreme hyperossification and (4) extreme hyperossification + osteoderms. Code for body size: (0) SVL 8.0–10.0 mm, (1) SVL 10.1–12.0 mm, (2) SVL 12.1–14.0 mm, (3) SVL 14.1-18.0 mm and (4) SVL > 30.0 mm. Code for advertisement call: (0) advertisement call I, (1) advertisement call II, (2) advertisement call III, (3) advertisement call IV, (4) advertisement call V and (5) advertisement call VI. Code for altitudinal range: (0) species occurrence in lowlands and highlands or (1) species occurrence restricted to highlands. Code to geographic distribution: (0) restricted to northern Serra do Mar, (1) restricted to southern Serra do Mar, (2) restricted to Serra da Mantiqueira, (3) restricted to Paranapiacaba Plateau, (4) restricted to riverine, marine and coastal plains, including islands, (4) restricted to Rio das Contas mountains and (5) widespread distributed in more than one region. Abbreviations of Brazilian states are: BA = Bahia, ES = Espírito Santo, PR = Paraná, RJ = Rio de Janeiro, SC = Santa Catarina, and SP = São Paulo.

Taxon	Locality	Body color	Hyperossification pattern	Body size	Advertisement call	Altitudinal range	Geographic distribution
B. alipioi	Vargem Alta, ES	1	1	2	0	1	2
B. brunneus	Campina Grande do Sul, PR	0	0	0	?	1	1
B. crispus	Cunha, SP	1	1	2	0	1	0
B. didactylus	Teresópolis, RJ	0	0	?	3	0	0
B. didactylus	P.E. Três Picos, RJ	0	0	0	3	0	0
B. didactylus	Vale da Revolta, RJ	0	0	0	3	0	0
B. didactylus	R.E. Guapiaçu, RJ	0	0	0	3	0	0
B. didactylus	Ilha Grande, RJ	0	0	0	3	0	4
B. didactylus	Bananal, SP	0	0	?	3	0	0
B. ephippium	P.N. Serra dos Órgãos, RJ	1	3	3	0	1	0
B. ephippium	Serrinha Alambari, Resende, RJ	1	3	?	0	1	2
B. ephippium	São Paulo, SP	1	3	2	0	1	0
B. ephippium	Guaratinguetá, SP	1	3	3	0	1	2
B. ephippium	Itanhaém, SP	1	3	3	0	1	0
B. ephippium	Vale da Revolta, RJ	1	3	2	0	1	0
B. ephippium	Resende, RJ	1	3	2	0	1	2
B. ephippium	Mogi das Cruzes, SP	1	3	3	0	1	0
B. ephippium	Serra do Japi, Jundiaí, SP	1	3	3	0	1	2
B. ephippium	São Miguel Arcanjo, SP	1	3	2	0	1	3
B. ephippium	R.E. Guapiaçu, RJ	1	3	2	0	1	0
B. ephippium	Joaquim Egydio, Campinas, SP	1	3	3	0	1	2
B. ferruginus	Morretes, PR	1	0	1	2	1	1
B. garbeanus	Nova Friburgo, RJ	1	3	3	?	1	0
B. garbeanus	Macaé de Cima, RJ	1	3	3	?	1	0
B. guarani	Ubatuba, SP	1	1	2	0	1	0

Appendix 2. Cont.

Taxon	Locality	Body color	Hyperossification pattern	Body size	Advertisement call	Altitudinal range	Geographic distribution
B. hermogenesi	Paraibuna, SP	0	0	0	3	0	0
B. hermogenesi	Ubatuba, SP	0	0	0	3	0	0
B. hermogenesi	Salesópolis, Boracéia, SP	0	0	0	3	0	0
B. izecksohni	Paranaguá, PR	1	0	1	?	1	1
B. margaritatus	Petrópolis, RJ	1	4	3	?	1	0
B. margaritatus	Paty de Alferes, Petrópolis, RJ	1	4	3	?	1	0
B. margaritatus	Castelo Country Club, Petrópolis, RJ	1	4	3	?	1	0
B. nodoterga	Salesópolis, Boracéia, SP	1	2	1	0	1	0
B. nodoterga	Santana do Parnaíba, SP	1	2	2	0	1	2
B. nodoterga	Mairiporã, SP	1	2	2	0	1	2
B. nodoterga	Ilhabela, SP	1	2	2	0	1	4
B. pernix	Quatro Barras, PR	1	0	1	2	1	1
B. pitanga	Natividade da Serra, SP	1	1	2	0	1	0
B. pitanga	São Luís do Paraitinga, SP	1	1	2	0	1	0
B. pombali	Guaratuba, PR	1	0	1	2	1	1
B. pulex	Camacan, BA	0	0	0	?	?	5
B. toby	Ubatuba, SP	1	1	2	0	1	0
B. vertebralis	Ubatuba, SP	1	1	2	0	1	0
B. sp 1	Santa Maria Madalena, RJ	0	0	0	3	0	0
B. sp 1	Mendanha, Rio de Janeiro, RJ	0	0	0	?	0	0
B. sp 2	Quatro Barras, PR	0	0	0	1	0	1
B. sp 2	Joinville, SC	0	0	0	1	0	1
B. sp 2	São Francisco do Sul, SC	0	0	0	1	0	4
B. sp 2	Barra do Turvo, SP	0	0	?	?	?	3
B. sp 2	Ilha do Cardoso, SP	0	0	0	1	0	4
B. sp 3	Garuva, SC	1	0	0	2	1	1
B. sp 4	Corupá, SC	1	0	1	?	1	1
B. sp 5	São Francisco do Sul, SC	1	0	1	2	0	4
B. sp 6	Joinville, SC	0	0	1	2	1	1
B. sp 7	Cachoeiras de Macacu, RJ	0	0	?	?	?	0

Appendix 2. Cont.

Taxon	Locality	Body color	Hyperossification pattern	Body size	Advertisement call	Altitudinal range	Geographic distribution
B. sp 7	Teodoro Oliveira, Nova Friburgo, RJ	0	0	3	?	?	0
B. sp 8	Santa Maria Madalena, RJ	1	2	2	0	1	0
B. sp 9	Cunha, SP	1	1	1	0	1	0
B. sp 9	Ubatuba, SP	1	1	1	0	1	0
B. sp 10	Mogi das Cruzes, SP	1	3	2	0	1	0
B. sp 10	Paranapiacaba, SP	1	3	2	0	1	0
B. sp 11	Bananal, SP	1	3	3	0	1	0
B. sp 11	Cunha, SP	1	3	2	0	1	0
H. binotatus	Ubatuba, SP	0	0	4	5	0	6
I. parva	P.N. Serra dos Órgãos, RJ	0	0	3	4	0	6

Appendix 3. Specimens examined in morphological analysis. Abbreviations of Brazilian states as in appendix 1.

B. alipioi, Santa Teresa, ES (MNRJ 25405, 25406, 25407); *B. alipioi*, Vargem Alta, ES (CFBH 25437-38, 24663-67; MNRJ 26042, 26044, 26046-50, 26052-55); *B. brunneus*, Campina Grande do Sul, PR (CFBH 7880, 7893-94; MNRJ 40289-91); *B. bufonoides*, Macaé, RJ (MZUSP 1458-59); *B. crispus*, Cunha, SP (CFBH 10698-99, 10701-03, 10705-08, 23894-903, 27857-68, 29659-61, 29822-27, 29829-30, 29832-40; MNRJ 17443-44); *B. didactylus*, Atílio Vivacqua, ES (CFBH 32636-40; MNRJ 58974, 66733, 66735-37, 66746, 66749, 66755, 66759, 66766); *B. didactylus*, Ilha Grande, RJ (MNRJ 31201, 49294-96, 49298, 55475, 59361-62, 70585-86); *B. didactylus*, Magé, RJ (MNRJ 56156-57, 60980, 62841-42); *B. didactylus*, Lídice Rio Claro, RJ (MNRJ 71334), *B. didactylus*, Parque Estadual dos Três Picos, Cachoeiras de Macacu, RJ (MNRJ 54402-03, 69868, 69875, 69877, 69880, 69884, 69891-93); *B. didactylus*, Reserva Ecológica de Guapiaçu, Cachoeiras de Macacu, RJ (MNRJ 53231-32, 55473-74, 57736, 68819-20, 72358-59); *B. didactylus*, Vale da Revolta, Teresópolis, RJ (MNRJ 70581, 39096); *B. didactylus*, Sacra Família do Tinguá, RJ (MNRJ 4062-73; MZUSP 58319-20, 64810-12, 94620-21); *B. ephippium*, Angra dos Reis, RJ (MNRJ 17458-59); *B. ephippium*, Atibaia, SP (MZUSP 104203-04; CFBH 16807-11, 16813, 16815, 16817-18, 16820-21, 16824, 16826); *B. ephippium*, Alto do Soberbo, Teresópolis, RJ (MZUSP 49991, 49996, 50008-09, 50011, 50014, 50022-23, 50028, 50032, 50039, 50043, 50051, 50054, 50055); *B. ephippium*, Cotia, SP (MZUSP 104149-58, 132267); *B. ephippium*, Guaratinguetá, SP (CFBH 31981-83); *B. ephippium*, Itanhaém, SP (CFBH 19348, 25809, 25816); *B. ephippium*, Joaquim Egydío, Campinas, SP (CFBH 994, 996, 1343, 2565-66, 2568-69, 4259-60; MNRJ 25347-52); *B. ephippium*, Juquitiba, SP (CFBH 32311); *B. ephippium*, Lídice, Rio Claro, RJ (MNRJ 25353-54, 66619-20, 71337, 76000, 81016); *B. ephippium*, Mangaratiba, RJ (MNRJ 2542, 11574-83, 13264-66, 13269); *B. ephippium*, Monteiro Lobato, SP (MNRJ 70620-22); *B. ephippium*, Mogi das Cruzes, SP (CFBH 28658-59, 28663-64, 28667-68, 28670; MZUSP 104208-10, 136758, 136762, 136764, 136787, 137319, 138775, 138776); *B. ephippium*, Parque Estadual Carlos Botelho, São Miguel Arcanjo, SP (MZUSP 136670); *B. ephippium*, Parque Nacional do Itatiaia, Itatiaia, RJ (MZUSP 4134, 7755, 104081, 104101-02; MNRJ 23583-86, 52428-29, 52439); *B. ephippium*, Parque Nacional da Serra dos Órgaos, Teresópolis, RJ (CFBH 22013-15, 24763-64, 32569-70; MZUSP 11205, 34646, 71707-08; MNRJ 17438-39, 17450, 51580-83); *B. ephippium*, Pedra Branca, Jacarepaguá, RJ (MNRJ 3327, 13818-19, 27577-79); *B. ephippium*, Pedra Branca, Paraty, RJ (ALMN 894, 896, 897-99; MNRJ 632, 2435, 10271); *B. ephippium*, Pindamonhangaba, SP (MNRJ 70616-18); *B. ephippium*, Piquete, SP (MZUSP 534, 851, 861, 864-66, 871, 3821-24, 3827-28, 3831-32, 3835, 3837-38, 3840, 3844-45, 4387, 54387); *B. ephippium*, Represa Billings, São Paulo, SP (CFBH 11623); *B. ephippium*, Resende, RJ (MNRJ 73096-99, 78465); *B. ephippium*, Reserva Ecológica do Guapiaçu, Cachoeiras de Macacu, RJ (MNRJ

38104, 56517-18, 59946); *B. ephippium*, Vale da Revolta, Teresópolis, RJ (MNRJ 50260-61, 70580); *B. ephippium*, Represa Rio Grande, RJ (MZUSP 9031, 100277, 100281, 100283, 100285-86, 100290, 100292, 100295, 100298-99, 100303-05, 100307, 100312, 100317, 100342-44, 100346, 100349, 100356, 100436, 100580, 100718); *B. ephippium*, Rio de Janeiro, RJ (MZUSP 100278-80, 103809, 103813, 103817, 103820, 103823, 103828, 103831, 103835, 103839, 103841, 103847, 103850, 103856, 103864, 103869); *B. ephippium*, Rócio, Petrópolis, RJ (CFBH 13946); *B. ephippium*, São Franscisco Xavier, SP (CFBH 16827-34; MNRJ 70619, 70623-25; MZUSP 2761-62); *B. ephippium*, Santo Antônio do Pinhal, SP (MNRJ 70628); *B. ephippium*, Serra do Japi, Jundiaí, SP (CFBH 9431, 16837, 16839, 16857, 16859, 16864, 16870, 30834-35, 30842; MZUSP 507; AP 1, 2, 3, TC 98, 99); *B. ephippium*, Taubaté, SP (MNRJ 70627); *B. ephippium*, Valença, RJ (MNRJ 73395); *B. ferruginus*, Morretes, PR (CFBH 8028, 8030, 8032; TC 142-44); *B. garbeanus*, Alto Caledonia, RJ (MNRJ 39585, 39602, 67498); *B. garbeanus*, Baixo Caledonia, RJ (MNRJ 57124, 57293); *B. garbeanus*, Nova Friburgo, RJ (MNRJ 17433, 27008-09; MZUSP 811, 1460-61); *B. garbeanus*, Alto de Macaé, RJ (CFBH 23564-67, 23569-71, 23574, 23576-77; MNRJ 17440-41, 25391, 25398, 39617); *B. garbeanus*, Serra Nevada, RJ (MNRJ 39342-43); *B. garbeanus*, Teodoro de Oliveira (MNRJ 51472-73); *B. guarani*, Prumirim, Ubatuba, SP (CFBH 27231-35, 27237); *B. hermogenesi*, Salesópolis, Boracéia, SP (CFBH 20125, 20128; MZUSP 138347; MNRJ 47932-34; TC 127, 129, 133, 135); *B. hermogenesi*, Capão Bonito, SP (A-RG 1-7); *B. hermogenesi*, Fazenda Capricórnio, Ubatuba, SP (MNRJ 18624-25, 18662-63); *B. hermogenesi*, Ibiúna, SP (MZUSP 142568); *B. hermogenesi*, Jambeiro, SP (CA 60, 64-65, 195-97, 208-10, 213-14, 221-27, 240, 248, 273, 280); *B. hermogenesi*, Juquitiba, SP (MZUSP 134806-07); *B. hermogenesi*, Cotia, SP (MZUSP 132257-62, 134377); *B. hermogenesi*, Paraibuna, SP (AAG-UFU 175-82); *B. hermogenesi*, Peruíbe, SP (CFBH 24086); *B. hermogenesi*, Picinguaba, Ubatuba, SP (CFBH 29793-95, 29797; MNRJ 18907; MZUSP 137219); *B. hermogenesi*, Registro, SP (MZUSP 138279); B. hermogenesi, Tapiraí, SP (A-TC 1-13, 15-16); *B. izecksohni*, Campina Grande do Sul, PR (CFBH 7375, 7384, 7386-87; MZUSP 132892-93); *B. margaritatus*, Castelo Country Club, Petrópolis, RJ (MNRJ 73700-02); *B. margaritatus*, Castelo Monte Belo, Petrópolis, RJ (MNRJ 70428-29); *B. margaritatus*, Paty de Alferes, RJ (MNRJ 76100-01); *B. margaritatus*, Petrópolis, RJ (MNRJ 60869-70, 60891, 62976-84); *B. margaritatus*, Quitandinha, Petrópolis, RJ (MNRJ 75174); *B. margaritatus*, Sacra Família do Tinguá, RJ (MNRJ 21675, 25373-76, 25385, 25387-89, 25401, 39312); *B. nodoterga*, Salesópolis, Boracéia, SP (MZUSP 30625-26, 30653; TC 128, 130-32, 136-38); *B. nodoterga*, Serra da Cantareira, Mairiporã, SP (CFBH 28413; MZUSP 809, 975, 112785-91; MNRJ 73674-75; TC 140); *B. nodoterga*, Ilhabela, SP (CFBH 7030, 7032-33, 7035, 7037-42, 7045-48; MNRJ 23633-36; 23638-40; TC 105-111); *B. nodoterga*, Santana de Parnaíba, SP (CFBH 9606; MZUSP A147711-22); *B. pernix*, Quatro Barras, PR (CFBH 28888-91; MNRJ 17343); *B. pitanga*, São Luis do Paraitinga, SP (CFBH 15758, 16746-47, 16750, 16755-56, 16760-66, 24299); *B. pitanga*, Natividade da Serra, SP (TC 115-121); *B. pombali*, Guaratuba, PR (CFBH 8048, 8051); *B. pulex*, Camacan, BA (MNRJ 69646; TC 180-184, 186-187,

189-194); *B. toby*, Corcovado, Ubatuba, SP (CFBH 12902-03, 20581-91, 20593-99, 23004-06; TC 113-114); *B. tridactylus*, Salto Morato, Guaraqueçaba, PR (MNRJ 67312-13; MZUSP 137952-57); *B. vertebralis*, Pedra Branca, Paraty, RJ (CFBH 3416-17; MNRJ 2053, 2338, 10599, 11095-96, 11098, 11100-07, 11111, 11113-15, 11117-18, 11120-21, 11124, 11127, 11132); *B. vertebralis*, Picinguaba, Ubatuba, SP (CFBH 3953-54, 3964, 7907-08, 7910, 8361-63, 29789-91); *B.* sp. 1, Mendanha, RJ (MNRJ 53167-68, 57008-09); *B.* sp. 1, Santa Maria Madalena, RJ (CFBH 28125-27, 27343); *B.* sp. 2, Parque Estadual do Jacupiranga, Barra do Turvo, SP (MZUSP 138640-43); B. sp. 2, São Francisco do Sul, SC (TC 251-252, 263-264); B. sp. 2, Joinville, SC (TC 234, 244-245); *B.* sp. 2, Quatro Barras, PR (TC 141); *B.* sp. 2, Ilha do Cardoso, SP (TC 267-268); *B.* sp. 3, Garuva, SC (MZUSP 132902, 132904-07, 132910, 132912, 132914, 132916, 132918, 132920, 132921, 132925-29; TC 202, 205, 208, 209, 213, 215, 216, 218, 220, 221, 225, 227-231); *B.* sp. 4, Corupá, SC (TC 246-248); *B.* sp. 5, São Francisco do Sul, SC (TC 232, 233, 253-262); *B.* sp. 6, Joinville, SC (TC 235-243); *B.* sp. 7, Teodoro Oliveira, RJ (MNRJ 51470-71, 56504-11, 56513-16, 66543); *B.* sp. 8, Santa Maria Madalena, RJ (CFBH 27342, 27344-45, 28048-49, 28124, 28128-38; MNRJ 42408-10, 52718-21); B. sp. 9, Cunha/Ubatuba, SP (CFBH 29843-55; TC 145-151); *B.* sp. 10, Parque das Neblinas, Mogi das Cruzes, SP (MZUSP 135753, 136870-79; PNG 4); *B.* sp. 10, Paranapiacaba, Santo André, SP (CFBH 27187-90, 28168, 28206-10; MNRJ 25367, 42873; MZUSP 93350-51); *B.* sp. 11, Bananal, SP (MNRJ 132166-81, 138647-56; TC 122-126); *B.* sp. 11, Cunha, SP (CFBH 27852-54, 27856, 27879, 29810-21, 29841; TC 158-162); *B.* sp., Serra do Ibitiraquire, Campina Grande do Sul, PR (MZUSP 132894-97, 132900); *B.* sp., Morro dos Perdidos, Guaratuba, PR (CFBH 20940, 20941, 20943-55).

Appendix 4. Advertisement call parameters of the analyzed species of *Brachycephalus*.

Species	Locality	N	Type	Notes/ call (N)	Note duration (s)	Internote duration (s)	Call rate (call/s)	Minimum frequency (Hz)	Maximum frequency (Hz)	Dominant frequency (Hz)	Pulses/ note (N)	Pulse duration (ms)	Pulse rate (pulse/s)
B. crispus	Cunha, SP	5	I	1	0.28 (± 0.0)	0.35 (± 0.0)	1.67	3.5 (± 0.2)	5.7 (± 0.1)	4.6 (± 0.1)	9 to 10	30.0 (± 0.0)	17.42
B. ephippium	São Miguel Arcanjo, SP	1	I	1	0.15 (± 0.0)	0.26 (± 0.0)	2.65	2.8 (± 1.6)	4.9 (± 1.6)	3.6 (± 1.3)	9	10.0 (± 0.0)	22.50
B. ephippium	Joaquim Egydio, SP	3	I	1	0.12 (± 0.0)	0.14 (± 0.0)	3.74	2.6 (± 0.1)	11.3 (± 2.30)	3.9 (± 0.0)	10 to 13	10.0 (± 0.0)	40.75
B. hermogenesi	Boracéia, SP	1	IV	1	0.04 (± 0.0)	4.14 (± 0.3)	0.29	4.9 (± 3.6)	9.7 (± 4.3)	7.0 (± 0.0)	1 to 2	20.0 (± 0.0)	0.58
B. hermogenesi	Boracéia, SP	2	IV	2	0.44 (± 0.0)	4.61 (± 0.6)	0.16	5.1 (± 0.1)	8.5 (± 0.1)	7.0 (± 0.3)	1 to 2	20.0 (± 0.0)	1.05
B. hermogenesi	Boracéia, SP	2	IV	4	0.93 (± 0.0)	3.85 (± 0.4)	0.30	5.6 (± 0.0)	8.4 (± 0.1)	6.7 (± 0.1)	1 to 2	10.0 (± 0.0)	2.08
B. hermogenesi	Paraibuna, SP	1	IV	1	0.02 (± 0.0)	3.45 (± 0.3)	0.37	4.5 (± 3.0)	9.3 (± 2.2)	6.9 (± 7.3)	1 to 2	10.0 (± 0.0)	0.74
B. hermogenesi	Paraibuna, SP	1	IV	2	0.46 (± 0.0)	3.91 (± 0.3)	0.27	4.7 (± 1.6)	8.5 (± 4.1)	6.4 (± 9.0)	2	20.0 (± 0.0)	1.10
B. hermogenesi	Paraibuna, SP	1	IV	3	0.60 (± 0.0)	2.44 (± 0.1)	0.43	4.5 (± 1.5)	8.6 (± 3.2)	6.7 (± 0.0)	2	10.0 (± 0.0)	2.60
B. hermogenesi	Paraibuna, SP	2	IV	4	0.79 (± 0.1)	2.03 (± 0.2)	0.45	4.1 (± 0.3)	10.6 (± 0.1)	6.9 (± 0.0)	1 to 2	10.0 (± 0.0)	3.53
B. hermogenesi	Ubatuba, SP	1	IV	1	0.03 (± 0.0)	3.91 (± 0.1)	0.33	5.2 (± 2.2)	9.9 (± 2.3)	8.3 (± 1.2)	2 to 3	10.0 (± 0.0)	0.84
B. hermogenesi	Ubatuba, SP	2	IV	2	0.29 (± 0.0)	2.83 (± 0.5)	0.43	5.6 (± 0.4)	10.4 (± 0.0)	8.2 (± 0.1)	1 to 2	10.0 (± 0.0)	1.89
B. hermogenesi	Ubatuba, SP	1	IV	3	0.58 (± 0.0)	2.74 (± 0.4)	0.44	5.2 (± 1.3)	9.7 (± 1.9)	7.8 (± 8.3)	1 to 2	10.0 (± 0.0)	2.64
B. nodoterga	Ilhabela, SP	1	I	1	0.17 (± 0.0)	0.28 (± 0.0)	2.39	4.3 (± 1.9)	6.0 (± 1.8)	5.0 (± 1.5)	10 to 11	20.0 (± 0.0)	22.75
B. nodoterga	Cantareira, SP	2	I	1	0.12 (± 0.0)	0.26 (± 0.0)	2.75	3.7 (± 0.1)	5.9 (± 0.2)	4.5 (± 0.0)	8 to 9	20.0 (± 0.0)	21.00
B. nodoterga	Boracéia, SP	3	I	1	0.19 (± 0.0)	0.27 (± 0.0)	2.35	3.8 (± 0.0)	5.6 (± 0.0)	4.6 (± 0.0)	10 to 12	20.0 (± 0.0)	26.07
B. pernix	Anhangava, PR	1	III	2	0.42 (± 0.1)	5.37 (± 0.5)	0.20	4.6 (± 2.6)	8.6 (± 3.7)	6.9 (± 3.5)	1 to 3	20.0 (± 0.0)	0.70
B. pernix	Anhangava, PR	1	III	3	0.83 (± 0.1)	4.57 (± 0.6)	0.23	5.4 (± 3.0)	7.6 (± 2.4)	6.4 (± 3.9)	1 to 3	10.0 (± 0.0)	1.20

Appendix 4. Cont.

