UNIVERSIDADE FEDERAL DO PARANÁ

CARINA RAUEN FIRKOWSKI

DIVERSIFICATION AND MICROENDEMISM IN MONTANE REFUGIA FROM THE BRAZILIAN ATLANTIC FOREST

CURITIBA

2013

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Dissertação apresentada como requisito parcial à obtenção do grau de Mestre em Ecologia e Conservação, no Curso de Pós Graduação em Ecologia e Conservação, Setor de Ciências Biológicas, Universidade Federal do Paraná.

Orientador: Marcio R. Pie

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ECOLOGIA E CONSERVAÇÃO

PARECER

Os abaixo-assinados, membros da banca examinadora da defesa da dissertação de mestrado, a que se submeteu **Carina Rauen Firkowski** para fins de adquirir o título de Mestre em Ecologia e Conservação, são de parecer favorável à **APROVAÇÃO** do trabalho de conclusão da candidata.

Secretaria do Programa de Pós-Graduação em Ecologia e Conservação.

Curitiba, 27 de fevereiro de 2014.

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Prof. Dr. Marcio Roberto Pie
Orientador e Presidente

Prof. Dr. Eduardo A. Botelho de Almeida
Membro

Prof. Dr. Ricardo Belmonte-Lopes
Membro

Visto:

Profª. Dra. Maria Regina Torres Boeger
Coordenadora do PPG-ECO

Centro Politécnico – Caixa Postal 19031 – CEP: 81531-990, Curitiba/PR
Telefones: (41) 3361-1595 – Fax (41) 3266-2042 – ecologia@ufpr.
www.ecologia.ufpr.br

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RESUMO

O estudo de padrões de diversidade e distribuição de espécies contribui para uma melhor compreensão da história evolutiva da Floresta Atlântica (FA) e dos mecanismos e processos responsáveis por gerar e moldar os padrões atualmente observados. Estudos baseados em espécies distribuídas em regiões de baixa altitude encontram suporte para instabilidade climática no sul da FA, com apenas recente expansão populacional a partir de refúgios climáticos presentes no sudeste da FA durante o Pleistoceno. Em contraste, evidências sugerem que as implicações das flutuações climáticas ao longo do passado evolutivo recente foram consideravelmente diferentes para espécies em regiões montanas da FA. A presente dissertação tem como objetivo investigar a diversidade dos anuros *Melanophryniscus* (Bufonidae) e *Brachycephalus* (Brachycephalidae), como modelo para compreender as dinâmicas evolutivas de especiação e endemismo em ambientes montanos no sul da FA. Dados genéticos compreendem fragmentos de seis genes: os mitocondriais 16S, citocromo b e ND2 e os nucleares β-fibrinogênio, tirosinase e RPL. Através de abordagem Bayesiana, inferimos as relações filogenéticas entre as populações amostradas, delimitando espécies e estimando tempos de divergência. Os resultados obtidos contribuem com 14 espécies de *Melanophryniscus* e 21 espécies de *Brachycephalus*, delimitadas e resolvidas filogeneticamente. Ambos os gêneros constituem clados que seguem padrão geográfico de diversificação. Datação para os clado montano de *Melanophryniscus* e *Brachycephalus* foi estimada em aproximadamente 3,5 e 4,5 milhões de anos, respectivamente. A divergência nas espécies atuais foi datada em 400-600 mil anos. Em resposta a pressões climáticas, populações ancestrais antes contínuas teriam se fragmentado, possibilitando evolução de novas espécies através de especiação alopátrica. A migração altitudinal é proposta como estratégia desses anuros para sobrevivência ao longo das flutuações climáticas do Pleistoceno, também contribuindo para o elevado grau de endemismo observado.

Palavras-chave: Filogeografia, Endemismo, Floresta Atlântica.

ABSTRACT

Studying patterns of diversity and distribution of species contributes to a better comprehension of the Brazilian Atlantic Forest (BAF) evolutionary history and the mechanisms and processes that have generated and modeled these patterns. Studies based in lowland species have found support for climatic instability over southern BAF, with only recent population expansion from Pleistocene climatic refugia present in southeastern BAF. However, evidence suggests that the implications of climatic fluctuations over the evolutionary past were considerably different for species of montane regions in southern BAF. The present thesis aims at investigating the anurans *Melanophryniscus* (Bufonidae) and *Brachycephalus* (Brachycephalidae) diversity as a model system to understand the diversification and endemism evolutionary dynamics in montane regions along southern BAF. Genetic data gathered for this study comprises six genes fragments: the mitochondrial 16S, cytochrome b and ND2 and the nuclear β-fibrinogen, tyrosinase and RPL. The inference of phylogenetic relationships, species delimitation and divergence time estimation is incorporated through a Bayesian analytical framework. The results contribute with 14 *Melanophryniscus* species and 21 *Brachycephalus* species, delimited and phylogenetically resolved. Both genera constitute clades that follow a geographic pattern of species diversification. Dating for the montane *Melanophryniscus* and *Brachycephalus* was estimated in approximately 3.5 and 4.5 million years, respectively. Divergence in the existing species was dated in 400-600 thousand years. As a response to climatic pressures, continuous ancestral populations would have been fragmented, allowing the evolution of new species through allopatric speciation. Altitudinal migration is proposed as these anurans strategy for survival across Pleistocene climatic fluctuations, contributing to the observed high level of endemism.

Key-words: Phylogeography, Endemism, Brazilian Atlantic Forest.

RESUMO EXPANDIDO

O processo evolutivo, contínuo e eterno, incorpora efeitos históricos e contemporâneos que interagem moldando a diversidade e distribuição de espécies. Um dos maiores desafios da ecologia e evolução é compreender quais mecanismos e processos são responsáveis por gerar e manter os padrões atualmente observados. Ao considerarmos um bioma hiper-diverso, complexo e ameaçado como a Floresta Atlântica (FA), essas questões constituem áreas fascinantes e em contínua expansão. Conhecimento e contribuições emergentes têm contribuído para uma melhor compreensão das dinâmicas de diversificação na FA. Estudos baseados em espécies distribuídas em regiões de baixa altitude encontraram suporte para a instabilidade climática no sul da FA durante o passado evolutivo, com apenas recente expansão populacional. Em contraste, evidências para espécies de média a elevadas altitudes sugerem um cenário de estabilidade climática no sul do bioma. No entanto, ainda pouco se sabe sobre os processos biológicos que permeiam os altos níveis de endemismo observados nesse bioma. Ainda, a amostragem defasada do bioma ao sul de São Paulo, particularmente em regiões montanas, é responsável por uma lacuna de conhecimento que compromete a compreensão plena da história evolutiva da FA e suas dinâmicas de diversificação. A complexidade topográfica, vegetacional e climática da FA, principalmente ao sul em sua distribuição, suporta inúmeros ambientes alto-montanos e de campos de altitude, isolados ao longo da Serra do Mar. Relatamos a descoberta de um notável número de espécies de anuros montanos e microendêmicos dos gêneros *Melanophryniscus* (Bufonidae) e *Brachycephalus* (Brachycephalidae), ocorrendo no sul da FA ao longo de um segmento de aproximadamente 250 km. *Melanophryniscus* é um gênero diverso de anuros, atualmente reconhecido por 26 espécies e de extensa distribuição geográfica que abrange o sudeste da América do Sul. Ao longo do sul da FA, sua ocorrência é caracterizada por duas espécies, sendo essas endêmicas e de distribuição restrita e isolada. O gênero *Brachycephalus* é endêmico à FA e atualmente compreende 20 espécies, das quais seis correspondem a espécies montanas de ocorrência ao longo do sul da FA. O presente estudo tem como objetivo investigar a diversidade de *Melanophryniscus* e *Brachycephalus* como modelo para compreender as dinâmicas evolutivas de especiação e endemismo em