Species	Locality	N	Type	Notes/ call (N)	Note duration (s)	Internote duration (s)	Call rate (call/s)	Minimum frequency (Hz)	Maximum frequency (Hz)	Dominant frequency (Hz)	Pulses/ note (N)	Pulse duration (ms)	Pulse rate (pulse/s)
B. pitanga	Natividade da Serra, SP	1	I	1	0.19 (± 0.0)	0.23 (± 0.0)	2.54	4.2 (± 1.5)	6.1 (± 1.4)	5.5 (± 3.7)	11 to 12	20.0 (± 0.0)	30.00
B. pitanga	São Luís do Paraitinga, SP	2	I	1	0.21 (± 0.0)	0.28 (± 0.0)	2.13	4.1 (± 0.1)	7.4 (± 0.5)	5.3 (± 0.3)	9 to 11	20.0 (± 0.0)	21.38
B. pombali	Igreja, PR	1	III	1	0.07 (± 0.0)	16.99 (± 1.1)	0.08	3.5 (± 5.6)	6.8 (± 4.0)	5.1 (± 1.6)	1 to 3	30.0 (± 0.1)	0.24
B. pombali	Igreja, PR	1	III	2	0.38 (± 0.1)	19.02 (± 0.4)	0.08	3.3 (± 2.2)	7.1 (± 4.7)	5.0 (± 1.5)	1 to 3	20.0 (± 0.1)	0.36
B. vertebralis	Ubatuba, SP	1	I	1	0.18 (± 0.0)	0.27 (± 0.0)	2.28	3.9 (± 1.7)	5.2 (± 1.0)	4.5 (± 3.7)	9 to 10	20.0 (± 0.0)	22.00
B. sp 2	Joinville, SC	2	II	5	2.22 (± 0.1)	5.96 (± 0.8)	0.16	5.1 (± 0.4)	8.2 (± 0.0)	6.4 (± 0.2)	6 to 13	20.0 (± 0.0)	7.58
B. sp 2	Ilha do Cardoso, SP	5	II	5	1.87 (± 0.1)	5.21 (± 1.2)	0.19	4.9 (± 0.3)	9.9 (± 0.3)	6.9 (± 0.1)	6 to 13	20.0 (± 0.1)	10.11
B. sp 2	São Francisco do Sul, SC	4	II	5	1.66 (± 0.1)	4.53 (± 1.7)	0.24	4.9 (± 0.3)	9.1 (± 0.2)	6.6 (± 0.1)	6 to 11	20.0 (± 0.1)	9.10
B. sp 3	Garuva, SC	6	III	2	0.43 (± 0.0)	11.54 (± 1.2)	0.11	2.4 (± 0.2)	11.0 (± 0.5)	6.4 (± 0.6)	1 to 4	10.0 (± 0.0)	0.47
B. sp 5	São Francisco do Sul, SC	3	III	1	0.05 (± 0.0)	4.15 (± 0.7)	0.31	4.4 (± 0.0)	8.7 (± 0.3)	6.6 (± 0.1)	2 to 3	20.0 (± 0.0)	0.73
B. sp 5	São Francisco do Sul, SC	1	III	2	0.55 (± 0.1)	5.21 (± 0.7)	0.21	4.3 (± 2.0)	8.7 (± 2.2)	6.5 (± 7.4)	1 to 3	20.0 (± 0.1)	1.20
B. sp 6	Joinville, SC	2	III	1	0.06 (± 0.0)	8.52 (± 0.6)	0.13	3.7 (± 0.1)	10.5 (± 0.4)	6.7 (± 0.1)	2 to 3	30.0 (± 0.0)	0.31
B. sp 6	Joinville, SC	4	III	2	0.37 (± 0.0)	7.43 (± 1.5)	0.15	3.4 (± 0.7)	10.9 (± 1.4)	6.8 (± 0.3)	1 to 3	20.0 (± 0.1)	0.53
B. sp 8	Desengano, RJ	4	I	1	0.28 (± 0.1)	0.21 (± 0.0)	2.33	3.9 (± 0.2)	7.0 (± 1.5)	4.8 (± 0.2)	11 to 19	20.0 (± 0.0)	28.88
B. sp 10	Paranapiacaba, SP	2	I	1	0.19 (± 0.0)	0.31 (± 0.0)	2.15	3.4 (± 0.1)	10.4 (± 0.4)	4.7 (± 0.4)	9 to 10	20.0 (± 0.0)	18.00
B. sp 11	Cunha, SP	3	I	1	0.25 (± 0.0)	0.27 (± 0.0)	2.02	4.1 (± 0.0)	6.10 (± 0.0)	4.8 (± 0.0)	18 to 20	10.0 (± 0.0)	36.83

CAPÍTULO III

Geographic patterns of diversification in the montane Atlantic forest revealed by the endemic anurans of the genus *Brachycephalus*

Geographic patterns of diversification in the montane Atlantic forest revealed by the endemic anurans of the genus *Brachycephalus*

Abstract

Historical areas of forest stability, tectonic activities, and geographic barriers are supposed to drive biological diversification in the Atlantic forest. The climatic stratification in high elevation Atlantic forest might also have influenced on spatial and temporal distribution of extant taxa and their evolutionary history. To contribute to the current knowledge of the evolutionary processes in high elevation areas of the Atlantic forest, we studied aspects of diversification in the endemic anurans of the genus *Brachycephalus*. The mtDNA analysis showed relatively older times of diversification for *Brachycephalus* compared to other anurans, being influenced by Oligocene, Miocene, and Quaternary events. Our findings corroborate phylogeographic studies in Atlantic forest in which species diversification was supposed to be driven by refugial isolation and geographic barriers, suggesting that both events were not mutually exclusive. Additionally, we found that climate and landscape resistances to gene flow increase reproductive isolation and consequently the opportunities for diversification in northern montane Atlantic forest. These results and the tropical higher genetic diversity compared to subtropical portion of biome evidenced a latitudinal gradient of diversification in *Brachycephalus*.

Introduction

The growing number of studies considering the evolutionary processes in Atlantic forest indicates forest refuges, tectonic activities, and geographic barriers as plausible explanations to biological diversification in this biome (DANTAS et al., 2011; TURCHETTO-ZOLET et al., 2013). According to the estimated dating of lineage divergence, one or several palaeoclimatic and palaeogeographic events together may have contributed to the current geographical structure of populations (TURCHETTO-ZOLET et al., 2013).

Mountain ranges of coastal Atlantic forest were originated in the late Cretaceous and their complex relief, formed by the uplift and rift of the ancient Brazilian crystalline shield, were attributed to tectonic activity reactivation dated to Miocene, Pleistocene, and Holocene (SIMPSON, 1979; ALMEIDA & CARNEIRO, 1998; RIBEIRO, 2006). These significant Tertiary tectonic activity and palaeogeographical reorganization influenced the relief and drainage patterns in coastal Brazil (POTTER, 1997; ALMEIDA & CARNEIRO, 1998; RIBEIRO, 2006). Tectonic faults and rivers are considered important geographic barriers for

plants (RIBEIRO et al., 2011), fishes (RIBEIRO, 2006), amphibians (FITZPATRICK et al., 2009; BRUNES et al., 2010; THOMÉ et al., 2010; AMARO et al., 2012), reptiles (SOUZA et al., 2003; PELLEGRINO et al., 2005; GRAZZIOTIN et al., 2006), and birds (RAPOSO et al., 2013) living in the Atlantic forest.

Between the late Tertiary and Quaternary, the Pliocene and Pleistocene climate shifts related to temperature, aridity and sea level fluctuations potentially impacted species survival, adaptability, and migration (OLIVEIRA et al., 2005; SUGUIO et al., 2005; TURCHETTO-ZOLET et al., 2013). The glacial and interglacial environmental fluctuations are correlated to past Atlantic forest fragmentation, expansion, and floristic composition changes (LEDRU et al., 2009). Areas climatically more stable, the so-called refuges, were considered important for diversity maintenance during cold and dry periods that resulted from past climatic changes (HAFFER, 1969; AB'SABER, 1977; BROWN & AB'SABER, 1979). Despite of contradictory evidences to historical tropical refuges (BUSH & DE OLIVEIRA, 2006), the classical theory supposes that the humid forests became restricted to topographic enclaves and that dryer climate regimes affected severely the lower and flatter areas in which forest was substituted by open formation, such as grasslands and savannas (HAFFER, 1969; BROWN & AB'SABER, 1979). Forest refuges have been extensively studied in Atlantic forest but their putative limits are not always coincident (e.g. CARNAVAL & MORITZ, 2008; CARNAVAL et al., 2009; THOMÉ et al., 2010). Historical stable areas are involved in the evolutionary histories of the Atlantic forest plants (PALMA-SILVA et al., 2009; PINHEIRO et al., 2013), amphibians (FITZPATRICK et al., 2009; THOMÉ et al., 2010; AMARO et al., 2012), reptiles (SOUZA et al., 2003; GRAZZIOTIN et al., 2006), birds (CABANNE et al., 2007; D'HORTA et al., 2011; MALDONADO-COELHO et al., 2012; RAPOSO et al., 2013), and mammals (LARA & PATTON, 2000).

Currently, despite of the intense habitat loss and forest fragmentation, the Atlantic forest remnants still maintain exceptional climatic and structural diversity (RIBEIRO et al., 2009). The latitudinal extension of the biome is accountable to its distribution across tropical and subtropical climates (ALMEIDA & CARNEIRO, 1998). The longitudinal variation of rainfall regimes drives the differences between forests from the interior and those near to the coast (CÂMARA, 2003). Furthermore, forest remnants were distributed since the lowlands up to 2700 meters above sea level (AB'SABER, 1954; TABARELLI et al., 2005). These latitudinal, longitudinal, and altitudinal gradients created a unique diversity of landscapes, which is congruent with the extraordinary biodiversity and endemism of this biome (SILVA & CASTELETI, 2003).

Several studies have addressed the latitudinal gradients for many taxonomic groups and geographic regions (GASTON, 2000). Speciation and extinction rates allied to colonization and range expansion were central issues to understanding the tropical diversity (MITTELBACH et al., 2007; PYRON & WIENS, 2013). Generally, species richness is higher in the tropics (GASTON & BLACKBURN, 1996; GASTON, 2000) and this trend is especially valid for tropical high elevation areas (GHALAMBOR et al. 2006; KOZAK & WIENS, 2007). Several montane tropical forests are considered hotspots of biodiversity (MYERS et al., 2000; RICKETTS et al., 2005). The high biodiversity of tropical montane areas is currently related to their high altitudinal and climatic stratification, which increase the opportunities for vicariant speciation (JANZEN, 1967; MORITZ et al., 2000; CADENA et al., 2011; GUARNIZO et al., 2009). Narrower physiologic tolerances of tropical species (WIENS, 2004; KOZAK & WIENS, 2007; HUA & WIENS, 2010) increases the costs of dispersal over environmental gradients, resulting in reduced gene flow and high rates of allopatric speciation, when compared to temperate analogous (JANZEN, 1967; GHALAMBOR et al., 2006; CADENA et al., 2011).

The high species diversity, levels of endemism, and the putative function as refuge have been favored evolutionary studies and latitudinal gradient investigations in mountainous regions (CADENA et al., 2011; WOLLEMBERG et al., 2011; PABIJAN et al., 2012). The Atlantic forest mountain ranges has been recently considered in evolutionary studies and not surprisingly revealed high levels of genetic diversity (D'HORTA et al., 2011; AMARO et al., 2012). For birds and amphibians these areas were considered historical refuges (D'HORTA et al., 2011; AMARO et al., 2012).

To contribute to the currently knowledge of the evolutionary processes that occurred in high elevation areas of the Atlantic Forest, we studied aspects of diversification in the montane anurans from the genus *Brachycephalus*. This genus is endemic to the Atlantic forest and comprises 21 formally named and valid species (FROST, 2014). Eighteen species of *Brachycephalus* exhibit geographic distribution restricted to mountain slopes with 600-1800 m of altitude (POMBAL et al., 1998; CONDEZ et al., 2014). Exceptionally, three *Brachycephalus* species have wide altitudinal ranges, occurring from the sea level up to the highlands (IZECKSOHN, 1971; GIARETTA & SAWAYA, 1998; NAPOLI et al., 2011). Species of *Brachycephalus* are leaf-litter inhabitants; exhibit direct development and an extremely small adult body size, with snout-vent length usually inferior to 18 mm (POMBAL et al., 1994; POMBAL, 1999; CLEMENTE-CARVALHO et al., 2011). The highly restricted geographical range of most species of *Brachycephalus*, their narrow climate niches (PIE et al.,

2013), and relative susceptibility to future climate changes (HADDAD et al., 2008) corroborate the disjunctive distribution pattern and geographic isolation within *Brachycephalus* populations. Herein, we inferred species relationships and described genetic diversity based on three mtDNA fragments (12S rRNA, 16S rRNA, and Cytochrome b) from several populations within the genus distribution. We compared the genetic variation patterns with geographic barriers and forest refuges to identify the influence of these mechanisms on species diversification. More specifically, we evaluated the genetic structure of *Brachycephalus* populations at northern and southern of Atlantic forest and tested the prediction of higher genetic diversity associated to areas of historical stability during the past environmental shifts. Finally, we consider the Atlantic forest latitudinal variation in climate and landscape conditions to evaluate species diversity gradients. We predict that highlands of tropical Atlantic forest exhibit higher environmental stratification, compared to subtropical areas in the same biome, which increases geographic isolation and consequently the opportunities to speciation. Considering these predictions, we evaluate genetic differentiation and spatial resistances to gene flow in northern and southern Atlantic forest.

Material and Methods

Population sampling

We obtained tissue samples from 246 individuals, from 18 formally named and 11 undescribed *Brachycephalus* species distributed in 57 localities along the Atlantic forest (AF) biome (Appendix 1). Our dataset comprises one to fourteen individuals per locality. Except for *B. atelopoide, B. bufonoides*, and *B. tridactylus,* for which we have not obtained tissues, our ingroup sampling represents the genus diversity throughout its distribution (FROST, 2014). Fieldwork from 2010 to 2014 provided most of the samples, but additional data were also obtained from tissue collections. Tissue samples analyzed were deposited in CFBHT (Célio F. B. Haddad Tissue collection, Departamento de Zoologia, I.B., Universidade Estadual Paulista, Campus de Rio Claro, São Paulo, Brazil), CTMZ (Tissue collection at Museu de Zoologia, Universidade de São Paulo, Brazil), and MNRJ (Tissue collection of Museu Nacional, Rio de Janeiro, Brazil).

DNA extraction, amplification and sequencing

The selected genes were the three mitochondrial fragments 12S rRNA (12S; 814 bp), 16S rRNA (16S; 864 bp), and Cytochrome b (Cytb; 694 bp). Total DNA was extracted from

ethanol-preserved muscle or liver tissues using DNeasy Tissue Kits (Qiagen Inc., Valencia, CA). The amplification and sequence strategy of mitochondrial fragments followed similar procedures as in a previous study (CLEMENTE-CARVALHO et al., 2011). We performed Polymerase Chain Reaction (PCR) for the amplification of the selected fragments using specific primers (Appendix 2) and PCR Master Mix (Taq Polymerase 0.05 u/ul, PCR reaction buffer with 4 mM $MgCl_2$, 0.4 mM of each dNTP) or PCR Grade Nucleotide Mix (Roche Diagnostics GmbH, Mannheim, Germany), according to the manufacturer's instructions. Fragments of the mitochondrial 12S gene were amplified using primer pair 12SJL and 12SKH (GOEBEL et al., 1999). Thermocycling conditions for dsDNA amplification began with a 5-min denaturation at 94°C followed by 35 cycles each of which had a 1-min denaturation at 94°C, a 1-min annealing at 50-52°C, and a 1-min extension at 72°C. A 5-min extention at 72°C followed the final cycle. A section of the mitochondrial 16S gene was amplified using primer pair 12SM and 16SA (DARST & CANNATELLA, 2004). Thermocycling conditions were similar to those for 12S except that annealing temperatures were 60-61°C. Partial sequences of Cytb gene were obtained using five primers. Primers MVZ15-L and CytbAR-H were previously published (MORITZ et al., 1992; GOEBEL et al., 1999) and primers BrachyCytb-F2, BrachyCytb-F3, and BrachyCytb-R1 were designed for this study and amplified a fragment just below 700 bp. Thermocycling conditions were similar to those for 12SrDNA except that annealing temperatures were 60-61°C for primer pair BrachyCytb-F2 and BrachyCytb-R1, 55°C for primer pair BrachyCytb-F3 and BrachyCytb-R1, and 50-55°C for primer pair MVZ15-L and CytbAR-H. PCR products were visualized in 1% agarose gels. PCR products were purified using Exonuclease and Shrimp Alkaline Phosphatase (Affimetrix/USB, Cleveland, OH), according to the manufacturer's instructions. Cycle sequencing reactions were completed with Applied Biosystems BigDye Terminator chemistry (Applied Biosystems). Sequencing was performed by Macrogen Inc. on an Applied Biosystems automated 3730 DNA analyzer or at Cornell University under the same conditions. Sequences were obtained in both directions with the same primers used for PCR amplification and subjected to BLAST searches in GenBank to verify amplification of desired sequences. Chromatograms were fully inspected, embedded primer sequences were deleted, and forward and reverse strands were compared before assembling consensus sequences with CodonCode Aligner 3.5 (Codon Code Corporation). Data also include sequences downloaded from GenBank, NCBI (Appendix 1). Sequences were aligned using CLUSTAL W (THOMPSON et al., 1994) in BioEdit software (HALL, 1999).

Phylogenetic and population genetic analysis

We used the concatenated mtDNA dataset (2372 bp) based on unique haplotypes extracted by DnaSP 5.0 (LIBRADO & ROZAS, 2009) to infer the phylogenetic relationship within *Brachycephalus*. Samples with missing fragments were excluded from the analysis, except for those not represented in the complete data set (Appendix 1). Molecular studies have established *Ischnocnema* as the sister group of *Brachycephalus* (HEDGES et al., 2008; PYRON & WIENS, 2011; CANEDO & HADDAD, 2012; PADIAL et al., 2014). According to these studies, we used *Ischnocnema parva* as outgroup and root the phylogeny with *Haddadus binotatus*. We used maximum parsimony (MP), maximum likelihood (ML), and Bayesian inference (BI) for phylogenetic reconstructions. We performed MP analysis in TNT 1.1, Willi Hennig Society Edition (GOLOBOFF et al., 2008) using New Technology Search at initial level 50, including sectorial searches, ratched, drift, and tree fusing; requested directed search to hit 15 times the best length; random addition sequence 1000; random seed 1; replace existing trees, auto-constrain. We also performed a total of 1000 jacknife replicates with the same specifications. We performed ML analysis in RAxMLGUI 0.9.3 (STAMATAKIS, 2006) using the GTR+I+G model of nucleotide substitution. We performed a total of 100 runs and assessed node support via 1000 bootstrap replicates (ML + thorough bootstrap; n. threads 10). We partitioned the dataset by gene (12S rRNA, and 16S rRNA) and codon position in protein-coding genes (Cytb). Saturation analysis of transitions and transversions against genetic distances were obtained in Dambe 4.1.19 (XIA & XIE, 2001). Because we did not detect any kind of saturation, all substitutions of these three genes were used for phylogenetic reconstructions. We performed BI analyses in MrBayes 3.1 (RONQUIST & HUELSENBECK, 2003) based on the best substitution models selected for each locus according to the Akaike Information Criterion (AIC) in PartitionFinder 1.0 (LANFEAR et al., 2012). The models of nucleotide evolution used in BI for the five partitions were: 12S (GTR+I+G), 16S (GTR+I+G), Cytb 1st codon (TVMef+I+G), Cytb 2nd codon (TVM+G) and Cytb 3rd codon (GTR+G). We performed analyses with 10 million generations, consisted of two independent runs, four Markov chains with default heating values, and default priors. Trees were sampled every 1000 generations and the first 25% of generated trees were discarded as burn-in. Output files were examined visually in Tracer 1.5 (RAMBAUT & DRUMMOND, 2007) and trees were visualized and edited using FigTree 1.1.2 program, available at http://tree.bio.ed.ac.uk/software/figtree/.

To assess the genetic diversity we used each mtDNA fragment individually and considered each locality as one population, using Arlequin 3.5.1.3 (EXCOFFIER et al., 2005).

For these analyses localities with less than two samplings were excluded. We calculated the number of haplotypes (*H*), number of polymorphic sites (*S*), haplotype diversity (*Hd*), nucleotide diversity (*Nd*), and the relationship between the mean number of pairwise differences and Theta (*Theta* π). We calculated genetic diversity indices for all populations, for mtDNA clades, and for population groups defined by geographic barriers, as described below.

Estimates of divergence times

Divergence dating estimations were implemented in BEAST 1.7.5 (DRUMMOND et al., 2012). We inferred the time to the most recent common ancestor (TMRCA) based on Cytb sequence data, which has been largely used in amphibian phylogeographic studies and currently presents relatively reliable divergence rates between 0.8% and 1.9% per million years (Ma; SANGUILA et al., 2011; GONZALEZ et al., 2014). Thus, we adopted this as the rate prior with a normal distribution and a mean rate of 1.4% per Ma (settings for mean = 0.014 and confidence interval = 0.008 to 0.019). We also used a calibration point based on the estimated divergence time for *Brachycephalus* and *Ischnocnema* provided by Heinicke et al. (2009). According to this study, these two genera were supposed to diverge in the early Cenozoic, 46.6 (32.4 to 64.2) Ma. We adopted these values as a normal distribution prior in the tree root, truncated to zero as lower bound. Since we used just one haplotype for each species we applied the Yule speciation tree prior and left all remaining priors as default. In order to simplify the number of parameters to be estimated we did not consider codon partitions. We also implemented the model of nucleotide substitution prioritizing the best fit according to the Akaike Information Criterion (AIC) obtained in JModelTest 2.1.4 (DARRIBA et al., 2012). We first adopted the best model GTR+I+G (AIC = 10210) but low ESS values for the prior and posterior distribution even when increasing chain lengths led us to decide for the second and less complex model of substitution TN93+I+G (AIC = 10212). This strategy has been used before in other studies and returned better parameter estimates without altering posterior age distributions (BLAIMER, 2012). We decided to run the analysis using a strict clock after access the low standard deviation and coefficient of variation when using an uncorrelated relaxed lognormal clock (DRUMMOND et al., 2007). Two independent runs of MCMC were done for 100 million generations, sampling every 10.000. After assessing convergence of all parameters using Tracer 1.5 (RAMBAUT & DRUMMOND, 2007), we discarded the first 10% of samples as burn-in and summarized as maximum clade credibility trees in TreeAnnotator 1.7.5 (BEAST package, DRUMMOND et al., 2012). Tree

was edited using FigTree 1.1.2 program, available at http://tree.bio.ed.ac.uk/software/figtree/. We adopted the International Chronostratigraphic Chart to evaluate divergence dates and ages (COHEN et al., 2013).

Isolation by distance, barriers, and forest stability

To characterize spatial distribution of species we used all georeferenced records available for the genus *Brachycephalus*. The geographic coordinates were checked individually and adjusted to UTM WGS 1984 datum to minimize projection errors. We calculated the pairwise geographic distance among all localities (using Euclidean distance) in the Network Analyst extension of ArcMap 10.0 (ESRI, 2010). We calculate the pairwise F_{ST} for the three mtDNA fragments individually using Arlequin 3.5.1.3 (EXCOFFIER et al., 2005) to test for genetic fixation and infer the average level of gene flow among populations. Statistical significance was assessed with 1000 permutations. To evaluate isolation by distance we tested for correlation between the genetic fixation index F_{ST} and the geographic distances applying a Mantel test with 10.000 replications in Ecodist 1.2.9 package for R (GOSLEE & URBAN, 2007; R CORE TEAM, 2013).

To identify genetic structure and geographic barriers we applied the Monmonier maximum difference algorithm in BARRIER 2.2 (MANNI et al., 2004). The algorithm uses a network between pairs of localities and searches for the genetically most differentiated sequences, which could be correlated to an existing effective barrier to gene flow. We used for input the genetic fixation index F_{ST} generated by the main mtDNA clades obtained in Arlequin 3.5.1.3 (EXCOFFIER et al., 2005) and the geographic coordinates of the centroid of the corresponding clade geographic distribution. Next, the geographic barriers suggested by the analysis were correlated to physical barriers (e.g. rivers, valleys, and tectonic faults) reported in literature and evaluated to be an explanation plausible or not for the genetic structure observed. At this time, we had two hypotheses of population clusters: (1) all populations individually structured and (2) populations clustered by mtDNA clades. To test the distribution of genetic variation in these different population structures we performed analyses of molecular variance (AMOVA) a posteriori in Arlequin 3.5.1.3 (EXCOFFIER et al., 2005). For each hypothesis, AMOVA calculates F-statistics by partitioning genetic variation among groups of populations, among populations within groups, and within populations (EXCOFFIER et al., 1992). These results were especially important to evaluate the portion of statistically significant genetic variation in population groups imposed by the geographic barriers.

To evaluate forest stability influence on the genetic diversity of *Brachycephalus* we overlapped the haplotype diversity (*Hd*) for all mtDNA fragments with the putative limits of AF refuges identified in previous studies. Additionally, we used an environmental layer of AF stability over the past 100.000 years (~800 m of resolution), in which each pixel was defined by the percentage of forest maintenance along this period. We overlapped this layer with the geographic coordinates of each population; delimited circular regions of 1 km radius buffering each sampled point; and extracted values of forest stability in the surroundings using ArcMap 10.0 (ESRI, 2010). To investigate if higher genetic diversity was correlated with the most stable areas, the average value of forest stability for each population was correlated with the previous calculated genetic diversity indices (*H*, *S*, *Hd*, *Nd*, and *Theta* π) using linear regression models in R (R CORE TEAM, 2013).

Latitudinal gradient of diversification

The northern and southern clades identified in mtDNA topology were used to evaluate the latitudinal gradient of diversification in *Brachycephalus*. The genetic structure was tested considering northern and southern populations by the analysis of molecular variance (AMOVA) in Arlequin 3.5.1.3 (EXCOFFIER et al., 2005). Once we found statistically significant genetic variation in these groups we described the genetic diversity by the same genetic indices calculated above. Due to differences in sampling size, to precisely determine differences in genetic diversity between northern and southern AF we used a rarefaction analysis in EcoSim 7 (SANDERS, 1968; GOTELLI & ENTSMINGER, 2004). The analysis was performed with number of haplotypes (*H*) based on 1000 randomized samples, using independent sampling, species richness index, and abundance level set with the lowest number of sampled individuals.

To address the hypothesis of latitudinal gradient of diversification we focused on spatial resistance to gene flow. We calculated the pairwise F_{ST} for the three mtDNA fragments, for both northern and southern groups of populations using Arlequin 3.5.1.3 (EXCOFFIER et al., 2005). The spatial resistances of climate and landscape features were calculated in CIRCUITSCAPE 3.5.1 (McRAE, 2006). CIRCUITSCAPE considers landscape heterogeneity and quantify the least cost path resistance matrices between all pairs of populations across their distribution, including the availability of all alternative pathways (McRAE & BEIER, 2007; McRAE et al., 2008). We estimated resistances in seven surfaces with 0.07-pixel size (with ~800 meters of resolution) between all pairs of focal points using a connection scheme that allowed connectivity among the four nearest cells. The layers were

previously reclassified according to the focal species biology. We used altitude layer (JARVIS et al., 2009), annual mean temperature, annual temperature range and precipitation (BIO1, BIO7, and BIO12 respectively, HIJMANS et al., 2005). Considering 650 meters as the lower limit of occurrence for the most species within the genus, our hypothesis is that habitats with high temperatures, low precipitation, and low altitudes can represent a scenario with high resistance for gene flow within *Brachycephalus* populations. Thus, we reclassified the altitude layer using all the values above 650 meters pooled and considered as low resistance to dispersal. We then attributed to these pixels the resistance value of 1, increasing resistance values as altitude decreases. Areas such as rocky formations located above 2200 meters, not suitable for *Brachycephalus* species, were not considered in our analysis. It represents less than 0.01% of the total area covered by the biome. In addition to altitude, we calculated a topographic complexity index based on a 1000 m digital elevation model of the study area using the raster calculator feature in ArcMap 10.0 (ESRI, 2010). Each pixel was defined as the standard deviation in elevation of the neighbor pixels (HUAXING, 2008). Our hypothesis is that high topographic complexity is related to high relief roughness and can be a costly route of dispersion among populations of *Brachycephalus*. We also used vegetation density (USGS & FAO, 2000) as an important measure of current landscape changes. The same layer of AF stability over the past 100.000 years was used to calculate the influence of historical stable areas to gene flow. Finally, we conducted partial Mantel tests (SMOUSE et al., 1986) for northern and southern groups of populations to examine the associations between pairwise F_{ST} and pairwise landscape resistances, controlled by the effect of spatial correlation between samples (pairwise simple Euclidean distance among populations). We estimated the Partial Mantel correlation coefficients with 10.000 matrix permutations and significance of 95% confidence intervals in Ecodist 1.2.9 package for R (GOSLEE & URBAN, 2007; R CORE TEAM, 2013).

Results

Phylogenetic and population genetic analysis

We obtained 227 unique haplotypes from the concatenated mtDNA data set and different numbers of unique haplotypes for the three mtDNA fragments (Table 1). For 12S we found 101 unique haplotypes, of which 18 were shared generally among individuals from the same locality. Exceptionally, populations of *Brachycephalus ephippium* (from Mogi das Cruzes and Guaratinguetá, SP), *B.* sp. 2 (from Cananéia, SP and Joinville, SC), *B. crispus* and *B. guarani* shared 12S haplotypes.

Table 1. Diversity indices for all populations and clades, considering each mtDNA fragment. Abbreviations were: number of haplotypes (*H*), number of polymorphic sites (*S*), haplotype diversity (*Hd*), nucleotide diversity (*Nd*), and the relationship between the mean number of pairwise differences and Theta (*Theta π*).

mtDNA	Group	Sample size	*H*	*S*	*Hd*	*Nd*	*Theta π*
12S	All populations	184	101	324	0.98	0.10	74.04
	Southern clade	56	18	217	0.90	0.06	42.38
	Northern clade	128	83	301	0.99	0.06	44.95
16S	All populations	194	112	550	0.98	0.15	121.30
	Southern clade	53	28	213	0.96	0.08	61.51
	Northern clade	141	84	531	0.99	0.11	89.99
Cytb	All populations	77	31	279	0.90	0.16	100.01
	Southern clade	37	12	200	0.73	0.06	31.27
	Northern clade	40	19	205	0.88	0.11	72.93

For 16S we found 112 unique haplotypes, of which 29 were shared generally among individuals from the same locality. Exceptions were *B. nodoterga* (from Santana do Parnaíba and Mairiporã, SP) and *B.* sp. 2 (from Cananéia, SP and Joinville, SC). For Cytb we found 31 unique haplotypes, of which 21 were shared within individuals from the same species or same mtDNA clade. From those, *B. garbeanus* (from Nova Friburgo and Macaé de Cima, RJ), *B. ephippium* (from Serrinha do Alambari and Resende, RJ; from São Miguel Arcanjo and Itanhaém, SP; from Joaquim Egydio, Serra do Japi and Mogi das Cruzes, SP), *B. nodoterga* (from Santana do Parnaíba and Salesópolis, SP) and the species *B.* sp. 9, *B. crispus*, *B. guarani* and *B. pitanga* share Cytb haplotypes. We found 324 polymorphic sites for 12S (814 bp), of which 255 were parsimony informative. For 16S (864 bp) we found 550 polymorphic sites, of which 390 were parsimony informative. For Cytb (694 bp) we found 279 polymorphic sites, of which 263 were parsimony informative and only 16 sites were autapomorphic. The sequence alignment for Cytb did not require gaps, as opposed to 12S and 16S alignments.

Phylogenetic reconstructions based on 153 concatenated mtDNA sequences confirmed that the genus *Brachycephalus* is monophyletic. Topologies inferred from the BI, ML, and MP were almost identical, except for the position of *B. pulex* and *B. hermogenesi* (haplotypes 1, 190, 191 and 194; Appendix 3). Here, we present the node support values for the three methods in the Maximum Likelihood tree (Figure 1).

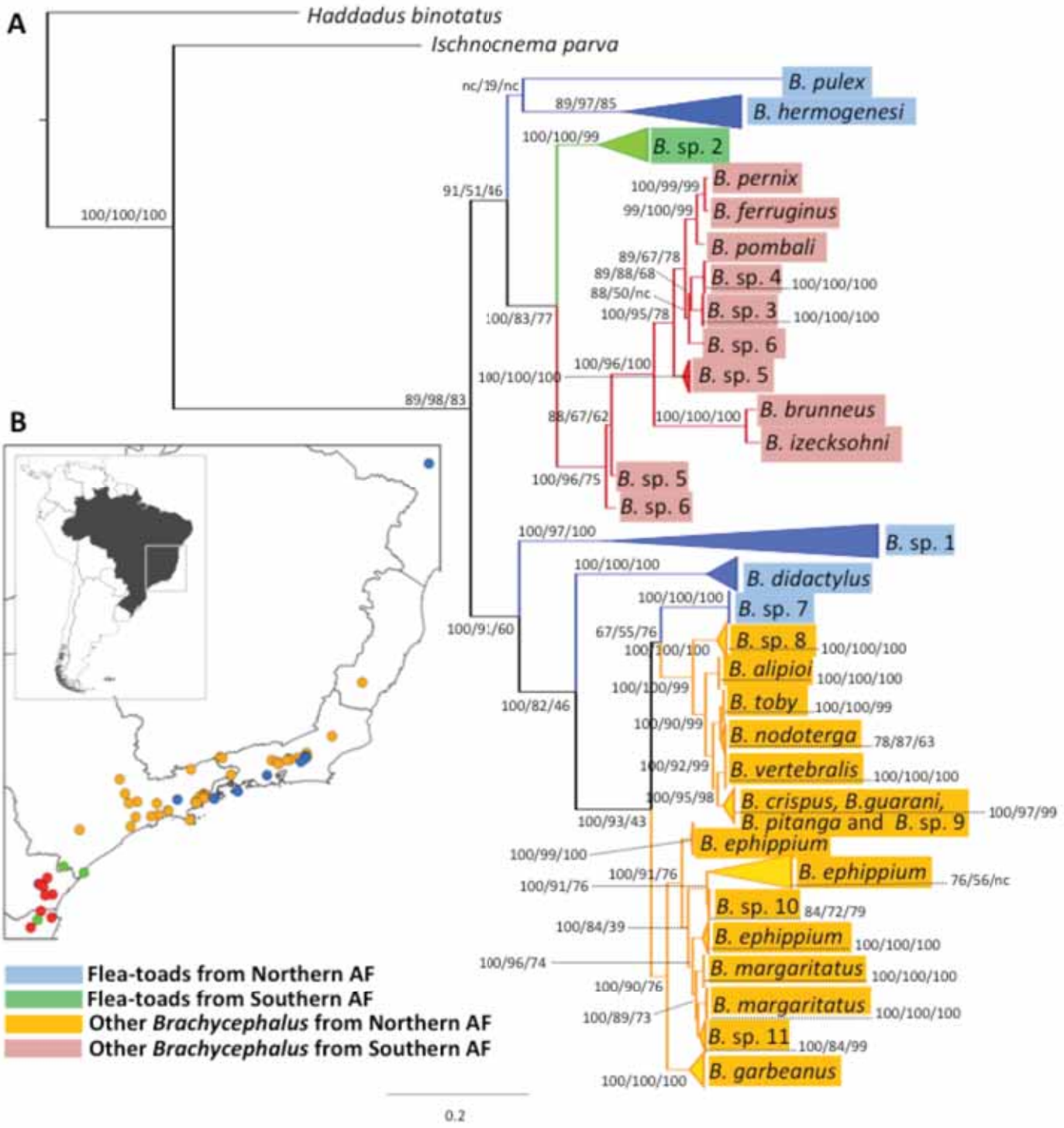

Figure 1. Phylogenetic relationships of *Brachycephalus* and spatial distribution of flea-toads and other species within the genus. A. Maximum Likelihood topology of concatenated mitochondrial fragments (12S, 16S and Cytb) with node support values displayed as (BI/ML/MP); Bayesian posterior probabilities (BI ≥ 0.90); Maximum likelihood bootstrap support (ML ≥ 70%) and Maximum Parsimony Jacknife support (MP ≥ 70%); nc = no such clade by this criterion. B. Spatial distribution of flea-toads and all other *Brachycephalus* species along Brazilian coast, considering northern and southern clades.

The topology did not show any evidence of distinction for the flea-toads, until recently allocated into the genus *Psyllophryne*, and all other *Brachycephalus* species. Except for *B. pulex* and *B. hermogenesi*, species for which the relationships were not clear, the first divergence in the topology refer to the division in two well-supported clades corresponding to northern and southern portion of AF (Figure 1). The northern clade is composed by four well-supported clades: first comprising the new species *B.* sp. 1; second comprising the flea-toad *B. didactylus*; third comprising the intermediate hyperossified skull and skeleton species *B. alipioi*, *B. crispus*, *B. guarani*, *B. nodoterga*, *B. pitanga*, *B. toby*, *B. vertebralis*, *B.* sp. 8 and *B.* sp. 9; and fourth comprising the extreme hyperossified species *B. ephippium*, *B. garbeanus*, *B. margaritatus*, *B.* sp. 10, and B. sp. 11. The relationship of the new species *B.* sp. 7 and the other species within the northern clade is not defined. Within the clade of species with intermediate hyperossified skull and skeleton the relationships among *B. crispus*, *B. guarani, B. pitanga*, and *B.* sp. 9 were not clearly resolved. The southern clade is composed by two well-supported clades: one comprising the flea-toads from the new species *B.* sp. 2, and other comprising the species *B. ferruginus*, *B. pernix*, *B. pombali*, *B. izecksohni*, *B. brunneus*, and the undescribed species *B.* sp. 3, *B.* sp. 4, *B.* sp. 5, and *B.* sp. 6. The relationship of *B.* sp. 5 and *B.* sp. 6 with the other species within this clade is not defined.

Estimates of divergence times

The BEAST topology was very similar to BI, ML, and MP trees (Figure 2). The positions of *Brachycephalus hermogenesi* and *B.* sp. 7 were the only with low node support values; all other relationships were equally recovered from Maximum Likelihood topology. The TMRCA of *Brachycephalus* was estimated to 40.5 Ma (95% Confidence Interval 27.3-54.5 Ma). Divergence between the northern and southern clades was estimated to have occurred in 28.7 Ma (95% Confidence Interval 19.2-39.7 Ma), at the Oligocene, end of the early Cenozoic. Despite of internal clade differences, northern and southern clades were supposed to diverge almost at the same time, at the Miocene, middle of Cenozoic: 14.9 Ma (95% Confidence Interval 9.7-21.1 Ma) and 15.5 Ma (95% Confidence Interval 10.3-22.0 Ma), respectively. One interesting result is that the deep divergences within each one of these clades correspond to nodes in which a flea-toad species is the oldest (*B.* sp. 2 in southern clade and *B. didactylus* in northern clade). In southern clade, the most recent species were supposed to have diverged at 2.0-0.2 Ma at Pleistocene, late Cenozoic.

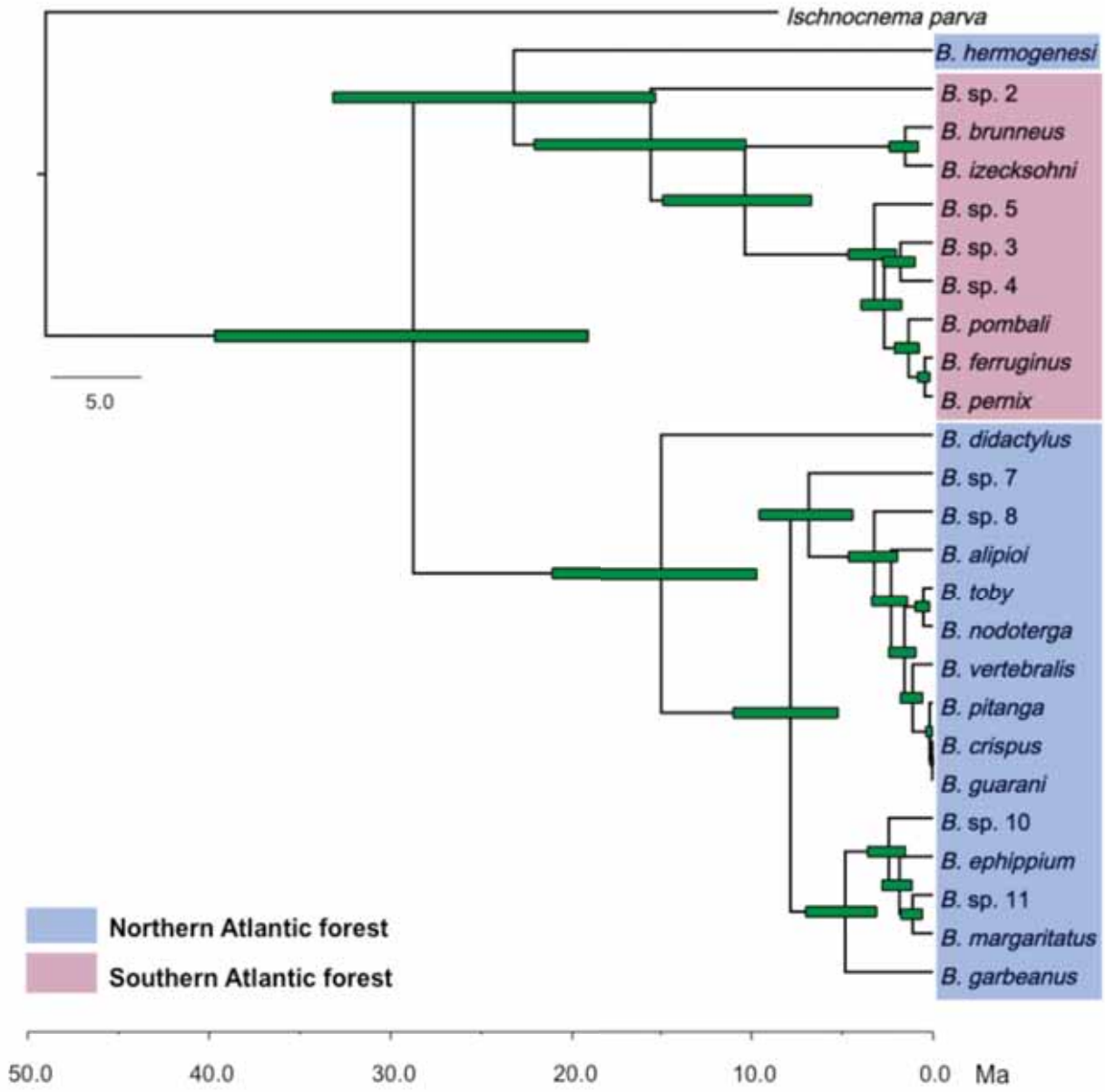

Figure 2. Divergence time estimation for *Brachycephalus* based on a strict molecular clock using BEAST. Boxes indicate the 95% confidence interval of the divergence time. Colors correspond to the northern and southern AF clades defined by the Guapiara lineament barrier (see Figure 3).

In northern clade, the divergence between the main internal nodes, corresponding to the intermediate and extreme condition of hyperossification of skull and skeleton was estimated to have occurred in 7.8 Ma (95% Confidence Interval 5.2-11.0 Ma), at the end of Miocene. Most recent evolution of the genus was estimated to have occurred in this clade at middle/upper Pleistocene, 0.1 Ma. It corresponds to *B. crispus*, *B. guarani*, *B. pitanga*, and *B.* sp. 9, the same species for which our phylogenetic reconstructions failed to resolve interspecific relationships.

Isolation by distance, barriers, and forest stability

The Mantel test indicated a significant association between genetic and geographical distances, considering the three mtDNA fragments (12S, $p < 0.01$, $r = 0.22$; 16S, $p < 0.01$, $r = 0.15$; Cytb, $p < 0.01$, $r = 0.15$). The isolation by distance corroborates the genetic structure and barriers proposed by BARRIER (Figure 3). The same five barriers were indicated by the analysis performed with each one of the three mtDNA fragments: A. Guapiara lineament barrier, splitting species from northern and southern AF clades; B. São Paulo and Rio de Janeiro barrier, splitting species from Serra do Mar mountain range (for example the flea-toads *Brachycephalus hermogenesi* from São Paulo and *B. didactylus* from Rio de Janeiro); C. Santa Maria Madalena barrier, splitting *B.* sp. 1 and *B.* sp. 8 from this locality and all others from Rio de Janeiro; D. Espírito Santo barrier, splitting the species *B. alipioi* from other species of *Brachycephalus*; and E. Southern Bahia barrier, splitting the species *B. pulex* (Camacan, Bahia state) from *B. alipioi* (Vargem Alta, Espírito Santo state). All identified barriers are correlated to physical elements that might be involved in *Brachycephalus* diversification, for example the Guapiara lineament (barrier A) between São Paulo and Paraná states, the forest discontinuity between São Paulo and Rio de Janeiro (barrier B), the Serra do Mar mountain range valleys in Rio de Janeiro (barrier C), the forest discontinuity between Espírito Santo and Rio de Janeiro states (barrier D) and the Doce river (barrier E), between Espírito Santo and Bahia states. Especially because of the deep divergences between northern and southern clades of *Brachycephalus*, the Guapiara lineament is supposed to be the most important physical barrier (Figures 2 and 3).

As we used mtDNA clades to search for the barriers, we also conducted AMOVA with this clusters of populations in order to evaluate genetic structure related to the geographic constraints. The results of AMOVAs were similar for the three mtDNA fragments according to genetic structure proposed (Table 2). The analysis comparing all populations revealed that more than 94% of the molecular variance existed among groups, whereas less than 1.4% was within populations. When the genetic structure was defined by mtDNA clades the molecular variance among groups was lower, 79% for 12S, 77% for 16S, and 78% for Cytb, whereas about 20% of molecular variance was related to differences among populations within groups and less than 5% was related to differences within populations. These results suggest that *Brachycephalus* populations are strongly structured. Due to this, the attempt to cluster populations in major groups, such as mtDNA clades or groups of populations isolated in one side of a geographic barrier, could result in less structured clusters of populations, in which the highest molecular variance should be found inside the groups and not among them.

Nevertheless, the mtDNA clade structure for *Brachycephalus* still indicates higher portion of genetic variance among groups, confirming the geographic barriers importance to genetic structure in *Brachycephalus*.