ambientes montanos no sul da FA. Amostras de tecido foram obtidas de espécimes coletados de 16 populações de *Melanophryniscus* e 26 populações de *Brachycephalus* ao longo da Serra do Mar, do sul de São Paulo ao sul de Santa Catarina. O material amostrado foi processado para a extração de DNA, amplificação por PCR e sequenciamento. Para a inferência das relações filogenéticas entre as populações estudadas, fragmentos de seis genes foram sequenciados: os mitocondriais 16S, citocromo b e ND2 e os nucleares β-fibrinogênio, tirosinase e RPL. Para genes nucleares, posições heterozigotas encontradas tiveram suas fases determinadas através do algoritmo PHASE, implementado no programa DNASP. As sequências foram alinhadas utilizando o MUSCLE. Genes codificadores de proteína foram traduzidos para aminoácidos previamente ao alinhamento e então re-traduzidos para nucleotídeos. Sítios de alinhamento ambíguo foram removidos utilizando o método GBLOCKS. Para avaliação da ocorrência de recombinação nos dados genéticos, utilizamos o programa TOPALI v2.5. Dada a ausência de suporte para recombinação, alinhamentos completos foram considerados nas análises posteriores. O conjunto de dados final compreende 4043 pares de base para *Melanophryniscus* e 1853 pares de base para *Brachycephalus*, constituindo 125 e 129 sequências únicas, respectivamente. Os grupos externos considerados para *Melanophryniscus* incluem *Rhinella icterica, Dendrophryniscus berthalutzae* e representantes dos grupos de espécies *M. moreirae*, *M. tumifrons* e *M. stelzneri*. Para *Brachycephalus*, grupos externos compreendem *Ischnocnema guentheri*, *B. ephippium* e *B. hermogenesi*. A análise filogenética para estimativa das árvores de gene mitocondriais e nucleares foi realizada através de inferência Bayesiana com o programa MRBAYES 3.2. A delimitação de espécies foi explorada através de três abordagens Bayesianas diferentes: BGMYC, BP&P e STRUCTURE. A inferência da árvore de espécies foi realizada através do método coalescente *BEAST implementado no programa BEAST v.1.7.5. A história demográfica evolutiva foi explorada através do método *Extended Bayesian Skyline Plots* (*EBSP*), também implementado no programa BEAST. Resultados para as árvores de gene mitocondriais e nucleares suportaram a identificação de grandes clados entre as populações de *Melanophryniscus* e *Brachycephalus* estudadas, reconhecendo quatro e três clados, respectivamente. Os resultados indicam um padrão geográfico de diversificação, moldando as relações filogenéticas entre populações. Ambos os gêneros constituem clados que seguem

um padrão norte-sul de diversificação, refletindo-se em um agrupamento de linhagens próximas em perspectiva geográfica e filogenética. A abordagem de delimitação de espécies BGMYC indicou suporte concordante aos clados definidos nas árvores de gene, embora fragmentos mitocondriais e nucleares diferem em contribuição para a delimitação. Em concordância com os grandes clados definidos, as análises realizadas através do BP&P e STRUCTURE apresentaram maior resolução para a delimitação de espécies e suas relações filogenéticas. BP&P definiu suporte para o reconhecimento de 14 espécies de *Melanophryniscus* e 21 espécies de *Brachycephalus*. Os resultados do STRUCTURE definiram a partição dos indivíduos em 13 e 17 espécies. Estimativa dos tempos de divergência foi obtida através da inferência da árvore de espécies. Os resultados indicam a datação do clado montano de *Melanophryniscus* em aproximadamente 3,5 milhões de anos (Ma), abrangendo o Plioceno. No entanto, o processo de diversificação em espécies microêndemicas foi datado para o Pleistoceno, há aproximadamente 0,4 Ma. Para *Brachycephalus*, a datação para o clado montano foi estimada em aproximadamente 4,5 Ma, também compreendendo o Plioceno Inferior. O processo de diversificação nas espécies atuais é estimado em aproximadamente 0,6 Ma, abrangendo o Pleistoceno. Por fim, demografia evolutiva estimada através do *EBSP* indicou padrões congruentes entre populações e gêneros, com uma história de estabilidade populacional. Incorporando diferenças entre métodos na delimitação de unidades evolutivas, o presente estudo propõem o reconhecimento de 14 e 21 espécies montanas e isoladas de *Melanophryniscus* e *Brachycephalus*, respectivamente. Esses dados revelam a excepcional unicidade do sul da FA, abrigando um elevado número de espécies ao longo de uma área geograficamente restrita. Os resultados do presente estudo indicam o alto grau de estabilidade climática em áreas montanas no sul da FA durante as flutuações climáticas do Pleistoceno, suportando o conceito de refúgio climático. Durante o passado evolutivo, os extremos de temperatura experimentados entre períodos glaciais e interglaciais teriam sido responsáveis pela extinção das atuais espécies. No entanto, a reorganização espacial através de uma estratégia de migração altitudinal teria sido responsável por permitir sua persistência em envelopes climáticos estáveis e adequados a sobrevivência. As estimativas para tempos de divergência e demografia evolutiva para *Melanophryniscus* e *Brachycephalus* são unicamente consistentes com esse panorama evolutivo. A elevada diversidade de espécie endêmicas atualmente observada é resultado da

evolução sob um cenário de pressões ambientais e superação de extremos climáticos, constituindo uma riqueza e história genética de valor imensurável. Espera-se, com este estudo, contribuir com informações que subsidiem e suportem medidas para a conservação na região, com a preservação dessas unidades evolutivas únicas e das comunidades ecológicas das quais fazem parte.

SUMÁRIO

1 INTRODUCTION

Evolution, as a continuous and everlasting process of change, incorporates historic and contemporaneous effects, the interplay of which shapes the diversity and distribution of species. Understanding patterns of diversity and the uneven distribution of species over space and time has always been a major challenge in evolution (DARWIN 1859, WALLACE 1870, MACARTHUR & WILSON 1967, HAFFER 1969) and comprehending the mechanisms and processes that have generated and modeled these patterns in complex and threatened biomes remains a compelling issue (MORITZ *et al.* 2000, MORITZ 2002, WIENS & DONOGHUE 2004, TURCHETTO-ZOLET *et al.* 2013). However, relatively little is known about the biological processes that underlie phylogenetic endemism. The concept refers to the degree to which the evolutionary history of major intra-specific lineages is spatially restricted, leading to regions in which unique species or populations are concentrated (EVANS *et al.* 2004, ROSAUER *et al.* 2009). The underlying evolutionary drivers of endemism patterns include lineage differentiation, lineage maintenance through time, and range restriction (TRIBSCH & SCHÖNSWETTER 2003), and by extension, features both historic and contemporary biotic and abiotic effects. Apart from the entangled knowledge, the identification of areas of endemism plays a central role in defining conservation strategies, given its immeasurable value to biodiversity. Microendemism refers to an even more extreme degree of strict specificity, reflecting the distribution over a mosaic of isolated small ranged areas that provide suitable stable microclimates and protection from the unfavorable regional environmental conditions. The concepts of endemism and microendemism are also critical under the consideration of climatic fluctuations, imposing an even greater risk over habitat loss (RULL 2008) and leading to more pronounced levels of fragmentation, with eventual species extinctions and a severe ecological disequilibrium (MYERS *et al.* 2000).

The Brazilian Atlantic Forest (BAF) has been identified as one of the hotspots for biodiversity, with exceptional diversity and concentration of endemic species (MYERS *et al.* 2000). It covers areas of complex topography and is characterized by strong seasonality and environmental gradients. This high heterogeneity in terms of

climatic zones is closely associated with vegetation formations (FUNDAÇÃO INSTITUTO BRASILEIRO DE GEOGRAFIA E ESTATÍSTICA 1988, 1993), creating complex scenarios for species diversification. However, it currently comprises a merely 12.5% of the original vegetation, having been subjected to an increase of 23% on the deforestation rate when compared to data for the past three years (FUNDAÇÃO SOS MATA ATLÂNTICA & INSTITUTO NACIONAL DE PESQUISAS ESPACIAIS 2013). Conservation Units currently protect 24% of BAF remnants and 97% of these protected areas constitute reduced and isolated forest fragments. Threatened and in the absence more effective conservation efforts, much more will be lost within near future (TABARELLI *et al*. 2005, SANDERSON *et al.* 2003).

There is growing knowledge and contribution towards a better comprehension of the dynamics underlying species diversification in the BAF (e.g. CARNAVAL & MORITZ 2008, CARNAVAL *et al.* 2009, MARTINS *et al.* 2011, TURCHETTO-ZOLET *et al.* 2013). These and many other studies seek to disentangle the historical processes responsible for the observed high levels of diversity and emphasize the role of climatic fluctuations over the evolutionary past in shaping species diversification and persistence through time (LOPES *et al.* 2009, MARTINS *et al.* 2009, RESENDE *et al.* 2010, D'HORTA *et al.* 2011, MARTINS *et al.* 2011, FOUQUET *et al.* 2012, DO AMARAL *et al.* 2013, TONINI *et al.* 2013, BATALHA-FILHO *et al.* 2013). They focus particularly on the effects over species diversity and distribution of glacial and interglacial cycles during the Pleistocene, more often during the Last Glacial Maximum. Along the evolutionary past, the distribution of forested habitats along the tropics was spatially and temporally variable, due to a profound influence of Pliocene and Pleistocene climatic fluctuating conditions (HEWITT 2000, 2004). The established fine-scale habitat heterogeneity was likely a magnifying effect on regionalized and variable responses to climate (SUGUIO & MARTIN 1978, CARNAVAL & MORITZ 2008). Current species occurrence along the BAF supports this notion, exhibiting non-homogeneous patterns of distribution. For this biome, studies on patterns of distribution, diversity and genetic variability, based on anuran and reptile species, mostly consider taxa with low to mid-altitude range and large geographic distribution. Results regarding these biological data have evinced climatic stability in northern BAF, with only recent population expansion through southward colonization (PELLEGRINO *et al.* 2005, GRAZZIOTIN *et al.* 2006, CARNAVAL *et al.* 2009, FITZPATRICK *et al.* 2009, BRUNES *et al.* 2010, MARTINS 2011). However,

when comparing results for lowland species and montane species, evidence suggests that the implications of climatic fluctuations over the evolutionary past were considerably different between them, with the impacts of environmental change expected to be strongest in low dispersal species. Thomé *et al.* (2010) and Amaro *et al.* (2012) provided support for climatic stability in southern BAF, with persistence of older lineages in montane regions, and a more complex scenario for the evolution of endemism in this biome. However, when considering anurans, in a phylogenetic and evolutionary perspective, sampling in the BAF south of São Paulo state is still poor, especially in montane regions. Such lack of data is responsible for a knowledge gap that compromises a full and comprehensive understanding of the biome's evolutionary history and dynamics of diversification and endemism. Moreover, contemporary climatic heterogeneity should not be overlooked, as it could act as a potential driver of lineage range restriction based on current climatic conditions. An approach incorporating both historic and contemporary components will best contribute to the determination of species' relative susceptibilities towards future climatic changes and for instance subside conservation actions.

The topographic, vegetational and climatic complexities of the BAF, which are particularly pronounced in the south of its distribution, support numerous and isolated montane sites over relatively short geographic distances along the Serra do Mar mountain range. We report herein on the discovery of a remarkable number of microendemic montane anuran populations belonging to the genera *Melanophryniscus* (Bufonidae) and *Brachycephalus* (Brachycephalidae), throughout a segment of the southern BAF approximately 250 km long. In addition to the elevated level of microendemism, the studied populations also encompass substantial phenotypic diversity, particularly with respect to coloration and skin rugosity (PIE *et al.* in prep). The genus *Melanophryniscus* itself has a broad geographic distribution over southeastern South America, extending for over 1,000,000 km^2. Its range includes south and southeastern BAF; the wetlands and grasslands regions of Brazil to the inter-andean valleys in Bolivia; and areas across Paraguay and Uruguay down to central Argentina (FROST 2011). Along the southern BAF, its occurrence is characterized by altitude endemic species with restricted and isolated distribution in cloud forests and grasslands (LANGONE *et al.* 2008, STEINBACH-PADILHA 2008). There are currently 26 described species along the entire genus distribution, with only two recognized altitude species, *M. alipioi* and *M.*

vilavelhensis. Both species are also distinguished for their association to bromeliads for phytotelm breeding (LANGONE *et al.* 2008, STEINBACH-PADILHA 2008) and the evolution of this new reproductive mode for the genus was proposed as a result of their altitudinal isolation (LANGONE *et al.* 2008). *Brachycephalus* is endemic to the BAF, with distribution extending nearly 2.000 km along the biome, occurring in isolated mountain tops from Bahia state in northeastern Brazil to Santa Catarina state in southern Brazil. Its distribution encompassed the Serra da Mantiqueira and Serra do Mar mountain ranges, including 20 recognized species. Out of these, only six correspond to described species for the montane regions along southern BAF. *Brachycephalus* comprises cryptic and aposematic anurans, which live in the forest leaf litter and are active during the day. The genus is characterized by miniaturization, establishing a marked influence on their life histories. Given the reduced dispersal ability imposed by the reduced size, allopatric speciation is an essential aspect when considering their diversification dynamics.

The goal of this study is to use montane species of *Melanophryniscus* and *Brachycephalus* as a model system to understand diversification and endemism in montane regions along southern BAF. In particular, we test two hypotheses: (i) the possibility of altitudinal migration buffering montane species from the shifts in vegetation experienced by lowland species and resulting in relatively constant population sizes in their recent evolutionary past; and (ii) both genera sharing the same mechanisms underlying their diversification, leading to concordant lineage timing of the divergence. In order to provide a phylogeographic and demographic perspective on the evolutionary history, three approaches have been applied: (i) the evaluation of these montane frogs diversity, phylogenetic relationship inference and species delimitation; (ii) phylogenetic approaches for estimating isolation and divergence times between populations; and (iii) the exploration of populations' historic patterns of demographic evolution. The phylogeographic study and phylogeny dating, together with paleoclimatic information, provide a comprehensive overview about the temporal and spatial evolutionary dynamics and constitute valuable knowledge regarding the conservation of this unique region.

2 MATERIALS AND METHODS

2.1 SAMPLE COLLECTION, AMPLIFICATION AND SEQUENCING

Tissue samples were obtained from field-collected specimens of 16 populations of *Melanophryniscus* and 26 populations of *Brachycephalus* (TABLE 1, FIGURES 1 and 2). Voucher specimens are deposited in the herpetological collection Departamento de Zoologia da UFPR (DZUP), in Curitiba, Brazil.

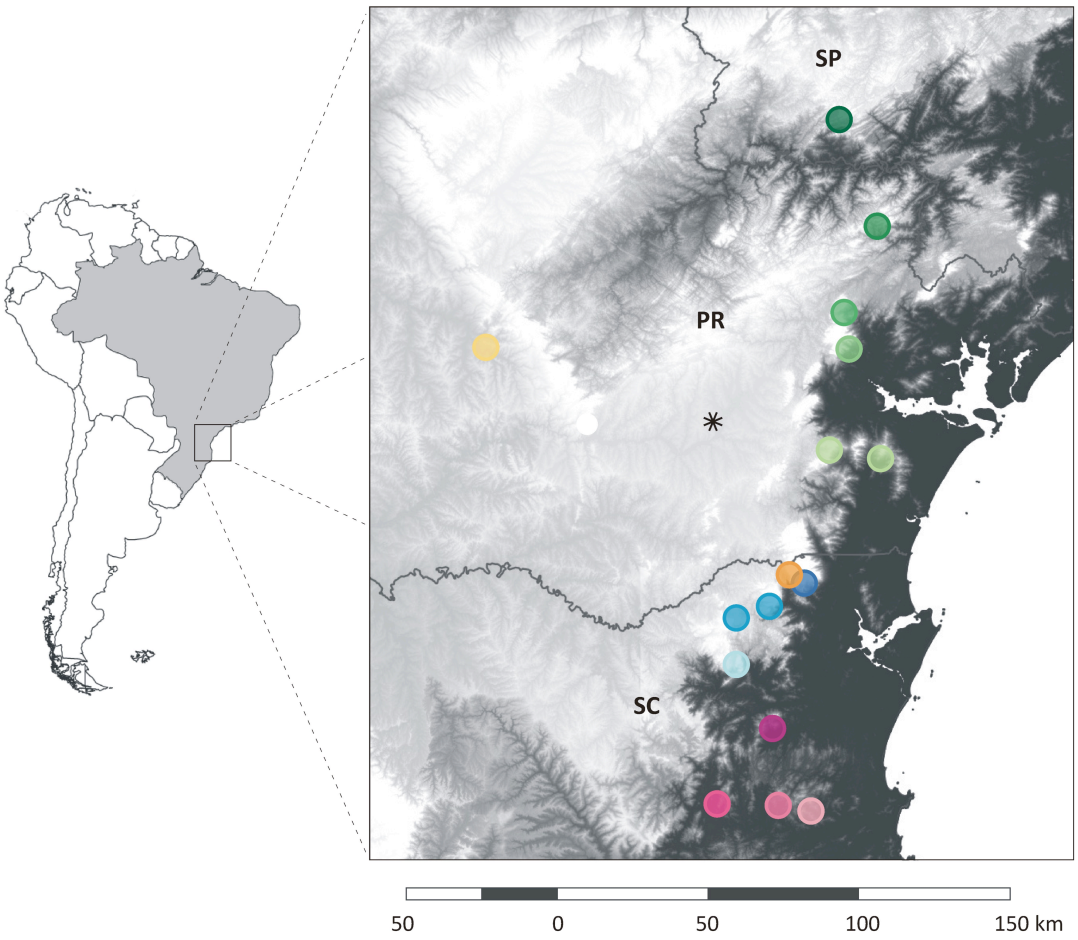

FIGURE 1. Map of southern Brazil with *Melanophryniscus* sampling localities. Coloring pattern is in congruence with diagram for species delimitation. The asterisk represents the city of Curitiba.

TABLE 1. Locality, coordinates and number of sampled individuals of *Melanophryniscus* and *Brachycephalus*. Superscript legend corresponds to described species.