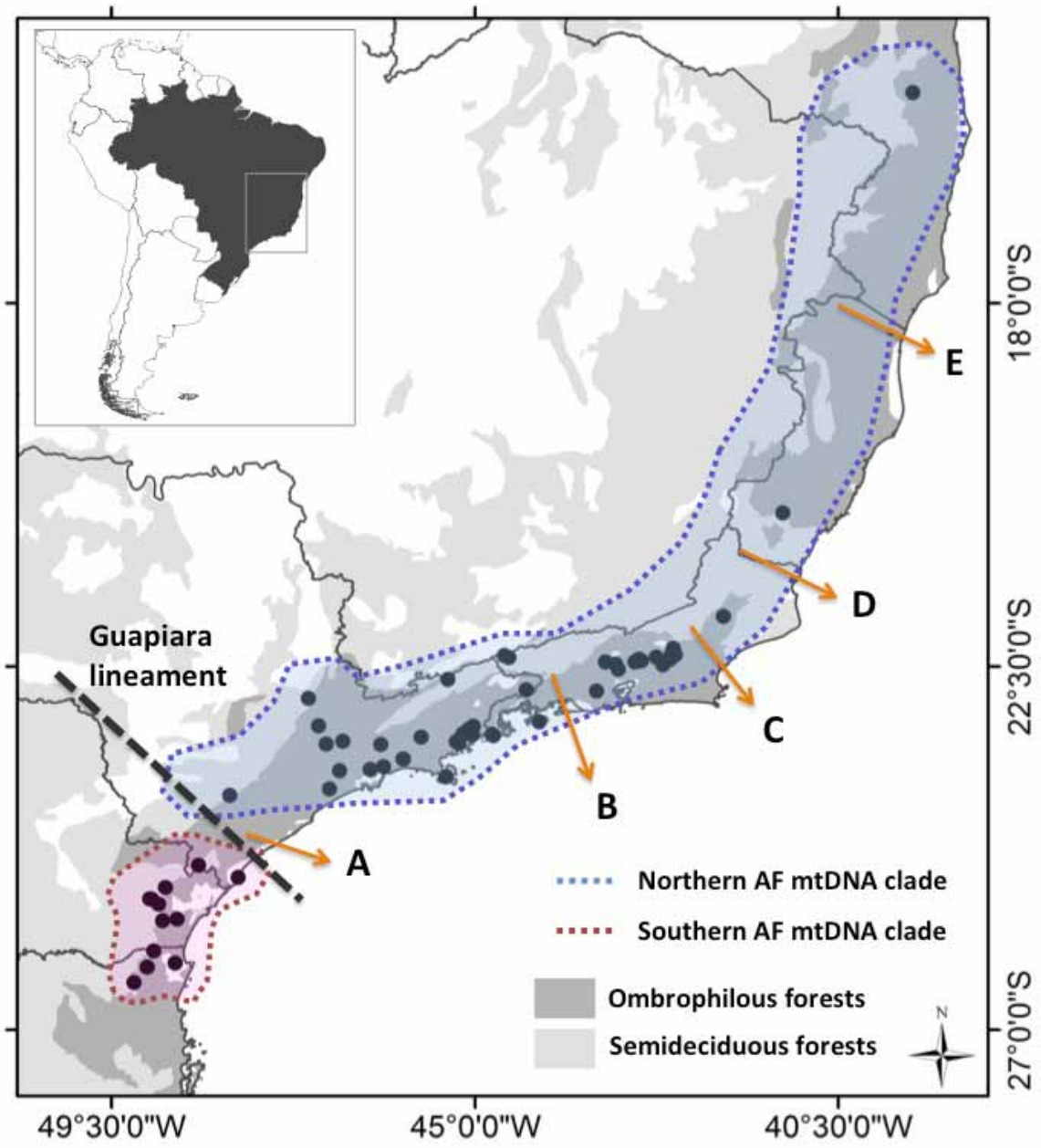

Figure 3. Spatial delimitation of southern and northern mtDNA clades of *Brachycephalus* along the Atlantic forest distribution. Orange arrows refer to genetic and geographic barriers identified by BARRIER: A. Guapiara lineament barrier; B. São Paulo and Rio de Janeiro barrier; C. Santa Maria Madalena barrier; D. Espírito Santo barrier; E. Southern Bahia barrier.

Table 2. Analysis of molecular variance (AMOVA) for *Brachycephalus* using three mtDNA fragments 12S, 16S and Cytb. Analyses were performed considering three distinct genetic structures: (1) all populations individually structured; (2) mtDNA clusters of populations; and (3) two groups composed by the northern and southern AF clades.

mtDNA	Genetic structure	Source of variation	*df*	Sum of squares	Variation (%)	Statistics	P-value
12S	(1) All populations	Among groups	56	6560.95	97.65	$F_{ST} = 0.97$	< 0.01
		Within populations	127	111.89	2.35	-	-
	(2) mtDNA clades	Among groups	10	5378.8	79.98	$F_{CT} = 0.79$	< 0.01
		Among populations within groups	46	1182.15	18.05	$F_{ST} = 0.98$	< 0.01
		Within populations	127	111.89	1.97	$F_{SC} = 0.90$	< 0.01
	(3) Northern/Southern AF	Among groups	1	3060.93	63.86	$F_{CT} = 0.63$	< 0.01
		Among populations within groups	55	3500.02	34.63	$F_{ST} = 0.98$	< 0.01
		Within populations	127	111.89	1.51	$F_{SC} = 0.95$	< 0.01
16S	(1) All populations	Among groups	57	11077.86	94.24	$F_{ST} = 0.94$	< 0.01
		Within populations	135	480.23	3.55	-	-
	(2) mtDNA clades	Among groups	10	9102.85	77.26	$F_{CT} = 0.77$	< 0.01
		Among populations within groups	47	1975.01	17.78	$F_{ST} = 0.95$	< 0.01
		Within populations	135	480.23	4.96	$F_{SC} = 0.78$	< 0.01
	(3) Northern/Southern AF	Among groups	1	3839.62	51.92	$F_{CT} = 0.51$	< 0.01
		Among populations within groups	56	7238.24	44.09	$F_{ST} = 0.96$	< 0.01
		Within populations	135	480.23	4	$F_{SC} = 0.91$	< 0.01
Cytb	(1) All populations	Among groups	52	8878.71	98.52	$F_{ST} = 0.98$	< 0.01
		Within populations	147	100.79	1.48	-	-
	(2) mtDNA clades	Among groups	8	6962.26	78.74	$F_{CT} = 0.78$	< 0.01
		Among populations within groups	44	1916.44	20.08	$F_{ST} = 0.98$	< 0.01
		Within populations	147	100.79	1.17	$F_{SC} = 0.94$	< 0.01
	(3) Northern/Southern AF	Among groups	1	2906.52	56.48	$F_{CT} = 0.56$	< 0.01
		Among populations within groups	51	5972.18	42.6	$F_{ST} = 0.99$	< 0.01
		Within populations	147	100.79	0.92	$F_{SC} = 0.97$	< 0.01

The importance of AF areas of historical stability in the maintenance of genetic diversity of *Brachycephalus* was confirmed by the correlation between 12S haplotype diversity (*Hd*) and historical forest stability ($p = 0.04$, $R^2 = 0.12$, $F = 4.57$, $df = 32$; Figure 4).

Nucleotide diversity (*Nd*) and *Theta* π indices presented marginal significative correlation with forest stability (p = 0.07, respectively) for the same mtDNA fragment. All other genetic indices for 16S and Cytb were not significantly correlated with forest stability. According to literature, geographic range of *Brachycephalus* overlaps three putative areas of historical stability: the Bahia refuge proposed by Carnaval & Moritz (2008), the Southeastern refuge, and the Serra do Mar refuge, proposed by Thomé et al. (2010). Most populations of *Brachycephalus* overlap these three areas, with just one area of high genetic diversity not coincident, located in São Paulo and Rio de Janeiro boundary (Figure 5). The forest stability layer revealed also three areas of historical stability in AF, but the limits are not the same predicted by previous studies. Our findings highlight a large stable forest region in the southern AF, from south of São Paulo to north of Santa Catarina states (Figure 5). Despite of overlapping with the refuges proposed, areas with high genetic diversity in *Brachycephalus* are widespread along the AF, mainly corresponding to high elevation areas. So, multiple and small historical stable areas might be responsible for the *Brachycephalus* diversity, rather than few large refuges.

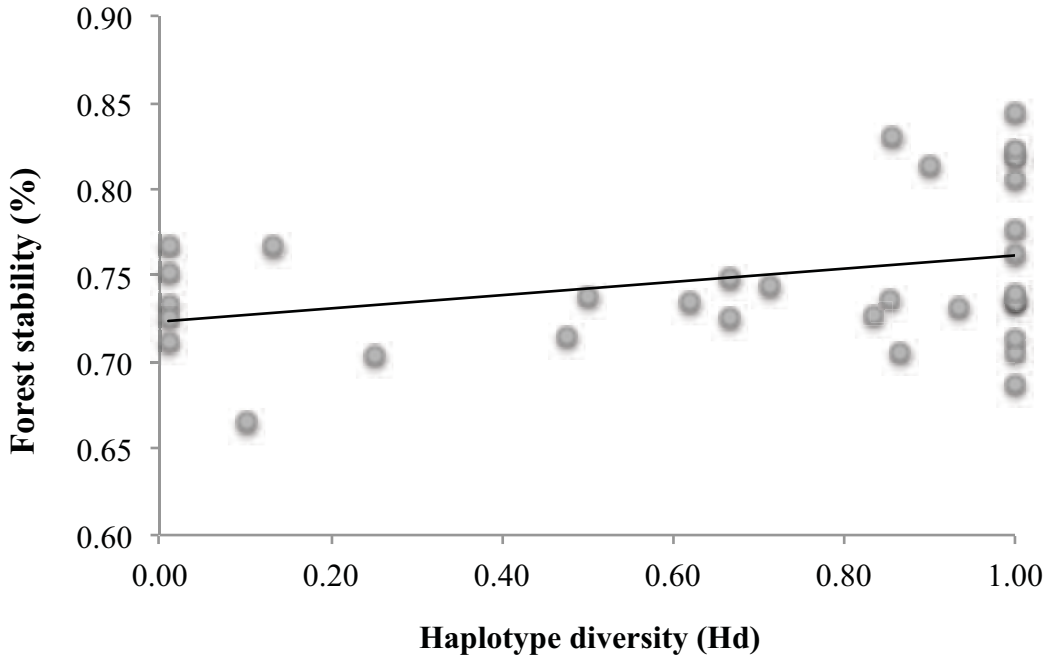

Figure 4. Relationship between 12S haplotype diversity and forest stability over the past 100.000 years for *Brachycephalus* species.

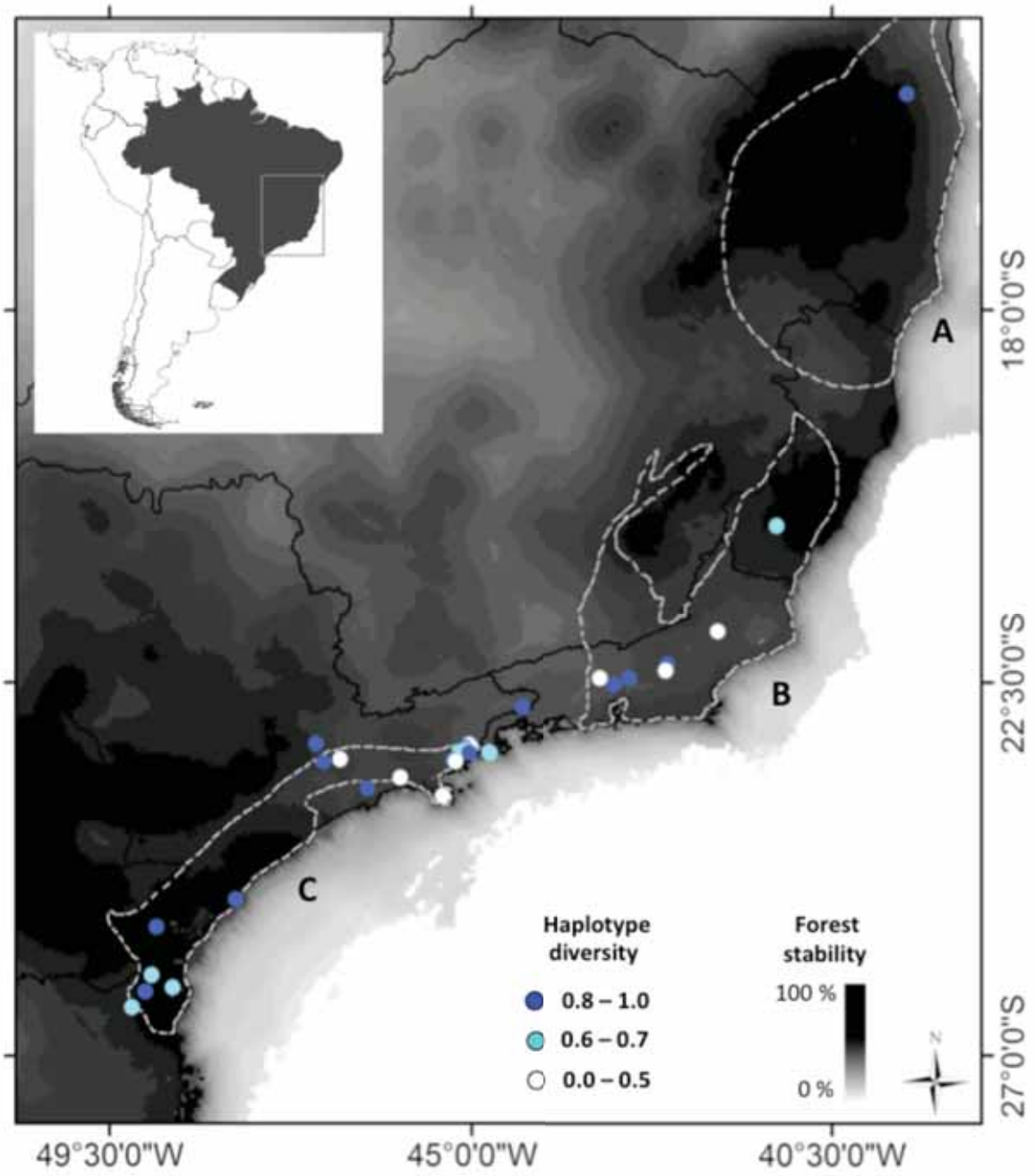

Figure 5. Genetic diversity of *Brachycephalus*, Atlantic forest stability, and putative refuges for the region, according to the literature. Haplotype diversity refers to 12S data. More stable areas over the past 100.000 years were represented in black. Dashed lines represent adapted limits for: A. Bahia refuge proposed by Carnaval & Moritz (2008), B. Southeastern refuge and C. Serra do Mar refuge proposed by Thomé et al. (2010).

Latitudinal gradient of diversification

As reported before, the latitudinal divergent mtDNA clades of *Brachycephalus* and the Guapiara lineament barrier corroborate the differences between northern and southern *Brachycephalus* populations. Despite of the low molecular variance attributed to this groups

(63%, 51%, and 56%, respectively for 12S, 16S, and Cytb), the major portion of genetic variance is found among groups and not among population within groups or within populations (Table 2). In the same way with mtDNA clades genetic structure, AMOVA confirm statistically significant genetic variation between northern and southern groups of *Brachycephalus*.

The northern AF clade presented larger number of haplotypes (*H*) and polymorphic sites (*S*), higher haplotype diversity (*Hd*), nucleotide diversity (*Nd*), and *Theta π* (Table 1). These results could be related to the larger geographic extension and larger number of samples in northern AF, but even adjusting the sample sizes the rarefaction analysis for 12S resulted in larger number of haplotypes in northern AF (H = 53, range = 50-55, 1000 randomizations of 56 samples) compared to southern AF (H = 18). For other mtDNA fragments the rarefaction analysis showed the same results: 16S, northern AF (H = 47, range 43-52, 1000 randomizations of 53 samples) and southern AF (H = 28), and Cytb, northern AF (H = 14, range 10-20, 1000 randomizations of 37 samples) and southern AF (H = 12). These results confirm that the genetic diversity in *Brachycephalus* is higher in northern AF.

The spatial resistances to gene flow were completely different in northern and southern AF (Table 3). In southern AF only the resistance to topographic complexity was correlated with F_{ST} (for Cytb p = 0.04, r = 0.270). In northern AF all climate and landscape resistances were correlated with F_{ST} index, considering the three mtDNA fragments (Table 3). The high *Mantel* coefficients *(r)* in northern AF were related to resistance to annual mean temperature, annual temperature range, forest instability, and altitude.

Discussion

Mitochondrial markers have been successfully used in phylogeographic studies, mainly due to the matrilineal inheritance, simple genetic structure, general low rates of recombination, relatively rapid rates of evolution and intrinsic sensitivity to detect population level processes (MORITZ et al., 1987; AVISE, 2000; RUBINOFF & HOLLAND, 2005). Even so, a multilocus approach is strongly recommended rather than a single data source to taxonomy and phylogenetic reconstructions (AVISE & WOLLENBERG, 1997), especially because mtDNA information could be confounded by introgression and the phylogenetic signal could be diminished (MORITZ et al., 1987; RUBINOFF & HOLLAND, 2005). In this study, we used mtDNA because our main goals were related to population evolutionary processes and we recognized that more inclusive data sets will led to an enhanced understanding of *Brachycephalus* phylogenetic relationships.

Table 3. Results of partial Mantel tests used to evaluate the relationship between pairwise genetic structure of *Brachycephalus* (F_{ST}) and models of climate and landscape spatial resistances in southern and northern AF. All models were controlled by geographic distance (Euclidean distance). *statistically significant results and **marginally significant results from $p < 0.05$.

mtDNA	Spatial resistance	Southern AF		Northern AF	
		p-valor	*Mantel (r)*	*p-valor*	*Mantel (r)*
12S	Altitude	0.88	-0.175	< 0.01*	0.193
	Annual mean temperature	0.88	-0.172	< 0.01*	0.236
	Annual temperature range	0.87	-0.152	< 0.01*	0.206
	Annual precipitation	0.7	-0.064	0.03*	0.138
	Topographic complexity	0.07**	0.201	0.10	0.099
	Vegetation density	0.92	-0.225	0.04*	0.108
	Forest instability	0.57	-0.047	0.02*	0.126
16S	Altitude	0.89	-0.161	0.01*	0.156
	Annual mean temperature	0.91	-0.176	0.01*	0.166
	Annual temperature range	0.93	-0.175	0.03*	0.151
	Annual precipitation	0.91	-0.169	0.33	0.041
	Topographic complexity	0.39	0.044	0.80	-0.095
	Vegetation density	0.94	-0.202	0.12	0.091
	Forest instability	0.61	-0.046	0.12	0.078
Cytb	Altitude	0.39	0.075	< 0.01*	0.177
	Annual mean temperature	0.37	0.078	< 0.01*	0.316
	Annual temperature range	0.31	0.082	0.27	0.025
	Annual precipitation	0.67	-0.041	< 0.01*	0.171
	Topographic complexity	0.04*	0.270	< 0.01*	0.160
	Vegetation density	0.48	0.024	< 0.01*	0.121
	Forest instability	0.28	0.080	< 0.01*	0.293

In spite of this, the mtDNA topology recovered the monophyly of *Brachycephalus*, the synonymy of the flea-toads, until recently allocated into the no longer recognized genus *Psyllophryne* (KAPLAN, 2002), and the main clades evidenced in the previous phylogenetic hypotheses (CLEMENTE-CARVALHO et al., 2011). Our results also provided the most complete sampling for this genus and constituted the base for this preliminary phylogeographic approach.

Populations of *Brachycephalus* presented high levels of genetic structure and mtDNA haplotypes were only shared across populations in *B. ephippium*, *B. garbeanus*, *B. nodoterga*, and *B*. sp. 2. These species, along with *B. brunneus, B. didactylus, B. hermogenesi*, and *B. margaritatus*, presented extended geographic ranges occurring in more than three localities (FROST, 2014). Exceptionally, we found shared mtDNA haplotypes among individuals from

the distinct species *B. crispus*, *B. guarani*, *B. pitanga*, and *B.* sp. 9. Despite of morphological evidence for the recognition of these species, our phylogenetic analysis of mtDNA haplotypes did not recovered monophyletic groups for each one of them. These species occur in nearby populations at Serra do Mar mountain range and constitute recently diverged taxa, according to our divergence time estimations. Possible explanations for shared mtDNA haplotypes, in this case, could be attributed to interspecific hybridization mediated by mitochondrial introgression or incomplete lineage sorting (e.g. SEQUEIRA et al., 2011), for example when mtDNA phylogeny of recently diverged species was affected by species physical contact or retained ancestral polymorphism (MORITZ et al., 1987). One unexpected result is that the three mtDNA fragments behave in different ways; Cytb haplotypes were shared among all of these species, while 12S haplotypes were shared only between *B. crispus* and *B. guarani*, and 16S haplotypes were not shared. If we consider the whole capture of mitochondrial genome by introgression, these lineages should present shared haplotypes from all mtDNA fragments (e.g. MELO-FERREIRA et al., 2005). We attribute these differences to 12S and 16S sequence alignment, which included several gaps sites and consequently might be overestimating the haplotypic variation (101 and 102 unique haplotypes, respectively for 12S and 16S, compared to Cytb, with 31 unique haplotypes). Once this level of phylogenetic resolution was not the main issue of this study, we just raised the question and propose that these species relationships must be properly investigated with contact-zone samplings and other sensitive molecular markers.

Brachycephalus species with restricted distribution in high elevation sites along the AF represent more than 85% of the currently known generic diversity (FROST, 2014). Generally, these species are known only from the type locality and its surroundings, being considered as microendemic (PIE et al., 2013). Moreover, the miniaturized body size of *Brachycephalus* reinforces its limited dispersal capability, a common situation in amphibians due to the limited osmotic tolerances and high levels of philopatry (VENCES & WAKE, 2007). Due to the complex mountain topography and climate variation in different altitudes, this disjunct distribution in mountainous regions allied to limited dispersal capability could facilitate genetic isolation among populations and support high levels of species diversification (GUARNIZO & CANATELLA, 2013). As predicted, we found high levels of genetic diversity among populations of *Brachycephalus* and our data also revealed a significant isolation by distance. This vicariant pattern suggests the influence of physical or climatic barriers determining the genetic structure of these populations. Physical barriers, such as rivers and mountains, and the gradient of climatic and ecological conditions in

montane regions, can represent important mechanisms involved in the interruption of gene flow and population divergences (JANZEN, 1967; GHALAMBOR et al., 2006; TURCHETTO-ZOLET et al., 2013).

Population clusters identified by BARRIERS presented genetic structure and were isolated from each other by five main geographic barriers, coincident with rivers, forest discontinuity, mountains, valleys, and tectonic faults. As the Monmonier algorithm identifies the largest differences between pairs of populations, most of the proposed geographic barriers correspond to barriers between two highly divergent populations (MANNI & GÉRARD, 2004), not necessarily correlated to the main divergences identified in mtDNA topology. Considering this, we compared proposed barriers with the deep divergences between mtDNA clades of *Brachycephalus* and then considered the Guapiara lineament as the most important geographic barrier to *Brachycephalus*. The Guapiara lineament is an ancient complex system of rifted blocks and tectonic faults mainly active during late Mesozoic (ALMEIDA & CARNEIRO, 1998; SAADI et al., 2002). These faults are coincident with one important interruption of Serra do Mar mountain range resulted from Ponta Grossa Arch uplift and the erosive action of Ribeira de Iguape river (ALMEIDA & CARNEIRO, 1998; RIBEIRO, 2006). Currently, the most interior configuration of the Serra do Mar along this extension can be attributed to the Ribeira de Iguape valley, embedded in the highly resistant escarpments of Paranapiacaba and Paraná (ALMEIDA & CARNEIRO, 1998). The opening of the Ribeira de Iguape valley is not precisely dated, but some estimates based on sedimentary deposits in grabens of Cananéia and Sete Barras suggested origin between Eocene and Miocene, about 56-13 Ma (ALMEIDA & CARNEIRO, 1998; RIBEIRO, 2006). The Pariquera-Açu deposits in Ribeira de Iguape river have been dated to the mid-Miocene, though this estimation is considered doubtful and very recent (ALMEIDA & CARNEIRO, 1998).

The divergence between *Brachycephalus* and the sister taxon *Ischnocnema* was estimated to have occurred in the early Cenozoic (HEINICKE et al., 2009). Sometime between 30-40 Ma the Serra do Mar mountain range has returned to the current position by tectonic, rivers, and ocean actions (ALMEIDA & CARNEIRO, 1998). Based on this information and our dating estimations, we suppose that right after this event, in mid-Eocene (40.5 Ma), *Brachycephalus* and *Ischnocnema* diverged (95% Confidence Interval 27.3-54.5 Ma). After that, divergence between the northern and southern clades in *Brachycephalus* was supposed to have occurred around 28.7 Ma (95% Confidence Interval 19.2-39.7 Ma), overlapping the age of Ribeira de Iguape valley formation. Thus, despite recognizing the uncertainty associated to the use of molecular clocks to species divergence dating, we

confirmed our expectations of the influence of Guapiara lineament in the deep *Brachycephalus* diversification.

The Guapiara lineament also coincides with phylogeographic breaks for several vertebrate species in the AF (GRAZZIOTIN et al., 2006; BRUNES et al., 2010; THOMÉ et al., 2010; RAPOSO et al., 2013). However, *Brachycephalus* divergence is the oldest reported (TURCHETTO-ZOLET et al., 2013), especially when compared to divergence estimation between northern and southern clades in *Bothrops jararaca* (GRAZZIOTIN et al., 2006), *Phyllomedusa burmeisteri* species group (BRUNES et al., 2010), and *Rhinella crucifer* species group (THOMÉ et al., 2010), assigned to Pliocene and Pleistocene, more recent than 5 Ma. These studies focused on one species or a group of related species, generally widespread distributed in AF. Our data set comprises several species of microendemic or restrict distributed amphibians, which also are montane specialists. Estimated divergences for the montane species *Proceratophrys boiei* are assigned to late Miocene, about 4.5-11.7 Ma (7.8 in average, AMARO et al., 2012), which suggest older and distinct diversification process in montane associated species. Estimated divergences for species with restricted distributions are even older, such as exemplified by the Amazonian terrestrial leaf-litter frog *Pristimantis ockendeni*, which presents lineage splitting dated to the mid-Miocene to Oligocene (20–25 Ma; ELMER et al., 2007).

The most recent divergences of *Brachycephalus* mtDNA topology were estimated to occur in the Miocene until the middle/upper Pleistocene. Diversification within northern and southern clades of *Brachycephalus* was estimated to have occurred at the same time, at the Miocene. This result suggests a widespread flea-toad ancestor, which probably differentiated into new species simultaneously in northern and southern AF. Shallower nodes of mtDNA topology occur in both northern and southern clades. During Miocene, until early Pliocene, warm and humid climate conditions were present in the Brazilian coast (VASCONCELOS et al., 1992; LARA & PATTON, 2000). Also, the Miocene was represented by intense tectonic activity and reactivation of faults, in which rocks less resistant to erosion constituted deformities in relief and drove the drainage patterns (ALMEIDA & CARNEIRO, 1998; RIBEIRO, 2006). The old faults from the end of Cretaceous were reactivated also in Tertiary and Quaternary and may have impacted the diversification processes of AF until recently (RIBEIRO, 2006; LARA & PATTON, 2000; BRUNES et al., 2010). The Pliocene and Pleistocene were characterized by paleoclimate variation arising from the cyclical alternation of glacial and interglacial periods accompanied by marine transgressions and regressions (OLIVEIRA et al., 2005; SUGUIO et al., 2005). At this time, the Pleistocene refuges were

supposed to be more stable areas responsible to maintain diversity (AB'SABER, 1977; BROWN & AB'SABER, 1979; CARNAVAL et al., 2009). The dryer climate regimes probably affected severely the lower and flatter areas, in which forest was substituted by open formations (BROWN & AB'SABER, 1979). At the Brazilian coast, humid forests became restricted to topographic enclaves and species occupying montane forests were probably less affected by Last Glacial Maximum conditions relative to lowland specialists (BROWN & AB'SABER, 1979; AMARO et al., 2012).

Brachycephalus current distribution matches the literature predicted historical stable areas in AF (CARNAVAL & MORITZ, 2008; THOMÉ et al., 2010; AMARO et al., 2012) and our results also indicated a historically stable region in southern AF. Positive correlation of genetic diversity and forest stability might be related to the restricted distribution of *Brachycephalus* species, mainly associated with high elevation sites along the AF. We suggest the existence of multiple refuges along the Brazilian AF instead of the prevalence of large northern refuges, as proposed by (CARNAVAL & MORITZ, 2008; CARNAVAL et al., 2009), corroborating other phylogeographic studies for the same region (AMARO et al., 2012). Additionally, the northern and southern simultaneous divergence times and widespread genetic diversity of *Brachycephalus* suggested that the diversification in this genus might be influenced by several small historical areas of stability along its distribution, defined as 'microrefuges' (TURCHETTO-ZOLET et al., 2013).

In the northern AF the genus *Brachycephalus* has high genetic diversity and we attribute this result to a latitudinal gradient of species diversification. The same pattern was observed for birds along AF (D'HORTA et al., 2011). Rather than only accumulate diversity due to paleoclimatic stability we argue that northern AF could present also higher species diversification, mainly related to the restricted physiologic tolerances of tropical species to disperse over climatic and environmental gradients (e.g. SCHNEIDER et al., 1999; MORITZ et al., 2000). Despite of being not considered an intentional approach to evaluate tropical species diversity, Janzen hypothesis of effective topographic barriers in the latitudinal gradient led directly to the prediction that species associated to high elevation will experience reduced gene flow, greater genetic divergence, and increased allopatric speciation in the tropics (JANZEN, 1967; GHALAMBOR et al., 2006). According to these predictions, we found that northern AF climate and landscape variation offered higher resistance to gene flow among *Brachycephalus* populations than in southern AF. In tropical AF all tested climate and landscape gradients were efficient barriers to *Brachycephalus* gene flow. On the other hand, gene flow in subtropical AF populations of *Brachycephalus* was just influenced by the

topographic complexity gradient. As *Brachycephalus* species were mainly associated to montane regions, it is reasonable to consider that topographic complexity gradient had an important influence in reproductive isolation along overall species distribution. The complex mountain topography exhibits an outstanding climate variation, which facilitates genetic isolation among populations and consecutively increases the probability of speciation (GUARNIZO & CANATELLA, 2013). Also, not considering the divergent climate conditions, the northern and southern AF mountain ranges are very similar in geology and relief complexity (ALMEIDA & CARNEIRO, 1998).

In northern AF, the reproductive isolation in *Brachycephalus* populations was also related to elevation, temperature, temperature fluctuation, precipitation, vegetation density, and forest instability. This result suggests that populations of *Brachycephalus* at highlands in northern AF exhibit narrow tolerances to high temperatures and low precipitation, which generally increase when elevation decreases (OLIVEIRA-FILHO & FONTES, 2000). The forest stability layer used in our analysis recovered historical stable areas during the Quaternary, representing only the recent past of AF. Even though, the last 100.000 years were marked by climatic changes that had severe impacts on AF distribution (LEDRU et al., 2005). We believe that northern AF *Brachycephalus* species, living in the tropical climate, were more susceptible to past climatic changes than colder adapted southern AF species. Because of this, the forest refuges might be more important to predict population connectivity in northern region. Our expectations followed the conclusions of Amaro et al. (2012), in which persistence in refuges might be more important to highland than lowland adapted species, mainly due to the tolerance to colder climates by the former. Currently, natural habitat fragmentation is an important concern to AF species conservation (RIBEIRO et al., 2009; BECKER et al., 2010). Our results evidenced that *Brachycephalus* distribution is deeply correlated with forested areas. The *Brachycephalus* reproductive isolation in northern populations increases as vegetation density in the surroundings decreases, indicating that these species are sensitive to landscape changes.

The *Brachycephalus* diversity is increasing considerably due to the herpetological surveys in high elevation AF (CLEMENTE-CARVALHO et al., 2012; GAREY et al., 2012; CONDEZ et al., 2014). Today, the northern AF harbors more *Brachycephalus* species than southern AF, besides the greater genetic diversity. Clearly, the AF tropical extension is much larger than the subtropical one and the area size is an important signal to be evaluated in diversity gradients (ROZENWEIG, 1995; ROZENWEIG & SADLIN, 1997). Since the ideas of Wallace (1876), ecologists and biogeographers consider the spatial and temporal

hypotheses that the tropical systems are larger in a realistic subdivision of the world (ROZENWEIG & SADLIN, 1997), and could have accumulated species over a longer period of time than temperate regions (MITTELBACH et al., 2007; WIENS, 2007). Great evidence of this, for example in hylids, is that older clades are present in tropical regions, whereas temperate faunas are dominated by younger clades (WIENS et al., 2006). We do not have enough evidences to infer that *Brachycephalus* originated at northern AF, according to the comparable depth of northern and southern mtDNA clades. Even so, the northern AF exhibits the major number of clades and the major genetic diversity within the genus.

The evaluation of contemporary and historical mechanisms involved in species diversification is essential to the comprehension of spatial distribution and evolutionary processes (GRAHAM et al., 2006). The climatic and landscape stratification in high elevation Atlantic forest notably have influenced spatial and temporal distribution of species and their evolutionary history. We contribute to the current knowledge of the of Atlantic forest species diversification by describing some aspects of the evolutionary history of the high elevation endemic species of the genus *Brachycephalus*. The increasing interest in historical and current patterns of species diversity should lead us to a better comprehension of the evolutionary processes associated to the montane regions of the Atlantic forest.

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Appendix 1. Haplotype names, respective species, vouchers, locality, identification in the map (ID), geographic coordinates and information on 12S rRNA, 16S rRNA and Cytb sequences used in this study. Haplotypes with asterisk were not used in phylogenetic reconstructions. Voucher acronyms CFBH, MNRJ, and CTMZ refer to scientific collection material (see Material and Methods section) and A, ARI, and TC refer to personal data, not yet deposited in scientific collections. Population State abbreviations: Bahia (BA), Espírito Santo (ES), Rio de Janeiro (RJ), São Paulo (SP), Paraná (PR), and Santa Catarina (SC).

Haplotype	Species	Vouchers	Locality	ID	Latitude	Longitude	12S	16S	Cyt b
Hap_1	*B. pulex*	TC 191	Camacan, BA	1	-15.394196	-39.568729	x	x	
Hap_2	*B.* sp. 2	TC 267	Cananéia, SP	2	-25.114730	-47.927880	x	x	x
Hap_3	*B.* sp. 2	TC 268	Cananéia, SP	2	-25.114730	-47.927880	x	x	x
Hap_4	*B.* sp. 2	TC 141	Quatro Barras, PR	3	-25.380718	-49.023979	x	x	x
Hap_5	*B.* sp. 2	TC 234	Joinville, SC	4	-26.228500	-49.057400	x	x	x
Hap_6	*B.* sp. 2	TC 244	Joinville, SC	4	-26.228500	-49.057400	x	x	x
Hap_7	*B.* sp. 2	TC 244	Joinville, SC	4	-26.228500	-49.057400	x	x	x
Hap_8	*B.* sp. 2	TC 251	São Francisco do Sul, SC	5	-26.175250	-48.712250	x	x	x
Hap_9	*B.* sp. 2	TC 264	São Francisco do Sul, SC	5	-26.175250	-48.712250	x	x	x
Hap_10	*B.* sp. 2	TC 252	São Francisco do Sul, SC	5	-26.175250	-48.712250	x	x	x
Hap_11	*B.* sp. 2	TC 263	São Francisco do Sul, SC	5	-26.175250	-48.712250	x	x	x
Hap_12*	*B. ferruginus*	TC 142	Morretes, PR	6	-25.447554	-48.914320	x	x	
Hap_13*	*B. ferruginus*	TC 143	Morretes, PR	6	-25.447554	-48.914320	x	x	
Hap_14*	*B. ferruginus*	TC 144	Morretes, PR	6	-25.447554	-48.914320	x	x	
Hap_15	*B. ferruginus*	CFBH 8025	Morretes, PR	6	-25.447554	-48.914320	x	x	x
Hap_16	*B.* sp. 3	TC 206	Garuva, SC	7	-26.025500	-48.973260	x	x	x
Hap_17	*B.* sp. 3	TC 207, TC 211	Garuva, SC	7	-26.025500	-48.973260	x	x	x
Hap_18*	*B.* sp. 3	TC 201	Garuva, SC	7	-26.025500	-48.973260	x	x	
Hap_19	*B.* sp. 3	TC 202, TC 203, TC 208, TC 209	Garuva, SC	7	-26.025500	-48.973260	x	x	x
Hap_20	*B.* sp. 3	TC 204	Garuva, SC	7	-26.025500	-48.973260	x	x	x
Hap_21	*B.* sp. 3	TC 205	Garuva, SC	7	-26.025500	-48.973260	x	x	x
Hap_22	*B.* sp. 3	TC 210	Garuva, SC	7	-26.025500	-48.973260	x	x	x
Hap_23*	*B.* sp. 3	TC 212	Garuva, SC	7	-26.025500	-48.973260	x		x
Hap_24	*B.* sp. 3	TC 213	Garuva, SC	7	-26.025500	-48.973260	x	x	x
Hap_25*	*B.* sp. 3	TC 214	Garuva, SC	7	-26.025500	-48.973260	x	x	
Hap_26	*B.* sp. 3	TC 215	Garuva, SC	7	-26.025500	-48.973260	x	x	x
Hap_27*	*B.* sp. 6	TC 235, TC 242	Joinville, SC	4	-26.228500	-49.057400	x	x	
Hap_28	*B.* sp. 6	TC 236	Joinville, SC	4	-26.228500	-49.057400	x	x	x
Hap_29	*B.* sp. 6	TC 237	Joinville, SC	4	-26.228500	-49.057400	x	x	x
Hap_30*	*B.* sp. 6	TC 238	Joinville, SC	4	-26.228500	-49.057400	x	x	

Haplotype	Species	Vouchers	Locality	ID	Latitude	Longitude	12S	16S	Cyt b
Hap_31*	*B.* sp. 6	TC 239	Joinville, SC	4	-26.228500	-49.057400	x	x	
Hap_32*	*B.* sp. 6	TC 241	Joinville, SC	4	-26.228500	-49.057400	x	x	
Hap_33*	*B.* sp. 6	TC 243	Joinville, SC	4	-26.228500	-49.057400	x	x	
Hap_34	*B.* sp. 4	TC 246	Corupá, SC	8	-26.415320	-49.219460	x	x	x
Hap_35*	*B.* sp. 4	TC 247	Corupá, SC	8	-26.415320	-49.219460	x	x	
Hap_36	*B.* sp. 5	TC 248	Corupá, SC	8	-26.415320	-49.219460	x	x	x
Hap_37*	*B.* sp. 5	TC 253	São Francisco do Sul, SC	5	-26.175250	-48.712250	x	x	
Hap_38*	*B.* sp. 5	TC 254	São Francisco do Sul, SC	5	-26.175250	-48.712250	x	x	
Hap_39	*B.* sp. 5	TC 232	São Francisco do Sul, SC	5	-26.175250	-48.712250	x	x	x
Hap_40*	*B.* sp. 5	TC 233	São Francisco do Sul, SC	5	-26.175250	-48.712250	x	x	
Hap_41	*B.* sp. 5	TC 255	São Francisco do Sul, SC	5	-26.175250	-48.712250	x	x	x
Hap_42	*B.* sp. 5	TC 256	São Francisco do Sul, SC	5	-26.175250	-48.712250	x	x	x
Hap_43	*B.* sp. 5	TC 257	São Francisco do Sul, SC	5	-26.175250	-48.712250	x	x	x
Hap_44*	*B.* sp. 5	TC 258	São Francisco do Sul, SC	5	-26.175250	-48.712250	x		x
Hap_45*	*B.* sp. 5	TC 260	São Francisco do Sul, SC	5	-26.175250	-48.712250	x	x	
Hap_46	*B.* sp. 5	TC 261	São Francisco do Sul, SC	5	-26.175250	-48.712250	x	x	x
Hap_47	*B.* sp. 5	TC 262	São Francisco do Sul, SC	5	-26.175250	-48.712250	x	x	x
Hap_48	*B. brunneus*	CFBH 7905	Campina Grande do Sul, PR	9	-25.241142	-48.829437	x	x	x
Hap_49	*B. izecksohni*	CFBH 7374	Guaratuba e Paranaguá, PR	10	-25.631213	-48.686147	x	x	x
Hap_50	*B. pernix*	CFBH 2597	Quatro Barras, PR	11	-25.388208	-49.003107	x	x	x
Hap_51	*B. pombali*	CFBH 8043	Guaratuba, PR	12	-25.650416	-48.866030	x	x	x
Hap_52*	*B. hermogenesi*	ARI 625	Picinguaba, Ubatuba, SP	13	-23.357556	-44.767278	x		
Hap_53	*B.* sp. 8	CFBH 13724	Santa Maria Madalena, RJ	14	-21.880306	-41.916917	x	x	x
Hap_54	*B.* sp. 8	CFBH 13725	Santa Maria Madalena, RJ	14	-21.880306	-41.916917	x	x	x
Hap_55	*B.* sp. 8	CFBH 13726	Santa Maria Madalena, RJ	14	-21.880306	-41.916917	x	x	x
Hap_56	*B.* sp. 8	CFBH 13727	Santa Maria Madalena, RJ	14	-21.880306	-41.916917	x	x	x
Hap_57*	*B.* sp. 8	CFBH 13728	Santa Maria Madalena, RJ	14	-21.880306	-41.916917	x	x	
Hap_58	*B.* sp. 8	CFBH 13729	Santa Maria Madalena, RJ	14	-21.880306	-41.916917	x	x	x
Hap_59	*B.* sp. 8	CFBH 13730	Santa Maria Madalena, RJ	14	-21.880306	-41.916917	x	x	x
Hap_60	*B.* sp. 8	CFBH 13731	Santa Maria Madalena, RJ	14	-21.880306	-41.916917	x	x	x
Hap_61	*B.* sp. 8	CFBH 13732	Santa Maria Madalena, RJ	14	-21.880306	-41.916917	x	x	x
Hap_62	*B.* sp. 8	CFBH 13733	Santa Maria Madalena, RJ	14	-21.880306	-41.916917	x	x	x
Hap_63	*B.* sp. 8	CFBH 13721	Santa Maria Madalena, RJ	14	-21.880306	-41.916917	x	x	x
Hap_64	*B. ephippium*	CFBH 12355	PARNA Serra dos Órgãos, RJ	15	-22.448522	-43.013471	x	x	x

Haplotype	Species	Vouchers	Locality	ID	Latitude	Longitude	12S	16S	Cyt b
Hap_65	*B. ephippium*	CFBH 12358	PARNA Serra dos Órgãos, RJ	15	-22.448522	-43.013471	x	x	x
Hap_66	*B.* sp. 10	CFBH 14576	Mogi das Cruzes, SP	16	-23.746933	-46.128450	x	x	x
Hap_67	*B.* sp. 10	CFBH 14586	Paranapiacaba, SP	17	-23.779340	-46.294891	x	x	x
Hap_68	*B.* sp. 10	CFBH 12909	Paranapiacaba, SP	17	-23.779340	-46.294891	x	x	x
Hap_69	*B.* sp. 10	CFBH 12910	Paranapiacaba, SP	17	-23.779340	-46.294891	x	x	x
Hap_70	*B.* sp. 10	CFBH 12911	Paranapiacaba, SP	17	-23.779340	-46.294891	x	x	x
Hap_71	*B.* sp. 10	CFBH 12912	Paranapiacaba, SP	17	-23.779340	-46.294891	x	x	x
Hap_72	*B. ephippium*	MNRJ 78465	S. Alambari, Resende, RJ	18	-22.392212	-44.574285	x	x	x
Hap_73	*B. margaritatus*	CFBH 2420	Petrópolis, RJ	19	-22.475194	-43.253194	x	x	x
Hap_74	*B. margaritatus*	CFBH 2424	Petrópolis, RJ	19	-22.475194	-43.253194	x	x	x
Hap_75	*B. ephippium*	CFBH 2978	São Paulo, SP	20	-23.793028	-46.675256	x	x	x
Hap_76	*B. ephippium*	CFBH 31982	Guaratinguetá, SP	21	-22.662720	-45.331881	x	x	x
Hap_77	*B. ephippium*	CFBH 55	Joaquim Egydío, SP	22	-22.900000	-47.060000	x	x	x
Hap_78	*B. ephippium*	CFBH 7926	Itanhaém, SP	23	-24.017477	-46.796771	x	x	x
Hap_79	*B.* sp. 2	CTMZ 2970	Barra do Turvo, SP	24	-24.966278	-48.418889	x	x	x
Hap_80	*B. ephippium*	MNRJ 50260	Vale da Revolta, RJ	25	-22.433333	-42.933333	x	x	x
Hap_81	*B. ephippium*	MNRJ 73097	Resende, RJ	26	-22.368516	-44.628845	x		x
Hap_82	*B. garbeanus*	MNRJ 57293	Nova Friburgo, RJ	27	-22.276000	-42.538000	x	x	x
Hap_83	*B. garbeanus*	MNRJ 51472	Nova Friburgo, RJ	27	-22.276000	-42.538000	x	x	x
Hap_84	*B.margaritatus*	MNRJ 76100	Paty de Alferes, RJ	28	-22.447658	-43.381944	x	x	x
Hap_85	*B.margaritatus*	MNRJ 76701	Paty de Alferes, RJ	28	-22.447658	-43.381944	x	x	x
Hap_86	*B. garbeanus*	CFBH 12688	Macaé de Cima, RJ	29	-22.358639	-42.565444	x	x	x
Hap_87	*B. garbeanus*	CFBH 12689	Macaé de Cima, RJ	29	-22.358639	-42.565444	x	x	x
Hap_88	*B.* sp. 11	TC 122	Bananal, SP	30	-22.794603	-44.359620	x	x	x
Hap_89	*B.* sp. 11	TC 123	Bananal, SP	30	-22.794603	-44.359620	x	x	x
Hap_90	*B.* sp. 11	TC 124	Bananal, SP	30	-22.794603	-44.359620	x	x	x
Hap_91	*B.* sp. 11	TC 125	Bananal, SP	30	-22.794603	-44.359620	x	x	x
Hap_92	*B.* sp. 11	TC 126	Bananal, SP	30	-22.794603	-44.359620	x	x	x
Hap_93	*B.* sp. 11	TC 158	Cunha, SP	31	-23.242806	-45.015861	x	x	x
Hap_94	*B.* sp. 11	TC 159	Cunha, SP	31	-23.242806	-45.015861	x	x	x
Hap_95*	*B.* sp. 11	TC 65	Cunha, SP	31	-23.242806	-45.015861	x		x
Hap_96*	*B. ephippium*	TC 14	Mogi das Cruzes, SP	32	-23.470529	-46.160675	x		x
Hap_97	*B. ephippium*	TC 07	Mogi das Cruzes, SP	32	-23.470529	-46.160675	x		x
Hap_98*	*B. ephippium*	TC 09	Mogi das Cruzes, SP	32	-23.470529	-46.160675	x		x