Locality	Coordinates		Number of sampled individuals	
	Latitude	Longitude	*Melanophryniscus*	*Brachycephalus*
SÃO PAULO (SP)				
Apiaí	24°30'53"S	48°49'57"W	4	1
PARANÁ (PR)				
Vila Velha, Ponta Grossa	25°14'51"S	50° 0'16"W	1[a]	-
Caratuval, Andrianópolis	24°51'21"S	48°42'26"W	14	1
Serra do Capivari, Campina Grande do Sul	25° 7'52"S	48°49'10"W	18[b]	-
Tupipiá, Antonina	25°14'31"S	48°47'47"W	-	5
Pico Paraná, Campina Grande do Sul	25°14'59"S	48°48'22"W	9	2
Caratuva, Campina Grande do Sul	25°14'35"S	48°50' 2"W	-	11[c]
Camapuã, Campina Grande do Sul	25°15'60"S	48°50'16"W	-	11
Corvo, Quatro Barras	25°20'17"S	48°54'56"W	-	8
Serra da Graciosa, Quatro Barras	25°20'50"S	48°54'25"W	-	3
Fazenda Thalia, Balsa Nova	25°30'58"S	49°40'12"W	-	4
Anhangava, Quatro Barras	25°23'15"S	49° 0'12"W	-	7[d]
Marumbi, Morretes	25°27' 1"S	48°54'56"W	-	3[e]
Morro do Canal, Piraquara	25°30'55"S	48°58'56"W	-	3
Morro do Vigia, Piraquara	25°30'33"S	48°58'58"W	-	1
Serra da Igreja, Morretes	25°36'10"S	48°51'50"W	17	11[f]
Serra da Prata, Morretes	25°37'31"S	48°41'28"W	2	11
Serra das Canavieiras, Morretes	25°34'23"S	48°40'17"W	-	2
Serra Malhada, São José dos Pinhais	25°42'43"S	49° 2'49"W	-	5
Morro dos Perdidos, Guaratuba	25°53'22"S	48°57'23"W	-	4
Araçatuba, Tijucas do Sul	25°54' 6"S	48°59'45"W	-	11
SANTA CATARINA (SC)				
Quiriri, Campo Alegre	26° 1'15"S	48°59'44"W	6	6
Pedra da Tartaruga, Garuva	26° 0'21"S	48°55'25"W	-	16
Quiriri, Garuva	26° 1'53"S	48°57'23"W	16	-
Serra Queimada, Joinville	26° 6'49"S	49° 3'42"W	3	11
Morro da Tromba, Joinville	26°12'44"S	48°57'29"W	-	12
Castelo dos Bugres, Joinville	26°14' 4"S	49° 2'53"W	-	5
Vale dos Lagos, Joinville	26° 8'48"S	49°10'43"W	3	-
Morro do Boi, Corupá	26°18'28"S	49°10'8"W	2	-
Morro Boa Vista, Jaraguá do Sul	26°30'59"S,	49° 3'15"W	6	-
Morro Azul, Timbó	26°46'40"S	49°14'23"W	6	-
Morro do Cachorro, Blumenau	26°46'43"S	49° 1'48"W	11	8
Morro do Baú, Ilhota	26°47'59"S	48°55'49"W	7	8

a) *Melanophryniscus vilavelhensis*, b) *Melanophryniscus alipioi*, c) *Brachycephalus brunneus*, d) *Brachycephalus pernix*, e) *Brachycephalus ferruginus* and f) *Brachycephalus pombali*.

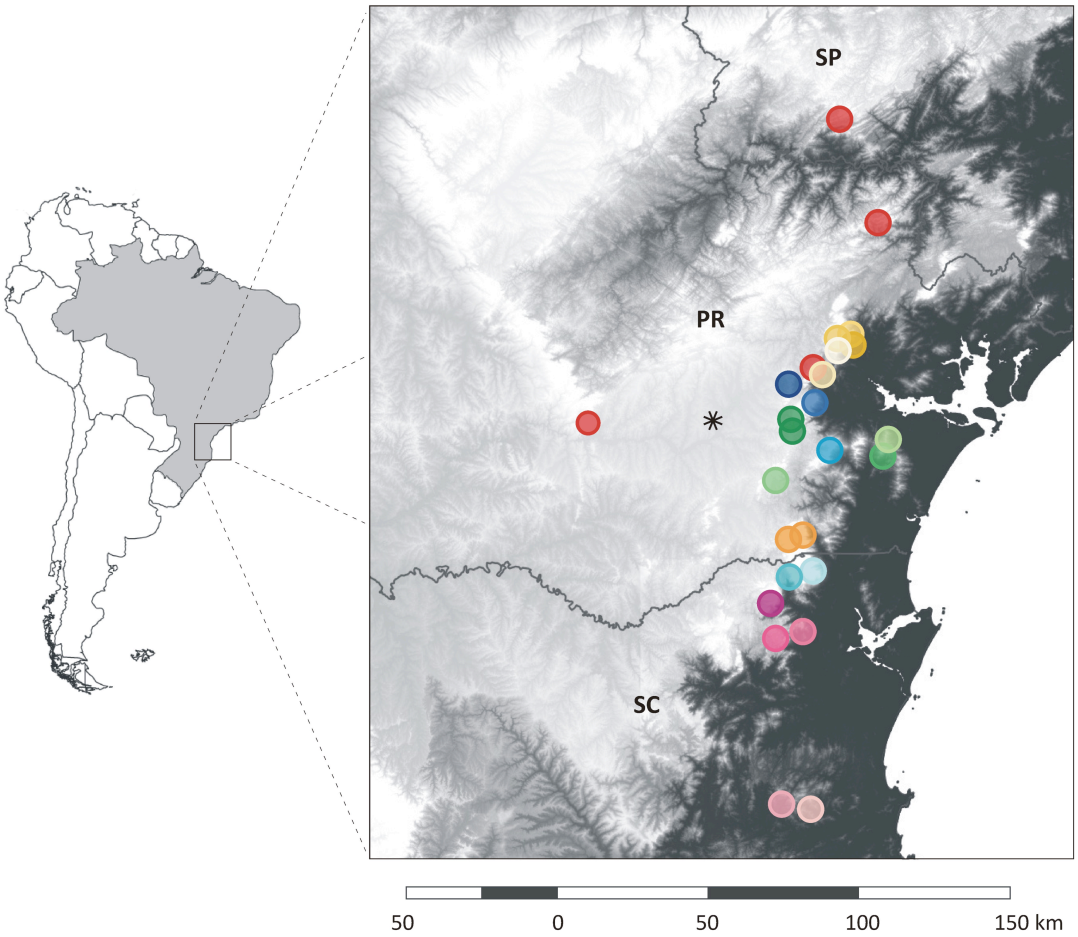

FIGURE 2. Map of southern Brazil with *Brachycephalus* sampling localities. Coloring pattern is in congruence with diagram for species delimitation. The asterisk represents the city of Curitiba.

Whole genomic DNA was extracted using PureLink ™ Genomic DNA kit (Invitrogen™, USA), according to the manufacturer's instructions. Three mitochondrial loci (16S, cytochrome *b* and NADH dehydrogenase subunit 2) and three nuclear loci (β-fibrinogen, Ribossomal Protein L3, and Tyrosinase exon 1; TABLE 2) were amplified by polymerase chain reaction (PCR). No single primer pairs were found to easily amplify a portion of the cyt*b* and ND2 genes in all *Brachycephalus* populations. Hence, for this genus, the final alignment comprised only four gene fragments. PCRs were performed in a final volume of 25 µL and consisted of 2U AmpliTaq DNA polymerase, 1X PCR buffer, 1.5 mM $MgCl_2$, 0.5 mM dNTPs, 1.0 µM each primer and approximately 30 ng of template DNA. Thermocycling conditions involved an initial denaturation at 94°C for 5 min followed by 35 cycles at 94°C for 1 min, 46-62°C for 35-50 s (TABLE 2) and 72°C for 1 min; and a final extension at 72°C for 5 min. PCR products were electrophoresed on 1.5%

TABLE 2. Amplified and sequenced genes, with primers' corresponding thermocycling condition for *Melanophryniscus* and *Brachycephalus*.

Gene	Primers	Sequence	Reference	Annealing duration and temperature	
				Melanophryniscus	*Brachycephalus*
16S	16SA-L 16SB-H	CGCCTGTTTATCAAAAACAT CCCGTCTGAACTCAGATCACGT	VENCES *et al.* 2000	56°C, 50s	
Cytochrome *b* (cyt*b*)	MVZ15-L Cytba-H	GAACTAATGGCCCACACWWTACG TCTTCTACTGGTTGWCCYCCRATTC	GOEBEL *et al.* 1999, MORITZ *et al.* 1992	58°C, 35s	
NADH dehydrogenase subunit 2 (ND2)	L4437 H5934	AAGCTTTCGGGCCCATACC ARGGTGCCAATGTCTTTGTGRTT	MACEY *et al.* 1997	58°C, 35s	
β-fibrinogen (β-fibr)	tmFibF1 tmFibR1	CCAGTAGTATCTGCCATTAGGGTTA TTCACAATGGCATGTTCTTCA	FITZPATRICK *et al.* 2009	52°C, 30s	56°C, 35s
Ribossomal protein L3 (RPL)	RPL35F RPL36RA	AAGAAGTCYCACCTCATGGAGAT AGTTTCTTTGTGTGCCAACGGCTAG	PINHO *et al.* 2010	46°C, 40s	
Tyrosinase exon 1 (Tyr)	Tyr1C Tyr1G	GGCAGAGGAWCRTGCCAAGATGT TGCTGGGCRTCTCTCCARTCCCA	BOSSUYT & MILINKOVITCH 2000	62°C, 35s	

agarose gels, with Kasvi Safer Dye fluorescent reagent for visualization under UV light. Positive PCR products were purified using PEG 8000. Sequencing reaction protocol was performed in a final volume of 10 µL, consisting of 0.7 µL ABI Prism® BigDye™ v3.1 (Applied Biosystems Inc., Foster City, CA), 1.0 µL 5X buffer and 1 µL each (3.2 pmol) primer and approximately 30 ng of template DNA. The cycle sequencing conditions included an initial denaturation step of 96°C for 1 min, followed by 35 cycles of 15 s at 96°C for denaturation, 15 s of annealing at 50°C and extension of 4 min at 60°C. Final DNA precipitation was conducted with isopropanol. Samples were sequenced in both directions and sequencing was performed in an ABI 3500 sequencer.