Haplotype	Species	Vouchers	Locality	ID	Latitude	Longitude	12S	16S	Cyt b
Hap_99*	*B. ephippium*	TC 10, TC 11	Mogi das Cruzes, SP	32	-23.470529	-46.160675	x		x
Hap_100*	*B. ephippium*	TC 12	Mogi das Cruzes, SP	32	-23.470529	-46.160675	x		x
Hap_101*	*B. ephippium*	TC 16	Mogi das Cruzes, SP	32	-23.470529	-46.160675	x		x
Hap_102	*B. ephippium*	CFBH 2083	Serra do Japi, Jundiaí, SP	33	-23.239417	-46.934917	x	x	x
Hap_103	*B. ephippium*	TC 103	Serra do Japi, Jundiaí, SP	33	-23.239417	-46.934917	x	x	x
Hap_104	*B. ephippium*	TC 104	Serra do Japi, Jundiaí, SP	33	-23.239417	-46.934917	x	x	x
Hap_105	*B. ephippium*	TC 99	Serra do Japi, Jundiaí, SP	33	-23.239417	-46.934917	x	x	x
Hap_106	*B. nodoterga*	TC 128	Salesópolis, Boracéia, SP	34	-23.650665	-45.882029	x	x	x
Hap_107	*B. nodoterga*	TC 130	Salesópolis, Boracéia, SP	34	-23.650665	-45.882029	x	x	x
Hap_108	*B. nodoterga*	TC 131	Salesópolis, Boracéia, SP	34	-23.650665	-45.882029	x	x	x
Hap_109	*B. nodoterga*	TC 136	Salesópolis, Boracéia, SP	34	-23.650665	-45.882029	x	x	x
Hap_110	*B. nodoterga*	TC 138	Salesópolis, Boracéia, SP	34	-23.650665	-45.882029	x	x	x
Hap_111	*B. nodoterga*	A147722	Santana do Parnaíba, SP	35	-23.465900	-46.837100	x	x	x
Hap_112	*B. nodoterga*	CFBH 2226	Santana do Parnaíba, SP	35	-23.465900	-46.837100	x	x	x
Hap_113	*B. nodoterga*	TC 41	Mairiporã, SP	36	-23.429167	-46.634833	x	x	x
Hap_114	*B. nodoterga*	TC 140	Mairiporã, SP	36	-23.429167	-46.634833	x	x	x
Hap_115	*B. nodoterga*	TC 105	Ilhabela, SP	37	-23.870570	-45.357744	x	x	x
Hap_116	*B. nodoterga*	TC 106	Ilhabela, SP	37	-23.870570	-45.357744	x	x	x
Hap_117	*B. nodoterga*	TC 107	Ilhabela, SP	37	-23.870570	-45.357744	x	x	x
Hap_118	*B. nodoterga*	TC 108	Ilhabela, SP	37	-23.870570	-45.357744	x	x	x
Hap_119	*B. nodoterga*	TC 109	Ilhabela, SP	37	-23.870570	-45.357744	x	x	x
Hap_120	*B. nodoterga*	TC 110	Ilhabela, SP	37	-23.870570	-45.357744	x	x	x
Hap_121	*B. nodoterga*	TC 111	Ilhabela, SP	37	-23.870570	-45.357744	x	x	x
Hap_122	*B. toby*	TC 113	Corcovado, Ubatuba, SP	38	-23.454932	-45.198414	x	x	x
Hap_123	*B. toby*	TC 114	Corcovado, Ubatuba, SP	38	-23.454932	-45.198414	x	x	x
Hap_124	*B. pitanga*	TC 115	Natividade da Serra, SP	39	-23.434531	-45.227830	x	x	x
Hap_125	*B. pitanga*	TC 116	Natividade da Serra, SP	39	-23.434531	-45.227830	x	x	x
Hap_126	*B. pitanga*	TC 117	Natividade da Serra, SP	39	-23.434531	-45.227830	x	x	x
Hap_127	*B. pitanga*	TC 118	Natividade da Serra, SP	39	-23.434531	-45.227830	x	x	x
Hap_128	*B. pitanga*	TC 119	Natividade da Serra, SP	39	-23.434531	-45.227830	x	x	x
Hap_129	*B. pitanga*	TC 120	Natividade da Serra, SP	39	-23.434531	-45.227830	x	x	x
Hap_130	*B. pitanga*	TC 121	Natividade da Serra, SP	39	-23.434531	-45.227830	x	x	x
Hap_131	*B. pitanga*	TC 163	São Luís do Paraitinga, SP	40	-23.338528	-45.148056	x	x	x
Hap_132	*B. pitanga*	CFBH 14544	São Luís do Paraitinga, SP	40	-23.338528	-45.148056	x	x	x

Haplotype	Species	Vouchers	Locality	ID	Latitude	Longitude	12S	16S	Cyt b
Hap_133	*B. pitanga*	CFBH 14545	São Luís do Paraitinga, SP	40	-23.338528	-45.148056	x	x	x
Hap_134	*B. pitanga*	CFBH 14546	São Luís do Paraitinga, SP	40	-23.338528	-45.148056	x	x	x
Hap_135	*B. pitanga*	CFBH 14547	São Luís do Paraitinga, SP	40	-23.338528	-45.148056	x	x	x
Hap_136	*B. pitanga*	CFBH 14548	São Luís do Paraitinga, SP	40	-23.338528	-45.148056	x	x	x
Hap_137	*B. pitanga*	CFBH 14865	São Luís do Paraitinga, SP	40	-23.338528	-45.148056	x	x	x
Hap_138	*B. pitanga*	CFBH 14866	São Luís do Paraitinga, SP	40	-23.338528	-45.148056	x	x	x
Hap_139	*B. pitanga*	CFBH 14867	São Luís do Paraitinga, SP	40	-23.338528	-45.148056	x	x	x
Hap_140	*B. pitanga*	CFBH 14868	São Luís do Paraitinga, SP	40	-23.338528	-45.148056	x	x	x
Hap_141	*B.* sp. 9	TC 151	Cunha, SP	41	-23.284972	-45.076222	x	x	x
Hap_142	*B.* sp. 9	TC 145	Cunha, SP	41	-23.284972	-45.076222	x	x	x
Hap_143	*B.* sp. 9	TC 146	Cunha, SP	41	-23.284972	-45.076222	x	x	x
Hap_144	*B.* sp. 9	TC 147	Cunha, SP	41	-23.284972	-45.076222	x	x	x
Hap_145	*B.* sp. 9	TC 149	Cunha, SP	41	-23.284972	-45.076222	x	x	x
Hap_146	*B.* sp. 9	TC 150	Cunha, SP	41	-23.284972	-45.076222	x	x	x
Hap_147	*B. vertebralis*	CFBH 1388	Ubatuba, SP	42	-23.341583	-44.781944	x	x	x
Hap_148	*B. vertebralis*	CFBH 1389	Ubatuba, SP	42	-23.341583	-44.781944	x	x	x
Hap_149	*B. vertebralis*	CFBH 1390	Ubatuba, SP	42	-23.341583	-44.781944	x	x	x
Hap_150	*B. vertebralis*	CFBH 1391	Ubatuba, SP	42	-23.341583	-44.781944	x	x	x
Hap_151	*B. vertebralis*	CFBH 1392	Ubatuba, SP	42	-23.341583	-44.781944	x	x	x
Hap_152	*B. vertebralis*	CFBH 1393	Ubatuba, SP	42	-23.341583	-44.781944	x	x	x
Hap_153	*B. crispus*	TC 34	Bacia B, Cunha, SP	43	-23.254111	-45.032722	x	x	x
Hap_154*	*B. crispus*	TC 35	Bacia B, Cunha, SP	43	-23.254111	-45.032722	x		x
Hap_155	*B. crispus*	TC 36	Bacia B, Cunha, SP	43	-23.254111	-45.032722	x	x	x
Hap_156	*B. crispus*	TC 67	Bacia B, Cunha, SP	43	-23.254111	-45.032722	x	x	x
Hap_157	*B.* sp. 9	TC 152	Ubatuba, SP	44	-23.311444	-45.059194	x	x	x
Hap_158	*B.* sp. 9	TC 153	Ubatuba, SP	44	-23.311444	-45.059194	x	x	x
Hap_159*	*B.* sp. 9	TC 154	Ubatuba, SP	44	-23.311444	-45.059194	x		x
Hap_160*	*B.* sp. 9	TC 155	Ubatuba, SP	44	-23.311444	-45.059194	x		x
Hap_161	*B.* sp. 9	TC 156	Ubatuba, SP	44	-23.311444	-45.059194	x		x
Hap_162	*B.* sp. 9	TC 157	Ubatuba, SP	44	-23.311444	-45.059194	x	x	x
Hap_163	*B. alipioi*	CFBH 11759	Vargem Alta, ES	45	-20.603611	-41.184722	x	x	x
Hap_164	*B. alipioi*	CFBH 11933	Vargem Alta, ES	45	-20.603611	-41.184722	x	x	x
Hap_165	*B. guarani*	CFBH 12996	Prumirim, Ubatuba, SP	46	-23.346633	-45.026688	x	x	x
Hap_166	*B. guarani*	CFBH 12997	Prumirim, Ubatuba, SP	46	-23.346633	-45.026688	x	x	x

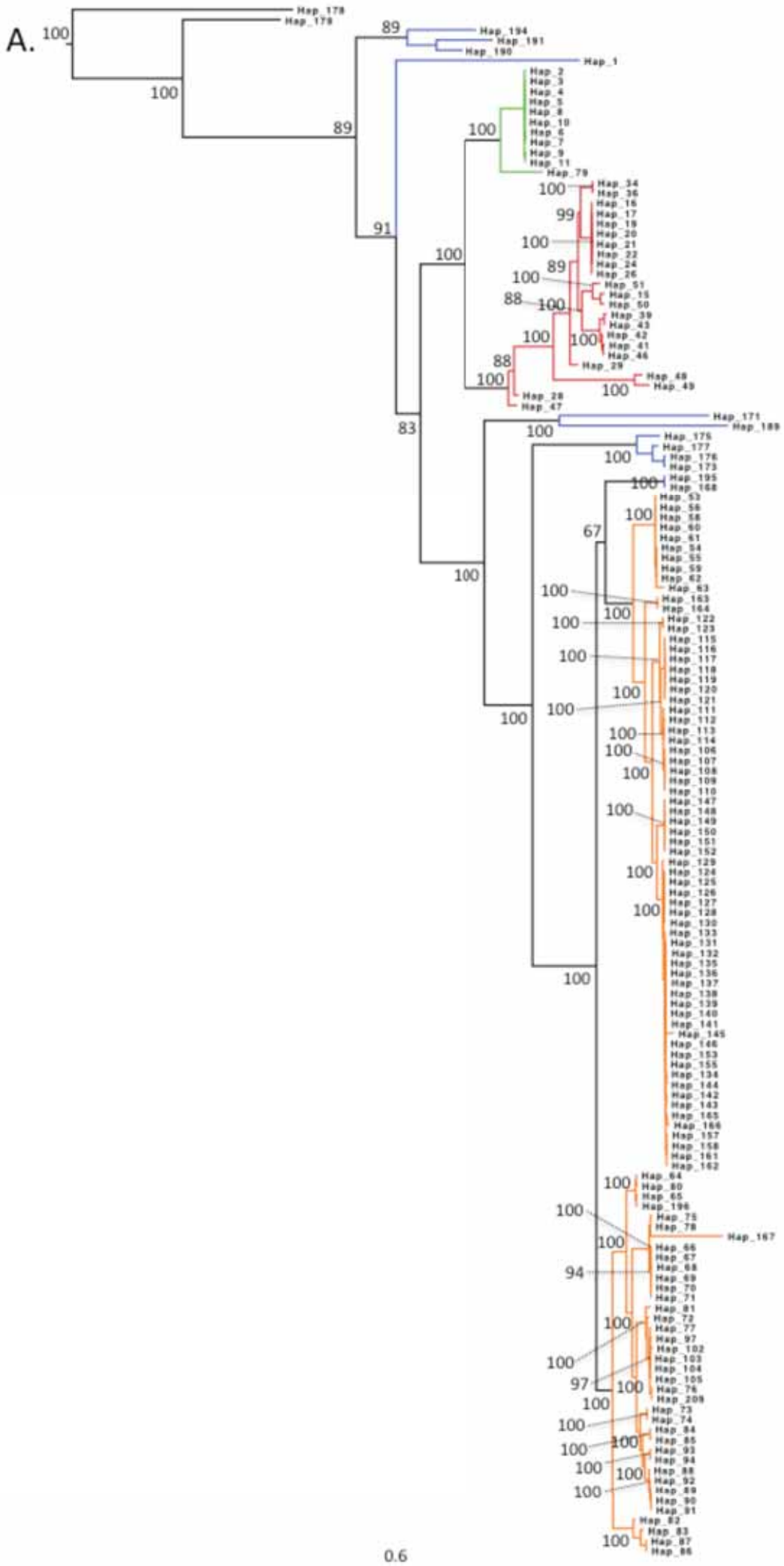
Haplotype	Species	Vouchers	Locality	ID	Latitude	Longitude	12S	16S	Cyt b
Hap_167	*B. ephippium*	CTMZ 2585	São Miguel Arcanjo, SP	47	-24.100000	-48.033333	x		x
Hap_168	*B.* sp. 7	MNRJ 56504	Cachoeiras de Macacu, RJ	48	-22.357209	-42.516112	x	x	x
Hap_169*	*B. margaritatus*	MNRJ 73702	Country Club, Petrópolis, RJ	49	-22.538210	-43.218411	x		
Hap_170*	*B.* sp. 11	CTMZ 1512	Bananal, SP	30	-22.794603	-44.359620	x		
Hap_171	*B.* sp.1	CFBH 13722	Santa Maria Madalena, RJ	14	-21.880306	-41.916917	x	x	
Hap_172*	*B.* sp. 1	CFBH 13723	Santa Maria Madalena, RJ	14	-21.880306	-41.916917	x	x	
Hap_173*	*B. didactylus*	MNRJ 37327	Teresópolis, RJ	50	-22.412319	-42.966486	x	x	
Hap_174*	*B. didactylus*	MNRJ 37326	Teresópolis, RJ	50	-22.412319	-42.966486	x		
Hap_175	*B. didactylus*	MNRJ 54402	P.E. Três Picos, RJ	51	-22.416667	-42.583333	x		
Hap_176	*B. didactylus*	CFBH 11506	Vale da Revolta, RJ	25	-22.433333	-42.933333	x	x	
Hap_177	*B. didactylus*	MNRJ 68818	R.E. Guapiaçu, RJ	52	-22.484444	-42.670556	x	x	x
Hap_178	*H. binotatus*	-	-	-	-	-	x	x	x
Hap_179	*I. parva*	MNRJ 51356	-	-	-	-	x		
Hap_180*	*B. pulex*	TC 180	Camacan, BA	1	-15.394196	-39.568729		x	
Hap_181*	*B. pulex*	TC 181	Camacan, BA	1	-15.394196	-39.568729		x	
Hap_182*	*B. pulex*	TC 183	Camacan, BA	1	-15.394196	-39.568729		x	
Hap_183*	*B. pulex*	TC 184, TC 185	Camacan, BA	1	-15.394196	-39.568729		x	
Hap_184*	*B. pulex*	TC 186	Camacan, BA	1	-15.394196	-39.568729		x	
Hap_185*	*B. pulex*	TC 192	Camacan, BA	1	-15.394196	-39.568729		x	
Hap_186*	*B. pulex*	TC 193	Camacan, BA	1	-15.394196	-39.568729		x	
Hap_187*	*B. didactylus*	CFBH 12907	Ilha Grande, RJ	53	-23.189853	-44.199154		x	x
Hap_188*	*B. didactylus*	CTMZ 1522	Bananal, SP	30	-22.794603	-44.359620		x	
Hap_189	*B.* sp. 1	MNRJ 58178	Mendanha, RJ	54	-22.806649	-43.490111		x	
Hap_190	*B. hermogenesi*	ARI 623	Paraibuna, SP	55	-23.381400	-45.662610		x	
Hap_191	*B. hermogenesi*	TC 47	Picinguaba, Ubatuba, SP	13	-23.357556	-44.767278		x	x
Hap_192*	*B. hermogenesi*	TC 48	Picinguaba, Ubatuba, SP	13	-23.357556	-44.767278		x	
Hap_193*	*B. hermogenesi*	TC 49	Picinguaba, Ubatuba, SP	13	-23.357556	-44.767278		x	
Hap_194	*B. hermogenesi*	TC 129	Salesópolis, Boracéia, SP	56	-23.650665	-45.882029		x	
Hap_195	*B.* sp. 7	MNRJ 56516	Teodoro de Oliveira, RJ	57	-22.366389	-42.554444		x	
Hap_196	*B. ephippium*	MNRJ 56517	R.E. Guapiaçu, RJ	52	-22.484444	-42.670556		x	x
Hap_197*	*B.margaritatus*	MNRJ 73701	Country Club, Petrópolis, RJ	49	-22.538210	-43.218411		x	x
Hap_198*	*B.* sp. 10	CTMZ 7052, CTMZ 7053	Paranapiacaba, SP	17	-23.779340	-46.294891		x	x
Hap_199*	*B.* sp. 10	CTMZ 7054, CTMZ 7121	Paranapiacaba, SP	17	-23.779340	-46.294891		x	x

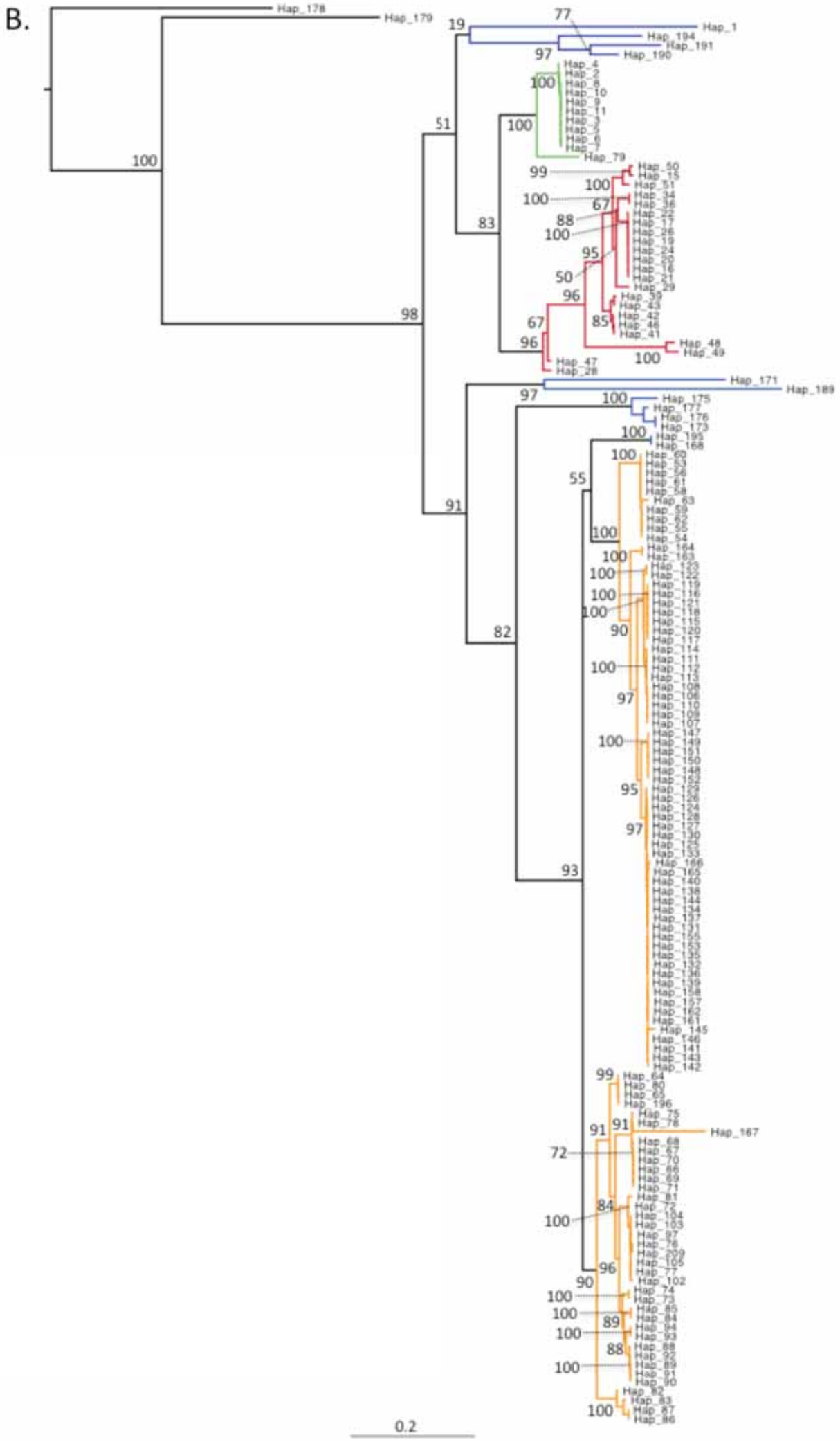
Haplotype	Species	Vouchers	Locality	ID	Latitude	Longitude	12S	16S	Cyt b
Hap_200*	*B.* sp. 10	CTMZ 7069	Paranapiacaba, SP	17	-23.779340	-46.294891		x	x
Hap_201*	*B. ephippium*	TC 98	Serra do Japi, Jundiaí, SP	33	-23.239417	-46.934917		x	x
Hap_202*	*B.* sp. 11	CTMZ 1654	Bananal, SP	30	-22.794603	-44.359620		x	x
Hap_203*	*B. nodoterga*	TC 132	Salesópolis, Boracéia, SP	34	-23.650665	-45.882029		x	x
Hap_204*	*B. nodoterga*	TC 137	Salesópolis, Boracéia, SP	34	-23.650665	-45.882029		x	x
Hap_205*	*B. crispus*	TC 66	Bacia B, Cunha, SP	43	-23.254111	-45.032722		x	x
Hap_206*	*B.* sp. 9	TC 168	Cunha, SP	41	-23.284972	-45.076222		x	x
Hap_207*	*B.* sp. 9	TC 97	Ubatuba, SP	44	-23.311444	-45.059194		x	x
Hap_208*	*B. ephippium*	MNRJ 73096	Resende, RJ	26	-22.368516	-44.628845			x
Hap_209	*B. ephippium*	CFBH 31981	Guaratinguetá, SP	21	-22.662720	-45.331881			x
Hap_210*	*B.* sp. 10	CTMZ 7125	Paranapiacaba, SP	17	-23.779340	-46.294891			x
Hap_211*	*B. ephippium*	CFBH 53	Joaquim Egydío, SP	22	-22.900000	-47.060000			x
Hap_212*	*B. ephippium*	TC 100, TC 101, TC 13, TC 08	Serra do Japi, Jundiaí, SP	33	-23.239417	-46.934917			x
Hap_213*	*B. ephippium*	TC 102	Serra do Japi, Jundiaí, SP	33	-23.239417	-46.934917			x
Hap_214*	*B.* sp. 11	TC 160, TC 162	Cunha, SP	31	-23.242806	-45.015861			x
Hap_215*	*B.* sp. 11	TC 161	Cunha, SP	31	-23.242806	-45.015861			x
Hap_216*	*B.* sp. 11	TC 82	Cunha, SP	31	-23.242806	-45.015861			x
Hap_217*	*B.* sp. 11	TC 83	Cunha, SP	31	-23.242806	-45.015861			x
Hap_218*	*B.* sp. 11	CTMZ 1636	Bananal, SP	30	-22.794603	-44.359620			x
Hap_219*	*B.* sp. 11	CTMZ 1643, CTMZ 1652, CTMZ1661	Bananal, SP	30	-22.794603	-44.359620			x
Hap_220*	*B. nodoterga*	A147716	Santana do Parnaíba, SP	35	-23.465900	-46.837100			x
Hap_221*	*B. nodoterga*	A147717, A147721	Santana do Parnaíba, SP	35	-23.465900	-46.837100			x
Hap_222*	*B. crispus*	TC 57, TC 68	Bacia B, Cunha, SP	43	-23.254111	-45.032722			x
Hap_223*	*B. crispus*	TC 58	Bacia B, Cunha, SP	43	-23.254111	-45.032722			x
Hap_224*	*B. crispus*	TC 60	Bacia B, Cunha, SP	43	-23.254111	-45.032722			x
Hap_225*	*B.* sp. 9	TC 95	Ubatuba, SP	44	-23.311444	-45.059194			x
Hap_226*	*B. vertebralis*	TC 44	Ubatuba, SP	42	-23.341583	-44.781944			x
Hap_227*	*B. vertebralis*	TC 45	Ubatuba, SP	42	-23.341583	-44.781944			x

Appendix 2. Primers used in this study.