2.2 HAPLOTYPE PHASING, TEST OF RECOMBINATION AND ALIGNMENT

If heterozygous positions were detected for a given nuclear sequence, genotypes were phased in DNASP (LIBRADO & ROZAS 2009) running PHASE algorithm (STEPHENS *et al.* 2001, STEPHENS & DONNELLY 2003) under default conditions for 1,000 iterations. A threshold posterior probability of 0.85 on phase calls was used to determine the most probable reconstructed haplotypes, with one arbitrarily chosen for further analyses. Sequence alignment was carried by MUSCLE v3.8.31 (EDGAR 2004) using default settings. Protein-coding genes were translated into aminoacids prior to the alignment and back-translated into nucleotides for later analyses. Ambiguous sites were removed using GBLOCKS (CASTRESANA 2000, TALAVERA & CASTRESANA 2007), allowing gap positions under stringent parameter settings.

In order to evaluate the possible effects of recombination on the phylogenetic inferences of data, intralocus recombination breaking points were detected with TOPALI v2.5 by applying the difference of sums of squares (DSS) method (MILNE *et al.* 2008). Following TOPALI authors' suggestion for parameter configuration, the considered values were those that maximized resolution and congruency within genes, while accounting for diminished statistical noise and signal stemming from confounding diverged region (MCGUIRE & WRIGHT 2000, HUSMEIER *et al.* 2005). Analyses were ran with step size set at 2 and 10, with evaluation of values 10, 50,

100, 150, 200, 300, 400 and 500, according to data. No evidence of recombination was detected in *Melanophryniscus* dataset, regardless of parameters settings. For *Brachycephalus*, TOPALI results were not consistent in breaking points detection across step and window size configurations, leading to the conclusion of false positive results. Given absence of supported evidence for recombination, full datasets were retained for analyses.

The resulting final alignments are 4043 bp long for *Melanophryniscus* and 1853 bp for *Brachycephalus*. The data set comprised 125 unique sequences for *Melanophryniscus* populations and 129 unique sequences for *Brachycephalus* populations (GenBank accession numbers XXXXX-XXXXXX). Final alignments are available on TreeBASE (XXXX; http://treebase.org/).

2.3 OUTGROUPS

Given that *Melanophryniscus* is currently considered the sister group of all remaining bufonids (FROST *et al.* 2006), we included *Rhinella icterica* and *Dendrophryniscus berthalutzae* as outgroups, as well as representatives of the *M. moreirae*, *M. tumifrons,* and *M. stelzneri* species groups. In the case of *Brachycephalus*, outgroup taxa included the brachycephalid *Ischnocnema guentheri*, given that its genus is currently understood to be the sister group of *Brachycephalus* (HEDGES *et al.* 2008). We also included *B. ephippium* and *B. hermogenesi* as representatives of two distinct lineages within *Brachycephalus* (CLEMENTE-CARVALHO *et al.* 2011, PIE *et al.* 2013). Outgroup sequences for each gene fragment were obtained as indicated in TABLE 3.

2.4 GENE TREE ESTIMATION

Phylogenetic analysis for mitochondrial and nuclear gene tree estimation was performed under Bayesian inference with MRBAYES 3.2 (RONQUIST *et al.* 2011).

TABLE 3. Locality and reference for sequenced outgroup samples and accession number for data obtained from GenBank.

Species	Locality	Reference	Voucher	GenBank Accession Nos.					
				16S	cyt*b*	ND2	RPL	β-fibr	Tyr
Melanophryniscus									
Bufo ictericus	Carapicuiba, SP	PRAMUK 2006	-	DQ158462	-	-	-	-	-
Bufo ictericus	Atibaia, SP	MACIEL *et al.* 2010	-	-	HM159230	-	-	-	-
Bufo ictericus	Bom Jardim da Serra, SC	THOMÉ *et al.* 2010	-	-	-	GU907269	-	-	-
Bufo ictericus	São Paulo, SP	SEQUEIRA *et al.* 2011	-	-	-	-	JN594606	-	-
Bufo ictericus	Joinville, SC	Present study	DZUP 243	-	-	-	-	-	-
Dendrophryniscus berthalutzae	Joinville, SC	Present study	DZUP 259	-	-	-	-	-	-
M. gr. moreirae	Itatiaia, SP	Present study	DZUP 499	-	-	-	-	-	-
M. gr. tumifrons	Rio Grande do Sul	Present study	DZUP 500	-	-	-	-	-	-
M. gr. stelzneri	San Luís, Argentina	Present study	DZUP 501	-	-	-	-	-	-
Brachycephalus									
Ischnocnema guentheri	Morretes, PR	Present study	DZUP 448	-	-	-	-	-	-
Brachycephalus hermogenesi	Piedade, SP	CLEMENTE-CARVALHO *et al.* 2011	-	-	-	-	-	-	HQ435738
Brachycephalus ephippium	Ilhéus, BA	Present study	DZUP 498	-	-	-	-	-	-

Models of molecular evolution were selected in PARTITIONFINDER (LANFEAR *et al.* 2012), based on the corrected Akaike information criterion (AKAIKE 1974). MRBAYES 3.2 analyses consisted in two independent runs, each with four chains, sampling every 1000th generation for $5x10^7$ generations. Each analysis was run twice to ensure convergence. Stationary distribution and effective sampling sizes (ESS) for all parameters were checked using the software TRACER v1.5 (RAMBAUT & DRUMMOND 2009). We disregarded 20% of trees as burn-in and using the remaining trees we estimated the maximum clade credibility consensus topology using median heights in TREEANNOTATOR v1.7.5 (DRUMMOND & RAMBAUT 2007, DRUMMOND *et al.* 2012). Congruence between consensus trees from both posterior samples was confirmed.

2.5 SPECIES DELIMITATION

Bayesian species delimitation was explored under three different approaches: BGMYC (REID & CARSTENS 2012), Bayesian Phylogenetics and Phylogeography v2.0 (BP&P; RANNALA & YANG 2003, YANG & RANNALA 2010) and STRUCTURE v2.3 (PRITCHARD *et al.* 2000). First, we applied the Bayesian GMYC model implemented in the R package BGMYC (REID & CARSTENS 2012). The analysis was performed separately for each gene fragment, using a subset of 100 random trees sampled from a posterior distribution of phylogenetic analyses BEAST v.1.7.5 (DRUMMOND & RAMBAUT 2007, DRUMMOND *et al.* 2012). Ultrametric gene tree estimation analysis was each performed with two independent runs of $5x10^7$ generations, sampling every 1000th generations and omitting the first 20% samples as burn-in. A speciation Yule process prior and an uncorrelated lognormal model of rate variation were used in the analysis. Convergence, stationary distribution of chains and ESS for all parameters were evaluated using TRACER v1.5. Consensus topology was obtained with TREEANNOTATOR v1.7.5, under the same previous configurations. In the BGMYC R package, Markov chain Monte Carlo (MCMC) chains were run for 10^5 generations, discarding the first $5x10^4$ as burn-ins and sampling every 100th generation.

BP&P delimits species using reversible-jump MCMC (rjMCMC). The method accommodates the species phylogeny and coalescent process of lineage sorting while calculating the posterior probabilities of potential species delimitations. A guide tree for species delimitation was obtained from gene trees estimate, with incongruences being resolved under this analytical framework. A gamma prior of (2, 1000) was assigned both for population size parameters (θ_s) and the age of the root in the species tree (τ_0), while the other divergence time parameters were assigned the Dirichlet prior (YANG & RANNALA 2010). To evaluate the impact of prior on θ_s and τ_0 we tested the following different gamma prior configurations: (i) (1, 10) for both θ_s and τ_0; (ii) (2, 2000) for both θ_s and τ_0; and (iii) (1, 10) for θ_s and (2, 2000) for τ_0. Overall species delimitation results were not affected by different prior settings. Heredity was adjusted for mitochondrial and nuclear genes accommodating in the model differences in mutation rates among loci. To ensure convergence and stability, both implemented algorithms were tested with three different starting trees: one-species model, fully resolved tree and one starting tree in between. Virtually no impact of differences in algorithm or starting tree was recovered. The effect of different fine-tuning parameters for ε, α and m were evaluated when needed. Fine-tuning parameters for optimal acceptance proportion were adjusted by trial and error, accordingly.

To assess population structure and better understand the genetic differences of geographically distinct populations, we applied the Bayesian clustering method implemented in STRUCTURE v2.3. To identify the number of distinct populations (K) and assign individuals to these clusters, a no-admixture model with correlated frequencies was specified (PRITCHARD *et al.* 2000). MCMC was run for 10^7 generations and 10^6 iterations for burn-in, testing the range of values $1 \leq K \leq 16$ for *Melanophryniscus* and $1 \leq K \leq 26$ for *Brachycephalus*. The most likely value of K was determined by comparing Ln $P(X|K)$ for the lowest value of K which effectively captured the major structure in data. The output barplot for individual membership to clusters was visualized using DISTRUCT1.1 (ROSENBERG 2004).

2.6 PHYLOGENETIC ANALYSIS FOR THE SPECIES TREE

Inference of population-level phylogeny was accomplished through a multispecies coalescent method to estimate the species tree based on multi-locus data under the *BEAST option implemented in BEAST v.1.7.5. Prior designation of species was based on sampling locality, considering previous results for species delimitation support under BGMYC, STRUCTURE v2.3 and BP&P v2.0. The analysis consisted of two independent runs, each of $2x10^8$ generations, sampling every 4000th generations and omitting the first 20% samples as burn-in. For molecular dating of divergence time estimation, calibration was based on the estimate for the mutation rate for ND2 of 0.957% divergence per million years (CRAWFORD 2003). The root of the tree and speciation times were estimated through an uncorrelated relaxed-clock model, with a Yule process set as speciation prior. For *Brachycephalus*, no molecular calibration was applied, dating rates were purely set to be estimated based on data. Yet, recovered estimations were congruent between genera. Convergence, stationary distribution of chains and ESS for all parameters were evaluated using TRACER v1.5. The consensus topology was obtained with TREEANNOTATOR v1.7.5, under the same previous configurations.

2.7 HISTORICAL DEMOGRAPHIC PATTERN

To explore the demographic evolutionary history of *Melanophryniscus* and *Brachycephalus* populations, we applied the coalescent-based Extended Bayesian Skyline Plots (EBSP; HELED & DRUMMOND 2008), implemented in BEAST v.1.7.5. Sequence partitioning, substitution models, divergence rate and molecular clock were the same as those used in BEAST for previously described analysis. We ran the analysis for $5x10^7$ generations, sampling every 1000th generations and discarding the first 20% as burn-in. Convergence, stationary distribution of chains and ESS for all parameters were evaluated using TRACER v1.5. Plots were generated and visualized in R 3.0.1 (R DEVELOPMENT CORE TEAM 2013).

3 RESULTS

3.1 GENE TREES

Phylogenetic gene trees analysis of mitochondrial and nuclear loci provided support in recognizing major clades. Main consistently and well-supported clades were identified when considering node posterior probability and concordance between gene trees.

For *Melanophryniscus* (FIGURE 3), results support four main clades: a) *M. vilavelhensis* and Quiriri – Campo Alegre; b) Apiaí, Caratuval, *M. alipioi*, Itapiroca, Serra da Prata and Serra da Igreja; c) Quiriri – Garuva, Joinville, Serra Queimada, and Morro do Boi; and d) Morro do Boa Vista, Morro Azul, Morro do Cachorro and Morro do Baú. Clade a) was identified as sister to b), c) and d), with clades b) and c) (together named northern clade) being sisters to d) (namely southern clade). At a finer scale, gene trees were unable to resolve relationships among populations with the exception of Morro do Boa Vista as sister to Morro Azul, Morro do Cachorro and Morro do Baú in 16S and ND2 gene trees. In *Melanophryniscus* dataset, one incongruence in a well-supported node was recovered between different gene trees. With the exception of Tyr gene tree, populations from Quiriri – Garuva, Joinville, Serra Queimada and Morro do Boi constituted a supported clade in all remaining gene trees.

In *Brachycephalus* (FIGURE 4), three major groups were recognized: a) Apiaí, Caratuval, Thalia and Corvo; b) Araçatuba, Morro dos Perdidos, Pico Paraná, *B. brunneus*, Tupipiá, Serra da Graciosa, Camapuã, Morro do Canal, Morro do Vigia, *B. izecksohni*, Serra Malhada and Canasvierias (northern clade); and c) *B. pernix, B. pombali*, *B. ferruginus*, Quiriri, Pedra da Tartaruga, Serra Queimada, Castelo dos Bugres, Morro da Tromba, Morro do Cachorro and Morro do Baú (southern clade). Clade a) was identified as sister to clades b) and c). For *Brachycephalus* gene tree estimates, one incompatibility was also defined. The gene tree for 16S supports a node that joins populations from Quiriri, Serra Queimada and Castelo dos Bugres. This configuration separates Quiriri from Pedra da Tartaruga, *B. pernix*, *B. ferruginus*

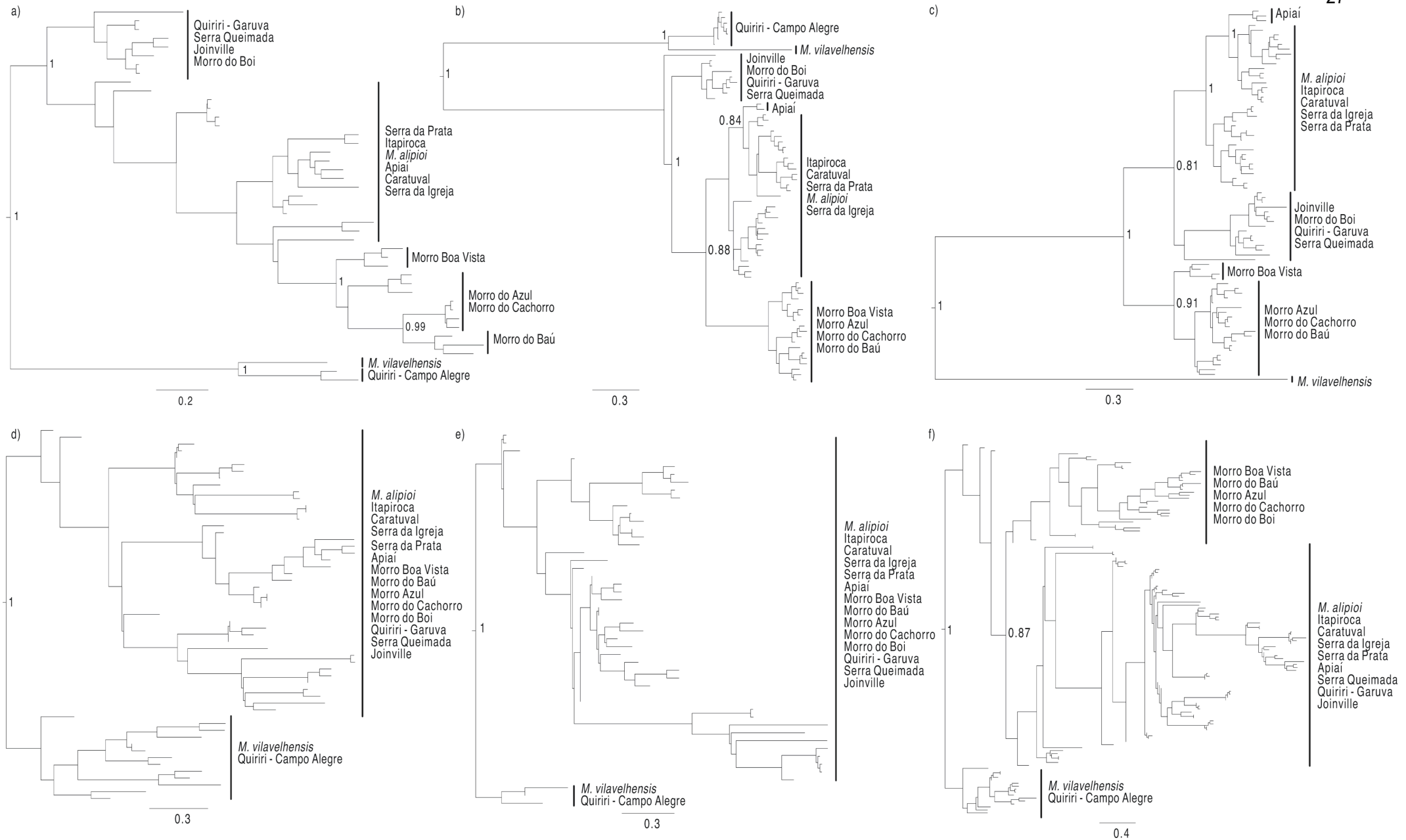

FIGURE 3. *Melanophryniscus* genetrees for a) 16S, b) cyt*b*, c) ND2, d) RPL, e) β-fibr and f) Tyr. Node values are Bayesian posterior probability for supported clades and branch lengths are shown in substitution per site.