Primer		Gene	Sequence	Reference
12SJL	F	12S rRNA	AAAGRTTTGGTCCTRRSCTT	Goebel et al., 1999
12SKH	R	12S rRNA	TCCRGTAYRCTTACCDTGTTACGA	Goebel et al., 1999
12SM	F	16S rRNA	GGCAAGTCGTAACATGGTAAG	Darst & Cannatella, 2004
16SA	R	16S rRNA	ATGTTTTTGGTAAACAGGCG	Darst & Cannatella, 2004
MVZ15-L	F	Cyt b	GAACTAATGGCCCACACWWTACGNAA	Moritz et al., 1992
CytbAR-H	R	Cyt b	TAWAAGGGTCTTCTACTGGTTG	Goebel et al., 1999
BrachyCytb-F2	F	Cyt b	AAGCAAATAAGAAGTATCAYTCTGG	this study
BrachyCytb-F3	F	Cyt b	CCGGACTCTTCCTAGCYATA	this study
BrachyCytb-R1	R	Cyt b	CCATCACCGGACTCTTCCTAGC	this study

Appendix 3. Bayesian inference (BI), Maximum Likelihood (ML), and Maximum Parsimony (MP) topologies based on 153 haplotypes of the concatenated mtDNA fragments (12S, 16S and Cytb) obtained for *Brachycephalus*. A. The 50% majority rule consensus tree from BI with posterior probability values (ns ≥ 0.90). B. The Maximum Likelihood tree with bootstrap support (ML ≥ 70%). C. Strict consensus from MP with Jacknife support (ns ≥ 70%). Color follows groups identified in Figure 1. Haplotypes names are in Appendix1.


A.
0.6


B.
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Hap_179
Hap_1
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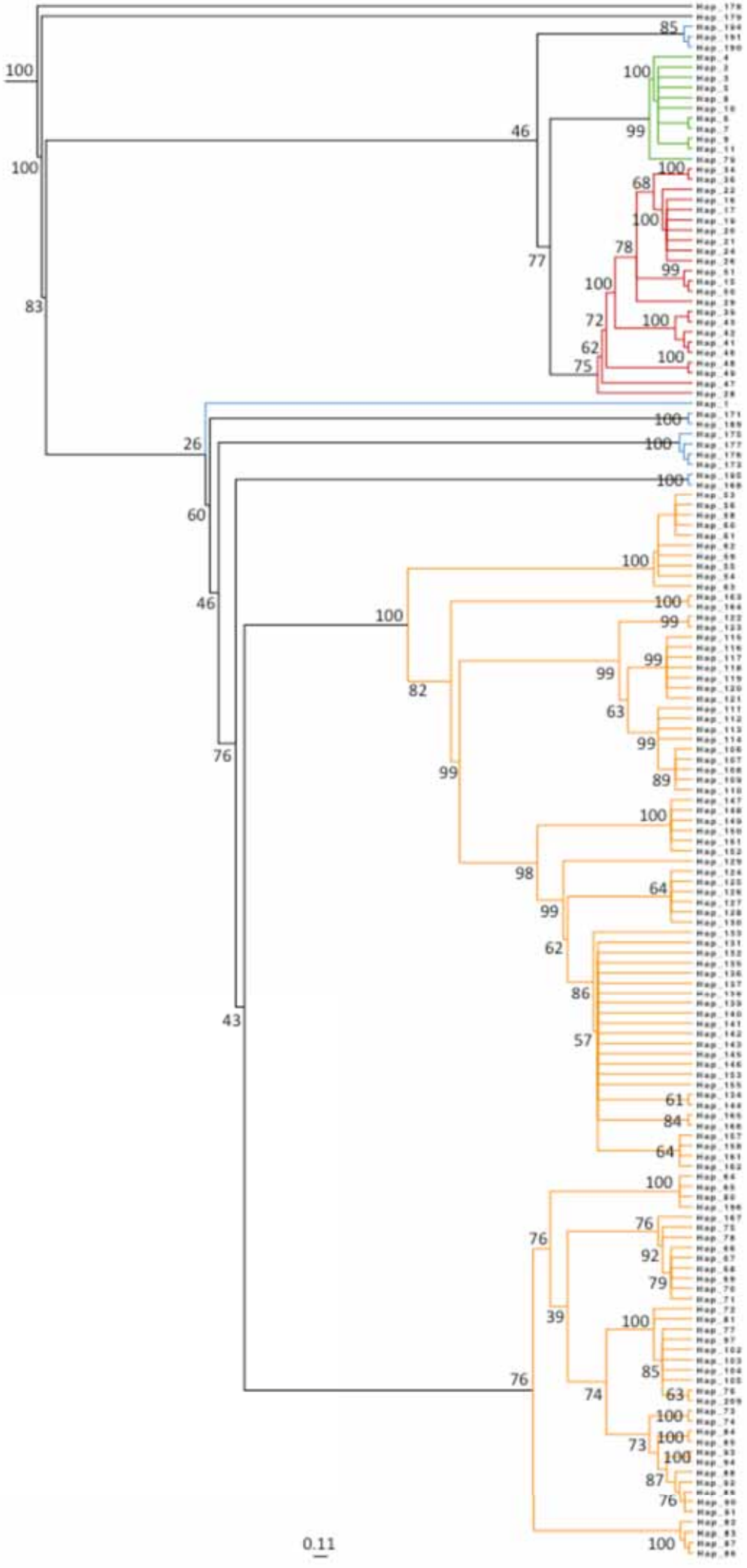
C.



CONCLUSÕES

- O gênero *Brachycephalus* constitui um modelo interessante para estudos de evolução em função de sua diversidade, características especializadas e distribuição geográfica geralmente restrita a áreas de altitude elevada na Mata Atlântica.

- Dois grupos de espécies são reconhecidos em *Brachycephalus* com base em diferenças morfométricas: (1) os sapos-pulga, anteriormente chamados de *Psyllophryne*, que apresentam ampla distribuição altitudinal e (2) as demais espécies do gênero, com distribuição restrita a áreas de altitude elevada.

- As principais diferenças morfométricas entre os sapos-pulga e as demais espécies de *Brachycephalus* referem-se ao tamanho do corpo e distância interorbital. Essas diferenças estão relacionadas à variação altitudinal e complexidade topográfica das localidades onde ocorrem.

- Entre as espécies de sapos-pulga estão registrados os menores valores de comprimento-rostro-cloacal; entretanto, este grupo apresenta pouca variação morfométrica.

- Entre as espécies de distribuição restrita a áreas de altitude elevada a variação de tamanho está principalmente relacionada a variações na temperatura, sendo que menores valores de comprimento rostro-cloacal são encontrados ao sul da distribuição, na porção subtropical da Mata Atlântica.

- A monofilia do gênero foi confirmada com o uso de marcadores de DNA mitocondrial e não encontramos evidências para a distinção entre os sapos-pulga e as demais espécies de *Brachycephalus*. A relação entre algumas espécies do gênero não foi esclarecida, evidenciando a necessidade de novos estudos com enfoque filogenético.

- Os clados principais correspondem a grupos de espécies estruturados geograficamente que compartilham características como o tamanho do corpo, coloração, grau de hiperossificação de elementos do esqueleto e canto de anúncio.

- As espécies atuais supostamente teriam evoluído a partir de um sapo-pulga ancestral, amplamente distribuído, com coloração críptica, sem hiperossificação de elementos do esqueleto e canto de anúncio curto, composto por uma a quatro notas pulsionadas de alta frequência.

- As linhagens de *Brachycephalus* apresentam um tempo estimado de divergência bastante antigo em comparação com outros anuros, sendo influenciadas por eventos históricos desde o Oligoceno.

- Barreiras geográficas como rios, falhas tectônicas e descontinuidades no relevo coincidem com quebras genéticas para as espécies do gênero *Brachycephalus*. O lineamento do Guapiara parece ter sido uma importante barreira, dividindo as espécies entre a porção tropical e subtropical da Mata Atlântica.

- Maiores índices de diversidade genética estão associados a áreas de estabilidade histórica na Mata Atlântica. Múltiplos refúgios em áreas de altitude elevada podem ter favorecido a persistência das espécies de *Brachycephalus* ao longo das flutuações climáticas do Quaternário.

- Nossos resultados sugerem um gradiente latitudinal de diversificação nas áreas de altitude elevada da Mata Atlântica. Na porção tropical do bioma, o maior isolamento entre as populações de *Brachycephalus* está relacionado a maiores resistências climáticas e da paisagem ao fluxo gênico, aumentando as oportunidades de especiação nestas áreas em comparação à porção subtropical do bioma.

ANEXO I

A new species of *Brachycephalus* (anura: brachycephalidae) from the highlands of the Atlantic forest, southeastern Brazil

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A NEW SPECIES OF *BRACHYCEPHALUS* (ANURA: BRACHYCEPHALIDAE) FROM THE HIGHLANDS OF THE ATLANTIC FOREST, SOUTHEASTERN BRAZIL

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ABSTRACT: A new species of brachycephalid frog is described from the highlands of the Atlantic Forest in Serra do Mar, municipality of Cunha, São Paulo State, southeastern Brazil. Specimens were collected amidst the leaf litter at approximately 920 m above sea level. The new species is distinguished from all of its congeners by the combination of the following characters: snout–vent length 9.5–15.6 mm; rough dorsum; general background color orange with a variable density of whitish spots on dorsum; skin on head and dorsum granular with dermal ossification; skull, spinal processes of sacral and presacral vertebrae, and process of the fourth vertebra hyperossified; absence of hyperossification in the central portion of the skull; dorsal surface of body covered by osteoderms. The new species was found active by day and is locally abundant. Males hold territories during the reproductive season. Females had few mature oocytes during the same period. The advertisement call of *B. crispus* is a long and low-intensity buzz with a regular repetition of notes.

Key words: Advertisement call; Atlantic rainforest; Cunha; Direct-developing frog

THE FAMILY Brachycephalidae contains 52 species allocated in two genera: *Brachycephalus* Fitzinger, 1826 and *Ischnocnema* Reinhardt and Lütken, 1862 (Hedges et al., 2008; Frost, 2013). Species of these genera share molecular and morphological features along with terrestrial breeding and direct development in which large eggs hatch into froglets, thereby bypassing the tadpole stage (Hedges et al., 2008).

Currently, the genus *Brachycephalus* contains 20 species (Frost, 2013). As a result of miniaturization (Trueb and Alberch, 1985; Hanken, 1993; Hanken and Wake, 1993; Yeh, 2002), species of *Brachycephalus* exhibit an extremely small adult body size and share the absence of bones such as the neopalatine and columellae in the skull, phalangeal loss in the manus and pes, and a reduced number of fingers and toes (Alves et al., 2006; Pombal and Gasparini, 2006). Another remarkable phenotypic feature among species of *Brachycephalus* is a gradient in the degree of mineralization of the skull and post-cranial skeleton, from a condition of the complete lack of hyperossification to a completely hyperossified condition, including a dorsal shield (Clemente-Carvalho et al., 2009).

Brachycephalus are endemic to the Atlantic Rain Forest of southern, southeastern, and northeastern Brazil (Ribeiro et al., 2005; Napoli et al., 2011; Frost, 2013). The greatest diversity of species occurs in the Serra do Mar mountain range in the states of Paraná and São Paulo (Garey et al., 2012). Most species of *Brachycephalus* have been described in the last decade (Alves et al., 2009; Haddad et al., 2010; Clemente-Carvalho et al., 2012) as a result of increased herpetological surveys in Atlantic Rain Forest highlands.

Seventeen species of *Brachycephalus* are associated with mountain slopes (600–1800 m elevation) along the main Atlantic Rain Forest mountain ranges. These species exhibit a generalized bufoniform morphology and frequently have conspicuous orange coloration and, in some cases, the skin contains toxic tetrodotoxin-like substances (Sebben et al., 1986; Pires et al., 2002, 2005; Hanifin, 2010). Three other species (*B. didactylus*, *B. hermogenesi*, and *B. pulex*) have a wide altitudinal

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range from sea level to 950 m (Izecksohn, 1971; Giaretta and Sawaya, 1998; Napoli et al., 2011). Morphological and biological characteristics have been used to support informal distinction of these two groups of species within *Brachycephalus* (Verdade et al., 2008; Napoli et al., 2011), although no evidence for this separation exists in the molecular phylogeny (Clemente-Carvalho et al., 2011). In addition to differences in advertisement call, the lowland species exhibit relatively smaller body sizes compared to the highland species, leptodactyliform morphology, inconspicuous coloration, and the ability to jump longer distances (Verdade et al., 2008; Napoli et al., 2011).

Natural history and ecological aspects of *Brachycephalus* species are poorly understood, especially because recent species descriptions lack natural history details. Collecting natural history data on these species is confounded by inaccessible study sites and possible activity under leaf litter. Natural history information is restricted to the reproductive behavior of *B. ephippium* (Pombal, 1999). Food habits and other ecological aspects are described for *B. brunneus* (Fontoura et al., 2011), *B. didactylus* (Almeida-Santos et al., 2011), and *B. garbeanus* (Dorigo et al., 2012). Advertisement calls are available for *B. ephippium* (Pombal et al., 1994), *B. pernix* (Wistuba, 1998), *B. hermogenesi* (Verdade et al., 2008), *B. pitanga* (Araújo et al., 2012) and *B. tridactylus* (Garey et al., 2012).

Herein we add to the known diversity of *Brachycephalus* by describing a new species collected in the mountain slopes of Serra do Mar, in the State of São Paulo, southeastern Brazil. The diagnosis of the new species is based on the external morphology and osteology of adults. Additionally, we provide the advertisement call and some aspects of reproductive biology.

Materials and Methods

Specimens were anaesthetized and killed in 5% lidocaine, fixed in 10% formalin, and preserved in 70% ethyl alcohol. Bones and cartilages were cleared and double-stained with alizarin red and alcian blue following procedures modified from Taylor and Van Dyke (1985). Scanning electron microscopy characterized hyperossification in the skeleton of the new species. Specimens were immersed in a solution of sodium hypochlorite for removal of soft tissues and air dried. Skeletons were then mounted on metal stubs, coated with gold in a Sputter Coater Balzers SCD050, and examined with a JEOL JSM 5800LV scanning electron microscope. Illustrations were made using a Zeiss stereomicroscope fitted with a drawing tube.

Fifteen traits were measured with a micrometric ocular fitted to the stereomicroscope. The abbreviations used were: SVL (snout–vent length); AGL (axilla–groin length); HL (head length; from tip of snout to angle of jaw); HW (head width; greatest width of head located between angle of jaw); ND (nostril diameter); IND (internostril distance; between inner margins of nostrils); ED (eye diameter); IOD (interorbital distance; between anterior corners of eyes); END (eye–nostril distance; from anterior corner of the eye to posterior margin of nostril); THL (thigh length); TBL (tibia length); FL (foot length; between the centrale and the larger toe); AL (arm length); FAL (forearm length); and HAL (hand length; between the centrale and the longest toe).

Specimens used in the description are deposited in the Célio F. B. Haddad (CFBH) amphibian collection, Departamento de Zoologia, I.B., Universidade Estadual Paulista, Campus de Rio Claro, São Paulo, Brazil. Specimens examined (Appendix 1) also included those from the herpetology collection of Museu de Zoologia, Universidade de São Paulo (MZUSP), São Paulo, Brazil and the herpetology collection of Museu Nacional, Rio de Janeiro (MNRJ), Brazil.

We described the habitat of the new species by measuring environmental characteristics at encounter sites. Altitude was determined using a barometric altimeter; air relative humidity and temperature were taken from the sampling locality with a hygro-thermometer. The average depth of leaf litter was measured with a ruler (±1 mm).

Five calling males were recorded with a Marantz PMD-660 digital recorder coupled to an external unidirectional Sennheiser ME-66 microphone. We analyzed 20 calls per individual using a sampling frequency rate of 44.1

FIG. 1.—Live adult female of *Brachycephalus crispus* sp. nov. CFBH 29839 (holotype), Parque Estadual da Serra do Mar, Núcleo Cunha, Municipality of Cunha, State of São Paulo, southeastern Brazil.

kHz and 16-bit resolution in the mono pattern. Bioacoustic analyses were performed using the program Raven Pro 1.4 (Bioacoustics Research Program, 2011). Audiospectrograms were produced with a fast Fourier transform of 256 points, overlap 50%, and hamming window type. Nine advertisement call variables were quantified: note duration (s); internote duration (s); note repetition rate (notes/s); minimum, maximum, and dominant frequency (Hz); number of pulses per note; pulse duration (ms), and pulse rate (pulses/s). Oscillograms and spectrograms were used to illustrate sound features.

We also collected fresh oocytes from both sides of oviducts of mature females and preserved them in 70% ethyl alcohol. To describe and measure aspect, number, and size of oocytes we used a Nikon Sight DS-U2 Camera fitted to a Zeiss stereomicroscope using NIS-Elements imaging software.

RESULTS

Brachycephalus **crispus** *sp. nov.* *(Fig. 1)*

Holotype.—CFBH 29839, adult female, one of a series collected in Bacia B, Parque Estadual da Serra do Mar, Núcleo Cunha, municipality of Cunha, São Paulo State, southeastern Brazil, approximately 920 m above sea level (23°15′14.8″S, 45°01′57.8″W; WGS84 datum), on 19 October 2011 by T. H. Condez, E. G. de Oliveira, and L. N. Bandeira (Fig. 1).

Paratopotypes.—CFBH 29822, 29824, 29826, 29827, 29829, 29830, 29833, 29836, 29838, 29840 adult females; CFBH 29823, 29828, 29834, 29835, 29837 adult males; CFBH 29825, 29831, 29832 juveniles; all collected with the holotype in Bacia B, Parque Estadual da Serra do Mar, Núcleo Cunha, on 19 October 2011. CFBH 27857, 27858, 27863, 27868 adult females; CFBH 27861, 27865–27867 adult males; CFBH 27862 juvenile; CFBH 27859, 27860 juveniles cleared and stained; and CFBH 27864 adult female prepared for scanning electron microscopy; all collected at the same locality of the holotype on 26 January 2011. CFBH 10699, 10708, 23895–23897, 29659, 29661 adult females; CFBH 10698, 10701, 10707, 23894, 23899, 23901–23902 adult males; CFBH 10702, 29660 juveniles; CFBH 10706, 23900 adult females; and 23898 adult male prepared for scanning electron microscopy; 10703 adult female; 23903 adult male; and 10705 juvenile cleared and stained; all collected in the same locality of the holotype on 19 October 2004, by D. M. Peccinini Seale.

Diagnosis.—*Brachycephalus crispus* is distinguished as an adult from all of its congeners by the combination of the following characters: adult SVL 9.5–15.6 mm; rough dorsum; general background color orange with variable density of whitish spots on dorsum; skin on head and dorsum granular with dermal ossification; skull, spinous processes of sacral and presacral vertebrae and process of the fourth vertebra hyperossified; absence of hyperossification in the central portion of the skull; dorsal surface of body covered by osteoderms.

Description of holotype.—*Brachycephalus crispus* is characterized by a robust, bufoniform body (Fig. 2); head as wide as long, slightly narrower than body; head length approximately 23% of SVL; snout short with length almost equal to eye diameter, rounded in lateral and dorsal views (Fig. 3A,B); nostrils protuberant, directed anterolaterally; canthus rostralis distinct and straight; loreal region weakly concave; lips nearly sigmoid; eye slightly protruding in dorsal and lateral views, eye diameter 48% of HL; tympanum absent;

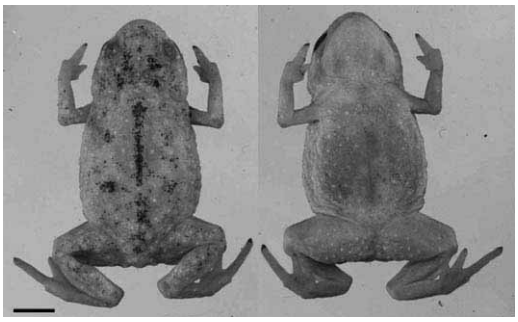

FIG. 2.—*Brachycephalus crispus* sp. nov. CFBH 29839 (holotype), adult female in ventral and dorsal views. Scale bar = 2 mm.

tongue longer than wide, posterior half not adherent to floor of mouth; choanae relatively small and round; vomerine odontophores absent. Upper arm slightly shorter than forearm; total arm length 39% of SVL; hands approximately as long as upper arm; fingers II and III robust and distinct; finger I and IV very small, vestigial; tip of fingers II and III slightly rounded; relative lengths of fingers II < III; subarticular tubercles and inner and outer metacarpal tubercles absent (Fig. 3C).

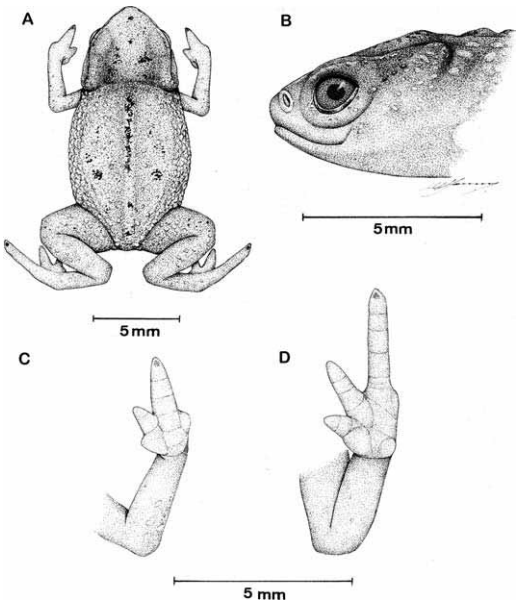

FIG. 3.—*Brachycephalus crispus* sp. nov. CFBH 29839 (holotype), adult female. (A) Dorsal view of head and body, (B) lateral view of head, (C) ventral view of left hand, and (D) ventral view of left foot.

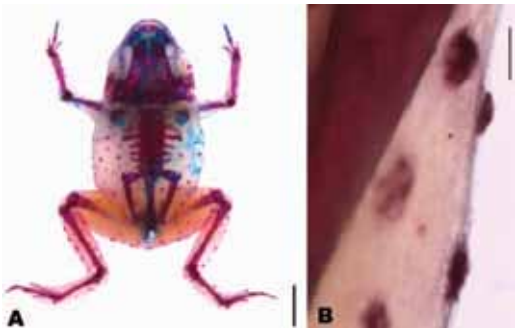

FIG. 4.—*Brachycephalus crispus* sp. nov. (paratopotype CFBH 27860) cleared and double-stained specimen. (A) Dorsal view of body covered by osteoderms, scale bar = 2 mm; and (B) detail of skin with osteoderms, scale bar = 0.2 mm.

Tibia shorter than thigh; total leg length 75% of SVL; foot length larger than thigh length; toes II, III, and IV distinct; toe II reduced, toes I and V externally absent; tips of toes II and III slightly rounded, tip of toe IV pointed; relative length of toes II < III < IV; subarticular tubercles and inner and outer metatarsal tubercles absent (Fig. 3D). Skin on head and dorsum granular with dermal ossifications; skin on dorsolateral surface of body, flanks, and dorsal surface of thighs granular; skin on venter and ventral surfaces of the legs smooth; granular skin on ventrolateral surfaces of body and area around the cloacal opening (Figs. 1, 2).