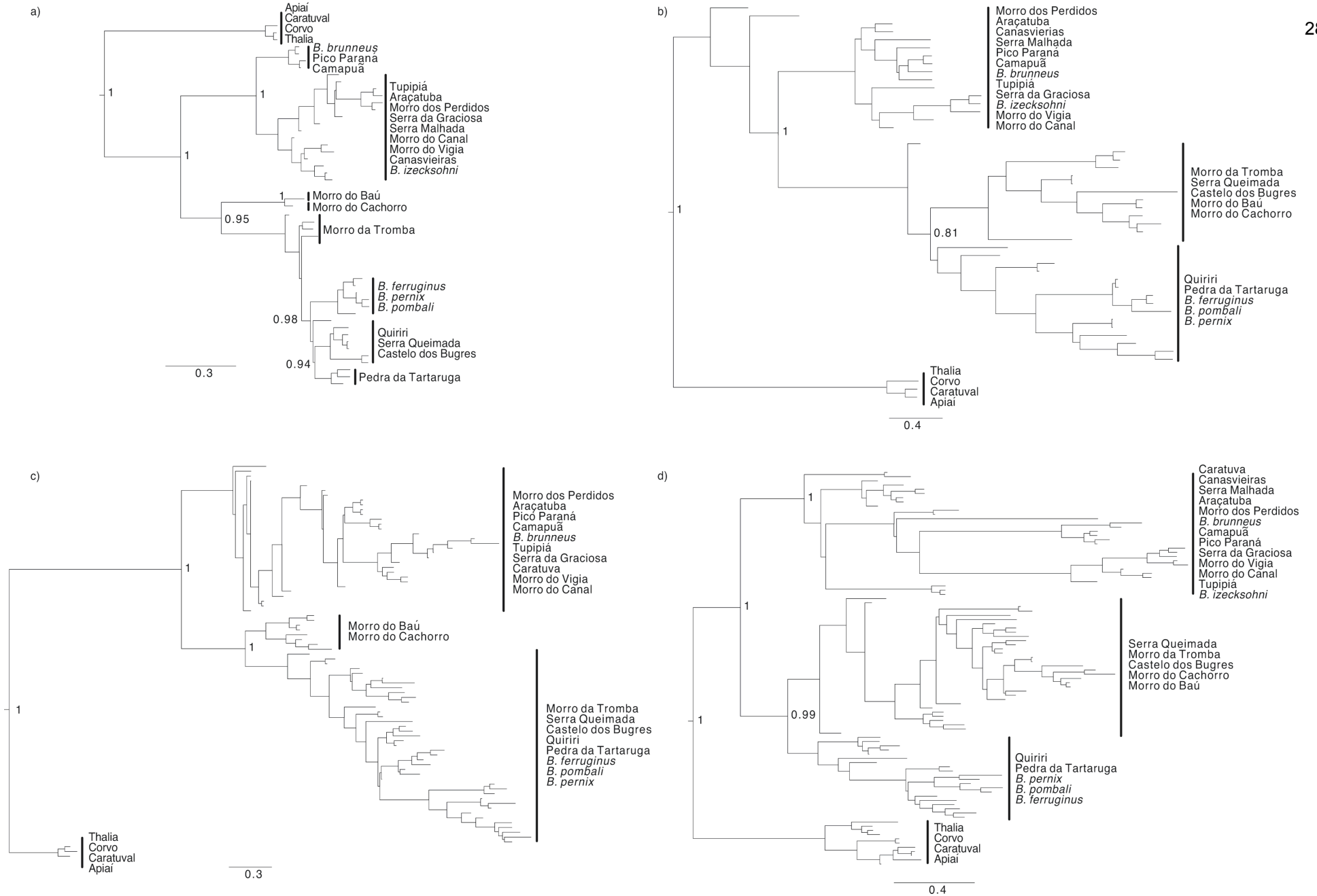

FIGURE 4. *Brachycephalus* gene trees for a) 16S, b) RPL, c) β-fibr and d) Tyr. Node values are Bayesian posterior probability for supported clades and branch lengths are shown in substitution per site.

and *B. pombali* and splits Serra Queimada and Castelo dos Bugres from Morro da Tromba. However, both clades constitute well supported nodes in RPL, β-fibr and Tyr gene trees.

3.2 PHYLOGEOGRAPHIC STRUCTURE AND SPECIES DELIMITATION

Concordance between all species delimitation approaches supported as unique phylogenetic units *M. vilavelhensis* and *Melanophryniscus* populations from Quiriri – Campo Alegre, Apiaí, Morro Boa Vista and Morro do Baú (FIGURE 5). For *Brachycephalus*, all species delimitation analysis joined populations from (i) Apiaí, Caratuval, Corvo and Thalia, and (ii) Araçatuba and Morro dos Perdidos. Different methods congruently partitioned populations from Quiriri, Pedra da Tartaruga, Serra Queimada, Castelo dos Bugres and Morro da Tromba (FIGURE 6).

When considering the Bayesian implementation of the GMYC model, although the analysis exhibits low power to distinguish relationships between individual populations, results supported delimitation of the major clades identified in the gene trees. Mitochondrial and nuclear gene trees differ in resolution and contribution in resolving different relationships among tips. For both genera, the mitochondrial locus 16S provided higher support for delimitation of individual species. *Melanophryniscus* and *Brachycephalus* results containing species-level probabilities > 0.95 are shown in FIGURES 5 and 6, respectively.

The Bayesian species delimitation analysis in BP&P supported 14 distinct genetic units in *Melanophryniscus* and 21 in *Brachycephalus*, recovering the highest number of species from the data among all applied methods. For *Melanophryniscus*, supported tips in the guide tree only exclude speciation events between Serra da Prata – Serra da Igreja and Joinville – Serra Queimada. In *Brachycephalus*, BP&P supported the guide tree with speciation in all nodes, with the exception of speciation events between individuals from (i) Apiaí, Caratuval, Corvo and Thalia, (ii) Araçatuba and Morro dos Perdidos and (iii) a Morro do Vigia and Morro do Canal. Supported species predominantly recovered posterior probability of 1.0 (FIGURES 7 and 8). When considering results for *Melanophryniscus*, posterior probabilities lower than 1.0 did not match incongruences for delimited species between either applied methods.