Measurements of holotype (in mm).—SVL 14.0; AGL 5.8; HL 3.1; HW 3.9; ND 0.3; IND 1.7; ED 1.6; IOD 2.9; END 0.9; THL 5.1; TBL 4.8; FL 7.9; AL 2.4; FAL 3.0; HAL 2.7.

Osteology of Brachycephalus crispus *sp. nov.*—The double-stained and scanning electron microscopy materials revealed that *B. crispus* has the dermal roofing bones of the skull ornamented (Figs. 4, 5). Nasal, sphenethmoid, frontoparietals, prootics, and exoccipitals are fused. Premaxillae broad, not fused medially; odontoids absent; alary process of premaxillae distinct and narrowly separated from the nasal. In ventral view, maxillae arched; odontoids absent. Quadratojugal and pterygoid present. Vomer completely fused, vomerine odontophores absent. Palatine absent. Parasphenoid and sphenethmoid fused and robust. Squamosal elongated in lateral view, anterior zygomatic ramus short

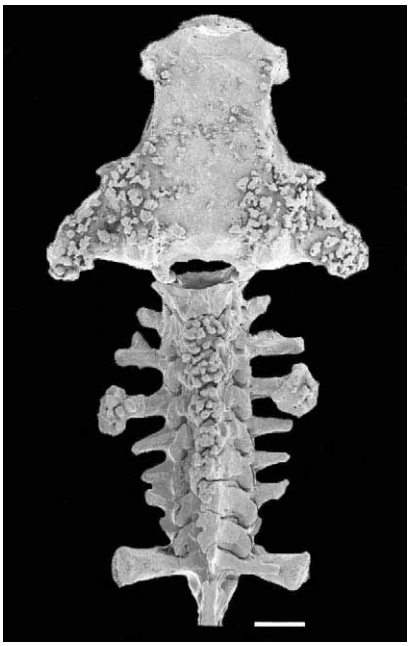

FIG. 5.—Scanning electron micrograph of the skull and vertebrae of *Brachycephalus crispus* sp. nov. (paratopotype CFBH 23900), adult female in dorsal view. Scale bar = 1 mm.

and ornamented. Tympanic annulus absent. Operculum present and partially cartilaginous. Mandible endentate. Pectoral girdle arciferal and robust; procoracoid and epicoracoid fused but not completely ossified; procoracoid and epicoracoid synostotically united with clavicle, coracoid, and scapula; suprascapula expanded, its anterior half ossified as cleithrum; omosternum present and cartilaginous; sternum absent. Vertebral column composed of eight presacral, nonimbricate vertebrae; spinous process of vertebrae hyperossified; first presacral vertebra (atlas) lacks transverse process; third presacral vertebra with forked extremity; fourth presacral vertebra with transverse process hyperossified and ornamented; all other vertebrae have slender transverse processes without hyperossification. Humerus slightly shorter than forearm; radius and ulna fused but distinguishable. Manus with distal carpals (I–V) fused with centrale; radiale and ulnare about the same size; one prepollical element; phalangeal formula 1–2–3–1; tips of the terminal phalangeal element of fingers I, II, and IV falciform and tip of finger III arrow shaped. Hindlimbs with tibia and fibula fused but distinguishable, forming the tibiafibula; tibiafibula slightly shorter than femur; tibiale and fibulare fused at their distal and proximal ends (medially not fused). Pes with distal tarsal element I, II, III present and IV–V absent; centrale present. One very-reduced prehallical element; phalangeal formula 1–2–3–4–1; tips of the terminal phalangeal elements of toes II, III, and IV arrow shaped, toes I and V reduced with terminal phalangeal element rounded in shape.

Coloration in life.—General background color orange; dorsal surface of head, body, arms, and legs with whitish spots; small and irregular brown spots on head and spine; finger III and toe IV with black tips; eyes black (Fig. 1).

Coloration in preservative.—General background color pale cream; dorsum with whitish spots; small and irregular brown spots on head and spine; finger III and toe IV with black tips; eyes black (Fig. 2).

Variation.—On average, females of *B. crispus* were larger than males (SVL: females = 14.0 ± 0.8 mm; males = 12.0 ± 0.3 mm; Welch's *t*-test, $t = 10.9$, $P < 0.01$; Table 1). Dermal ossification of head and dorsum varies ontogenetically. Juveniles can lack hyperossification or present skull and post-cranial skeleton less ornamented than in adults. Aspect in life varies among specimens in roughness and density of whitish spots on the dorsum (Fig. 6). Some specimens have the dorsum almost entirely orange with some sparse whitish spots, generally associated with the granular aspect of the skin (Fig. 6A,B); some specimens have irregular brown spots concentrated on head and spine (Fig. 6C,D) and, in some specimens, the whitish color can cover extensive areas of the dorsum (Fig. 6E,F). The holotype is intermediate between the last two groups.

Comparison with other species.—Like other members of the genus, *Brachycephalus crispus* is diminutive in size and displays loss of phalangeal elements (Fig. 3C,D); the pectoral girdle is completely ossified, with epicoracoids closely juxtaposed and articulating throughout their lengths, omosternum present, and sternum absent. The skin on

TABLE 1.—Morphometric traits of the type series of *Brachycephalus crispus* sp. nov. (values [mm] are reported as means ±1 SD).

	Males (n = 22)		Females (n = 25)		Juveniles (n = 6)	
Trait[1]	Mean ± 1 SD	Range	Mean ± 1 SD	Range	Mean ± 1 SD	Range
SVL	12.0 ± 0.3	11.5–12.9	14.0 ± 0.8	11.9–15.6	10.6 ± 0.6	9.5–11.3
AGL	4.3 ± 0.5	3.0–4.9	5.3 ± 0.8	3.8–7.2	3.9 ± 0.3	3.4–4.2
HL	2.8 ± 0.2	2.1–3.1	3.1 ± 0.2	2.8–3.5	2.5 ± 0.3	2.1–2.9
HW	3.5 ± 0.2	3.0–3.9	3.9 ± 0.3	3.2–4.2	3.0 ± 0.2	2.8–3.4
ND	0.3 ± 0.0	0.3–0.4	0.4 ± 0.0	0.3–0.5	0.3 ± 0.0	0.3–0.4
IND	1.6 ± 0.1	1.5–1.9	1.7 ± 0.1	1.5–1.9	1.5 ± 0.1	1.3–1.6
ED	1.4 ± 0.1	1.3–1.6	1.5 ± 0.1	1.4–1.6	1.3 ± 0.1	1.2–1.4
IOD	2.7 ± 0.2	2.5–3.1	3.0 ± 0.2	2.5–3.3	2.5 ± 0.2	2.3–2.8
END	0.9 ± 0.1	0.7–1.0	1.0 ± 0.1	0.8–1.1	0.8 ± 0.0	0.8–0.9
THL	4.9 ± 0.3	4.4–5.7	5.4 ± 0.2	4.9–5.7	4.4 ± 0.4	3.9–4.9
TBL	4.4 ± 0.2	4.2–4.9	4.9 ± 0.2	4.2–5.3	4.0 ± 0.4	3.4–4.5
FL	6.8 ± 0.6	5.8–7.7	8.0 ± 0.7	5.8–9.0	5.7 ± 0.7	4.9–7.0
AL	2.2 ± 0.2	1.9–2.4	2.6 ± 0.2	2.0–2.7	1.9 ± 0.2	1.7–2.1
FAL	2.6 ± 0.2	2.2–2.9	3.0 ± 0.2	2.5–3.3	2.3 ± 0.1	2.1–2.4
HAL	2.3 ± 0.2	1.9–2.7	2.6 ± 0.2	2.1–2.9	2.0 ± 0.2	1.8–2.3

[1] SVL = snout–vent length; AGL = axilla–groin length; HL = head length, from tip of snout to angle of jaw; HW = head width, greatest width of head located between angle of jaw; ND = nostril diameter; IND = internostril distance, between inner margins of nostrils; ED = eye diameter; IOD = interorbital distance, between anterior corners of eyes; END = eye–nostril distance, from anterior corner of the eye to posterior margin of nostril; THL = thigh length; TBL = tibia length; FL = foot length, between the centrale and the larger toe; AL = arm length; FAL = forearm length; HAL = hand length, between the centrale and the longest toe.

head and dorsum of *B. crispus* has dermal ossification. This characteristic distinguishes the new species from *B. brunneus*, *B. didactylus*, *B. ferruginus*, *B. hermogenesi*, *B. izecksohni*, *B. pernix*, *B. pombali*, *B. pulex*, and *B. tridactylus*, whose texture of skin on head and dorsum is smooth, with no dermal ossification. *Brachycephalus crispus* also differs from *B. didactylus*, *B. hermogenesi*, and *B. pulex* by being larger in SVL, having a rounded snout shape in dorsal view, and an orange background color. These three species have pointed snouts in dorsal view and brownish body color. Compared to *B. ephippium*, *B. garbeana*, and *B. margaritatus*, the new species has smaller SVL and lacks the dorsal bony shield. The roughness of the dorsum distinguishes this new species from *B. alipioi*, *B. atelopoide*, *B. bufonoides*, *B. guarani*, *B. nodoterga*, *B. pitanga*, *B. vertebralis*, and *B. toby*, whose general texture of the dorsum is smoother. Whitish spots on the dorsum distinguishes *B. crispus* from *B. alipioi*, *B. atelopoide*, *B. bufonoides*, and *B. vertebralis*, whose general aspect of dorsum is orange without colored spots; from *B. guarani* which is orange with a dark brown stripe; from *B. nodoterga* which has a dark yellow dorsum; from *B. pitanga* which has a reddish dorsum; and from *B. toby* which has a greenish dorsum.

The scanning electron micrographs revealed that *B. crispus* has an intermediate condition of hyperossification compared with

FIG. 6.—Intrapopulation variation in color of *Brachycephalus crispus* sp. nov. in life, Parque Estadual da Serra do Mar, Núcleo Cunha, Municipality of Cunha, State of São Paulo, southeastern Brazil. Paratopotypes: (A) CFBH 27860, (B) CFBH 29830, (C) CFBH 29824, (D) CFBH 29822, (E) CFBH 29828, (F) CFBH 27861.

FIG. 7.—*Brachycephalus* species with osteoderms in dorsal surface of skin. (A) *B. crispus* sp. nov., (B) *B. nodoterga*, and (C) *B. margaritatus* (photo credit: I. Nunes).

other *Brachycephalus* species. In *B. crispus*, the spinous processes of sacral and presacral vertebrae are hyperossified as is the process of the fourth vertebra (Fig. 5). Hyperossification of the spinous processes and presacral vertebrae in *B. crispus* is similar to that seen in *B. alipioi*, *B. guarani*, *B. nodoterga*, *B. pitanga*, *B. toby*, and *B. vertebralis* (Haddad et al., 2010). The absence of hyperossification in the central portion of the skull distinguishes *B. crispus* from these species. In the new species, hyperossification is concentrated on the lateral and posterior portions of the skull; the nasal, sphenethmoid, and frontoparietals region lack hyperossification; and prootics are completely ornamented. *Brachycephalus crispus* differs from *B. ephippium, B. garbeana*, and *B. margaritatus* by the presence of a hyperossified dorsal shield in these latter species. Again, *B. brunneus*, *B. didactylus*, *B. ferruginus*, *B. hermogenesi*, *B. izecksohni*, *B. pernix*, and *B. pombali* lack hyperossification in the spinous processes and vertebrae.

The cleared and double-stained material revealed that *B. crispus* has small bony elements spread on the skin, characterized as osteoderms (Ruibal and Shoemaker, 1984; Campos et al., 2010). Osteoderms in *B. crispus* are generally <0.2 mm and are sparsely distributed on the skin of dorsum, including head and dorsal surface of arms and legs (Fig. 4). Osteoderms are also found in *B. nodoterga* (RBGC-C, personal observation) and *B. margaritatus* (Pombal and Izecksohn, 2011), but these species are not known to have as many osteoderms as *B. crispus* (Fig. 7).

Natural history.—Specimens of *B. crispus* were found in forest leaf litter at ≈800–1100 m elevation. The new species was found active by day and is locally abundant. When surveying 6-m^2 plots ($n = 4$) we found a mean (±1 SD) of 3.5 ± 2.1 individuals per plot. Average leaf litter depth in these plots was 8.6 ± 0.4 cm, air temperature was 20.8 ± 1.7°C, and relative humidity of the air was 78 ± 1.2%. Some individuals were found under downed timber where moisture is higher. Generally males were heard calling under the leaves, but they can be found calling exposed and active on the surface litter, especially on cloudy and rainy days. Males of *B. crispus* can perform visual signaling to hold territories in the reproductive season similar to that described for *B. ephippium* (Pombal et al., 1994). Females were also more active on days with higher humidity and were usually found exposed, walking on the leaf litter. We analyzed six reproductive females collected in October 2011. The mean SVL was 14.4 ± 0.6 mm and each female contained 9 ± 1.9 oocytes, distributed in both oviducts. Most of the females had two large, mature, depigmented oocytes in each oviduct measuring an average 2.0 × 1.9 mm (Fig. 8). Remaining eggs were generally smaller and embedded in a follicular mass. Our observations suggest that the oviposition in *B. crispus* should involve about four oocytes, resulting in a small clutch, similar to that described by Pombal (1999) for *B. ephippium*.

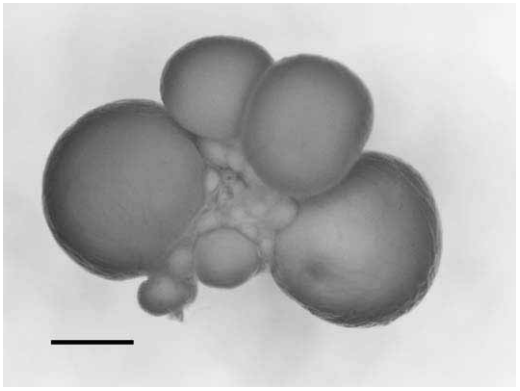

FIG. 8.—Oocytes found in the left oviduct of *Brachycephalus crispus* sp. nov. (paratopotype CFBH 29829), mature female. Scale bar = 1 mm.

Call description.—The advertisement call of *B. crispus* is a long and low-intensity buzz with a regular repetition of notes, with each note lasting 0.28 ± 0.02 s ($n = 100$; Fig. 9). The minimum and maximum frequencies were 3.5 ± 0.19 and 5.7 ± 0.17 kHz, respectively, with

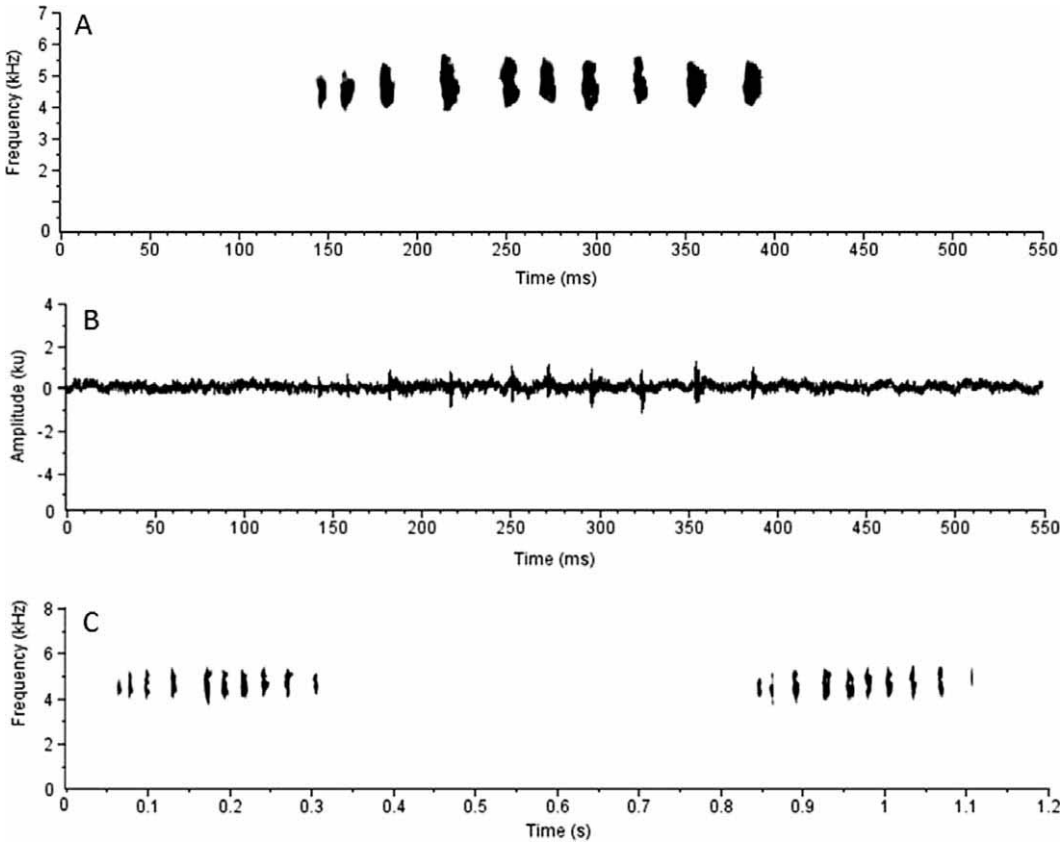

FIG. 9.—Advertisement call of *Brachycephalus crispus* sp. nov. (19 October 2011, 0930 h; air temperature = 15.8°C, air relative humidity = 77%). (A) Spectrogram of one call. (B) Oscillogram of one call. (C) Spectrogram of a sequence of two calls, showing the interval.

a dominant frequency of 4.6 ± 0.19 kHz (n = 100 for each parameter). Harmonics were not recorded. Each note can have 7–12 pulses ($\bar{X}$ = 10 ± 1.19, n = 100; Fig. 9A). Pulses have nearly the same frequency and pulse duration is 27.1 ± 4.0 ms (n = 517; Fig. 9B). Pulse rate is 17.4 ± 2.12 pulses/s (n = 5). Intervals between notes were 0.35 ± 0.02 s (n = 100; Fig. 9C). Males of *B. crispus* can call continuously for up to 5 min with a repetition rate of 1.67 ± 0.09 notes/s (n = 5). Call duration is variable and males usually stop calling in response to any disturbance.

Etymology.—The specific epithet is a Latin adjective meaning rouged or rugous. The name is used in allusion to the roughness of the dorsum of this species.

Distribution.—*Brachycephalus crispus* is known only from the type locality at Bacia B, Parque Estadual da Serra do Mar, Núcleo Cunha, Cunha municipality, São Paulo State, southeastern Brazil (Fig. 10).

DISCUSSION

Mountain slopes of Serra do Mar harbor high diversity and endemism of anurans in Brazil, which may be attributed to the habitat complexity associated with topographic and climatic variation (Cruz and Feio, 2007). Of the nine *Brachycephalus* species occurring in the state of São Paulo, five are restricted to the highlands of the Parque Estadual da Serra do Mar, one of the most important protected areas of the region. *Brachycephalus crispus* is one of these species, occurring geographically close to *B. guarani*, *B. pitanga*, *B. toby*, and *B. vertebralis* in the northeastern area of São

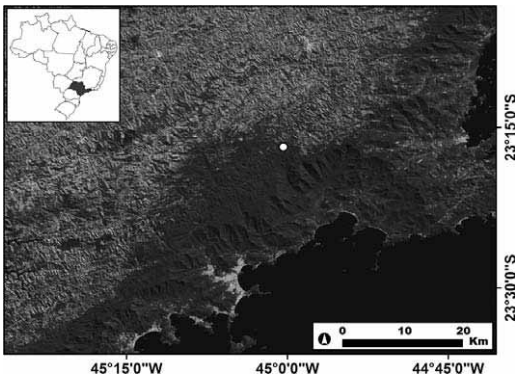

FIG. 10.—Type locality (white dot) of *Brachycephalus crispus* sp. nov. (23°15′14.8″S, 45°01′57.8″W; WGS84 datum), Parque Estadual da Serra do Mar, Núcleo Cunha, municipality of Cunha, São Paulo State (darkened area of inset), southeastern Brazil.

Paulo State. Despite this geographic proximity, highland species of *Brachycephalus* have more-restricted ranges and are completely isolated in different mountains. Considering the disjunctive biogeographic pattern, high diversity of species, and endemism within *Brachycephalus*, additional surveys in mountainous areas will probably increase knowledge of their taxonomic diversity and help to understand the biology of this genus.

Advertisement calls of *Brachycephalus* described in the literature were compared to that of the new species (Table 2). The structure of the call of *B. crispus* is similar to that described for *B. pitanga*. Despite having the same frequency range, *B. pitanga* has shorter notes with more pulses and higher dominant frequency compared with *B. crispus*. Also, the advertisement call of the new species differs from *B. ephippium* in spectral and temporal aspects and is very distinct from the acoustic parameters described for *B. hermogenesi*, *B. pernix*, and *B. tridactylus*. Aside from similarities between the advertisement calls of *B. tridactylus* and *B. hermogenesi*, the differences in advertisement calls are congruent with the recognized phylogenetic lineages in the genus (Clemente-Carvalho et al., 2011) and with the highland and lowland groups of species (Verdade et al., 2008; Napoli et al., 2011). Furthermore, the specific variations of temporal and spectral parameters of the advertisement call indicate that this kind of analysis can help to diagnose species. The use of vocalizations, allied to variation in general aspect of skin and coloration, morphometric variation, and skeletal characters can increase our comprehension on the diversity and evolution of this genus in the Brazilian Atlantic Rain Forest.

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TABLE 2.—Advertisement call variables of *Brachycephalus* species.

Call parameters	*Bcri*[1]	*Beph*[2]	*Bper*[3]	*Bher*[4]	*Bpit*[5]	*Btri*[6]
Note duration (s)	0.28	0.12	0.06	0.20	0.17	0.10
Internote duration (s)	0.35	0.13	0.14	—	—	—
Note repetition rate (notes/s)	1.67	—	—	1.09	—	—
Minimum frequency (kHz)	3.5	3.4	4.5	—	3.5	3.2
Maximum frequency (kHz)	5.7	5.3	6.7	—	5.7	6.4
Dominant frequency (kHz)	4.6	—	—	6.8	4.9	4.8
Number of pulses per note	10	12	3	—	11	—
Pulse duration in average (ms)	27.1	—	—	—	—	—
Pulse rate (pulses/s)	17.4	—	—	—	—	—

[1] *Brachycephalus crispus* sp. nov.
[2] *B. ephippium* (Pombal et al., 1994).
[3] *B. pernix* (Wistuba, 1998).
[4] *B. hermogenesi* (Verdade et al., 2008).
[5] *B. pitanga* (Araújo et al., 2012).
[6] *B. tridactylus* (Garey et al., 2012).

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Appendix

Specimens Examined

Brachycephalus alipioi: Espírito Santo, Vargem Alta MNRJ 26042, 26044–50, 26052–55 (paratopotypes), CFBH 24633–67, 25437–38; *B. guarani*: São Paulo, Prumirim, Ubatuba CFBH 27233 (holotype), 27231–32, 27234–35, 29563–64 (paratopotypes); *B. nodoterga*: São Paulo, Serra da Cantareira MZUSP 0975 (holotype), 0809, 112785–91, CFBH 28413, São Paulo, Santana do Parnaiba CFBH 9606, São Paulo, Ilhabela CFBH 7030–42, 7045–48; *B. pitanga:* São Paulo, São Luís do Paraitinga CFBH 16746 (holotype), 16747, 16750, 16755–56, 16758, 16760–16766 (paratopotypes), 24299, 09567; *B. vertebralis:* Rio de Janeiro, Parati CFBH 3416–17 (paratopotypes), São Paulo, Pico do Cuscuzeiro, Ubatuba 7907–08, 7910, 8361–63; *B. toby*: São Paulo, Pico do Corcovado, Ubatuba CFBH 20598 (holotype), 20581–91, 20593–97, 20599, 23004–06 (paratopotypes), 12902–03.