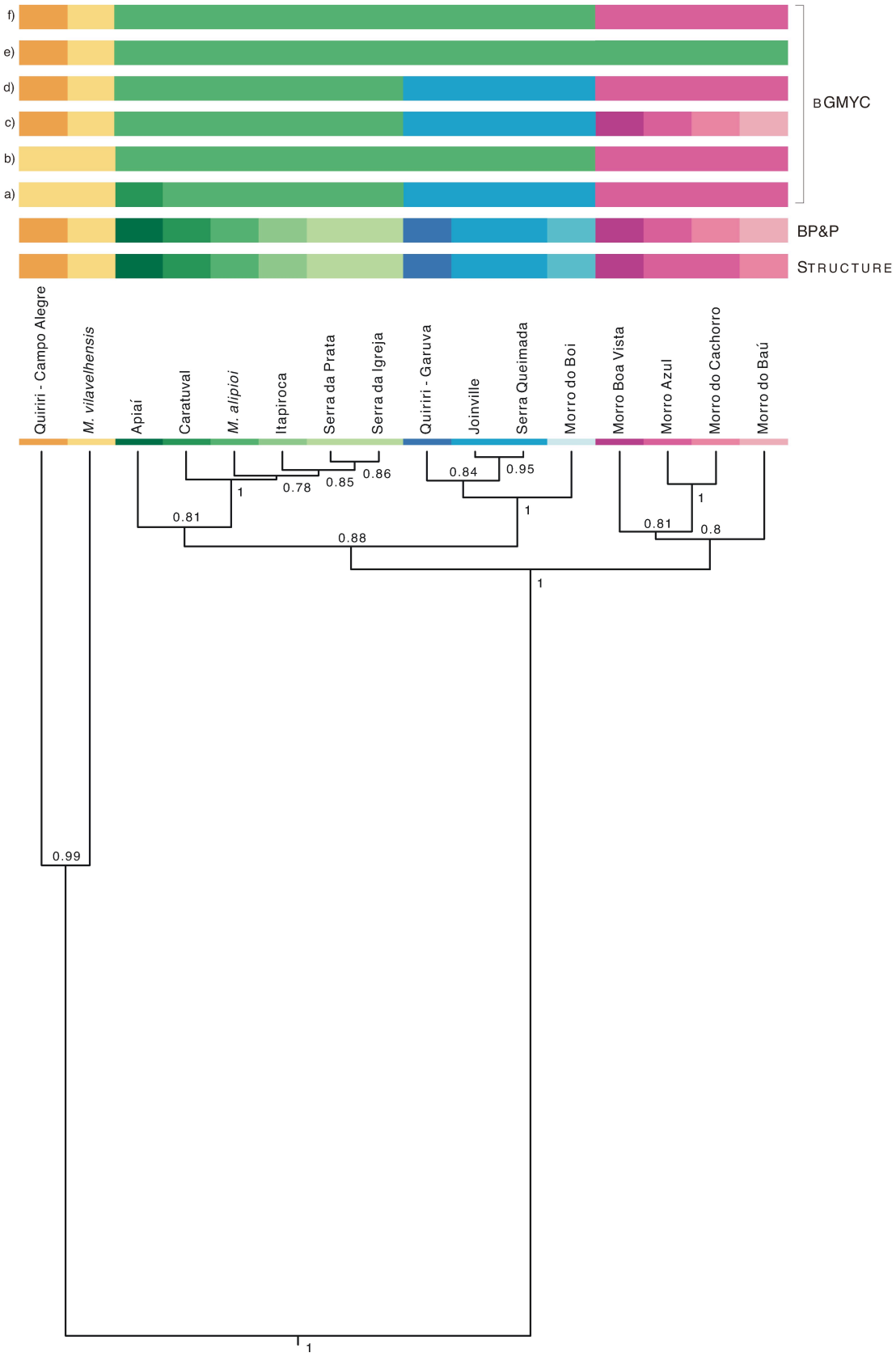

FIGURE 5. *Melanophryniscus* diagram for species delimitation. Summarized results from species delimitation analyses under BGMYC, STRUCTURE and BP&P, represented on a *BEAST tree with posterior probabilities shown under each node. Values in parenthesis represent the number of delimited lineages using each method. Colored bars correspond to partitioning of populations, with each grouping represented by a different color. Colored lines above the phylogeny represent congruency among approaches and proposed species delimitation. For BGMYC legend represents a) 16S, b) cyt*b*, c) ND2, d) RPL, e) β-fibr and f) Tyr.

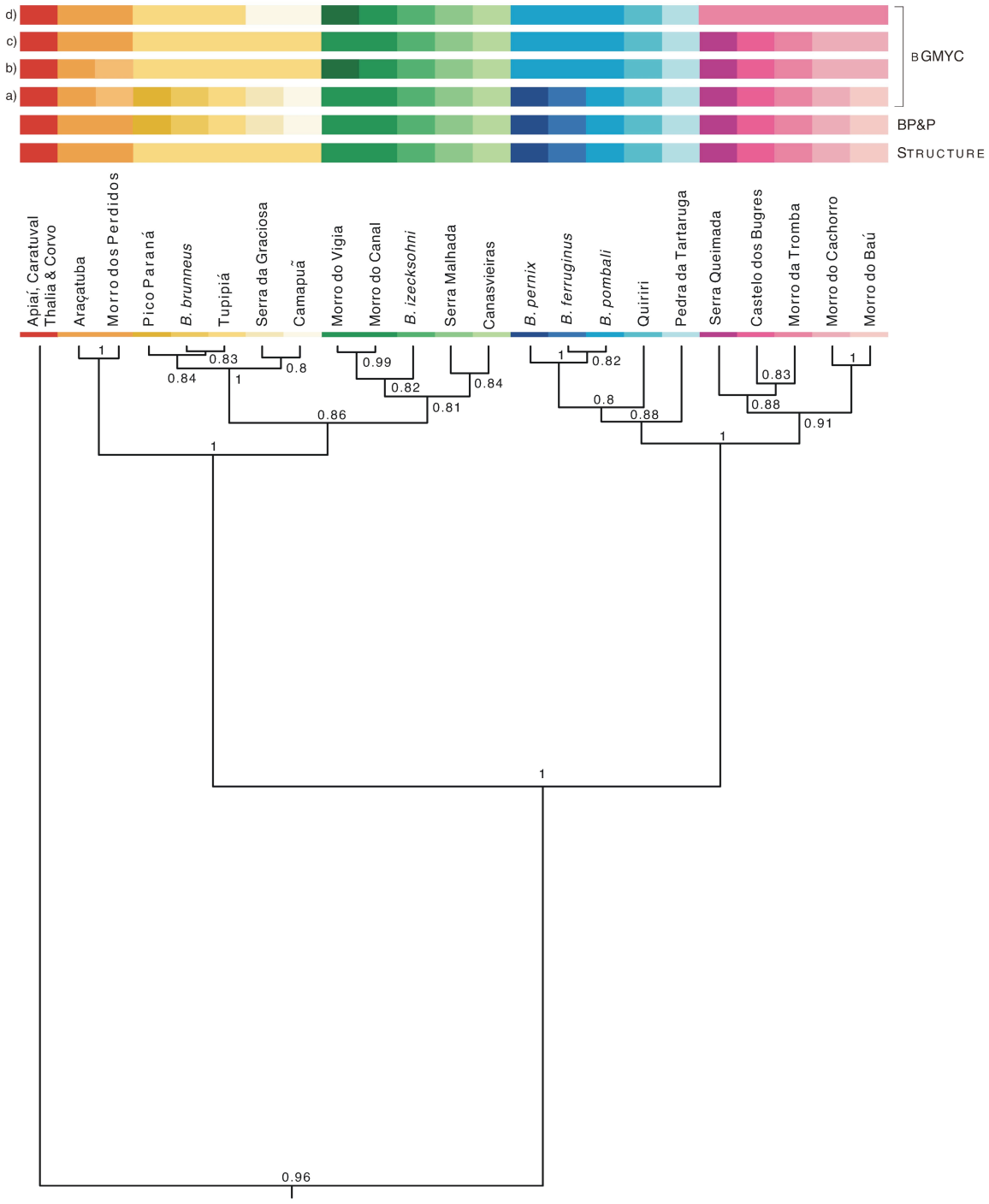

FIGURE 6. *Brachycephalus* diagram for species delimitation. Summarized results from species delimitation analyses under BGMYC, STRUCTURE and BP&P, represented on a *BEAST tree with posterior probabilities shown under each node. Colored bars correspond to partitioning of populations, with each grouping represented by a different color. Colored lines above the phylogeny represent congruency among approaches and proposed species delimitation. For BGMYC, legend represents a) 16S, b) RPL, c) β-fibr and d) Tyr.

All performed runs recovered posterior probability of 1.0 for these populations and only the node for Quiriri – Garuva yield a lower posterior of 0.98. In *Brachycephalus*, only conflicting results between applied methods retrieved speciation probability lower than 1.0. These diverging results supported the distinct genetic entities with posterior values ranging from 0.95-0.99 and include *B. brunneus* and populations from Pico Paraná, Tupipiá, Serra da Graciosa and Camapuã.

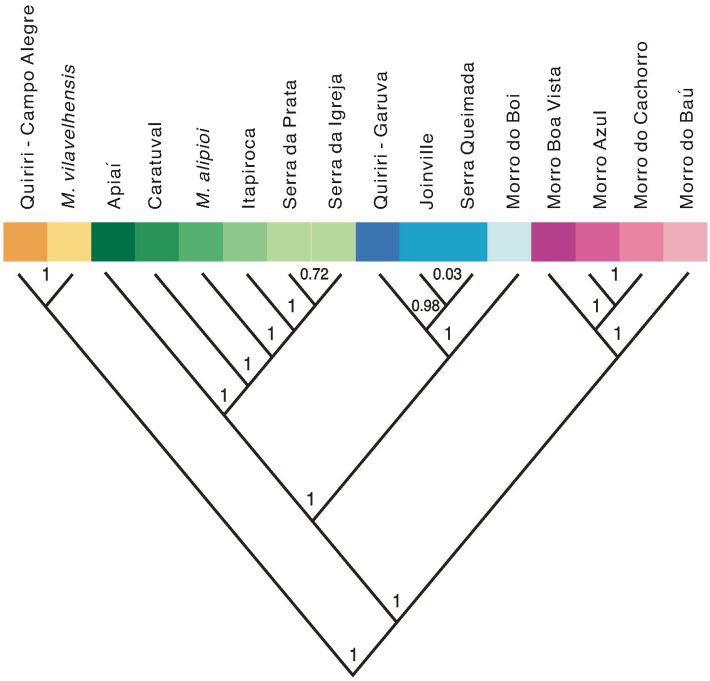

FIGURE 7. BP&P species delimitation for *Melanophryniscus*. Number above nodes represents speciation probabilities values. Coloring pattern is in congruence with diagram for species delimitation.

STRUCTURE analysis results supported individuals' partitioning into 13 *Melanophrynicus* lineages and 17 *Brachycephalus* lineages. In order to evaluate the potential reduced power of reliably in inferring the optimal number of clusters and define the most likely value of *K*, we compared Ln *P*(*X*|*K*) for the range of values under consideration (FIGURES 9 and 10). For *Melanophryniscus* and *Brachycephalus*, we include the barplot for individual membership to clusters corresponding to $8 \leq K \leq 13$ and $15 \leq K \leq 19$, respectively (FIGURES 11 and 12). Most individuals had very high posterior probability assignment to a cluster. Exceptions as source of error include (i) individual membership split into different clusters that do not share haplotypes due to modeling from a unique or few samples and (ii) cases of clusters splitting into two or more groups, with every individual being admixed from each cluster, as a response to low population genetic variability.

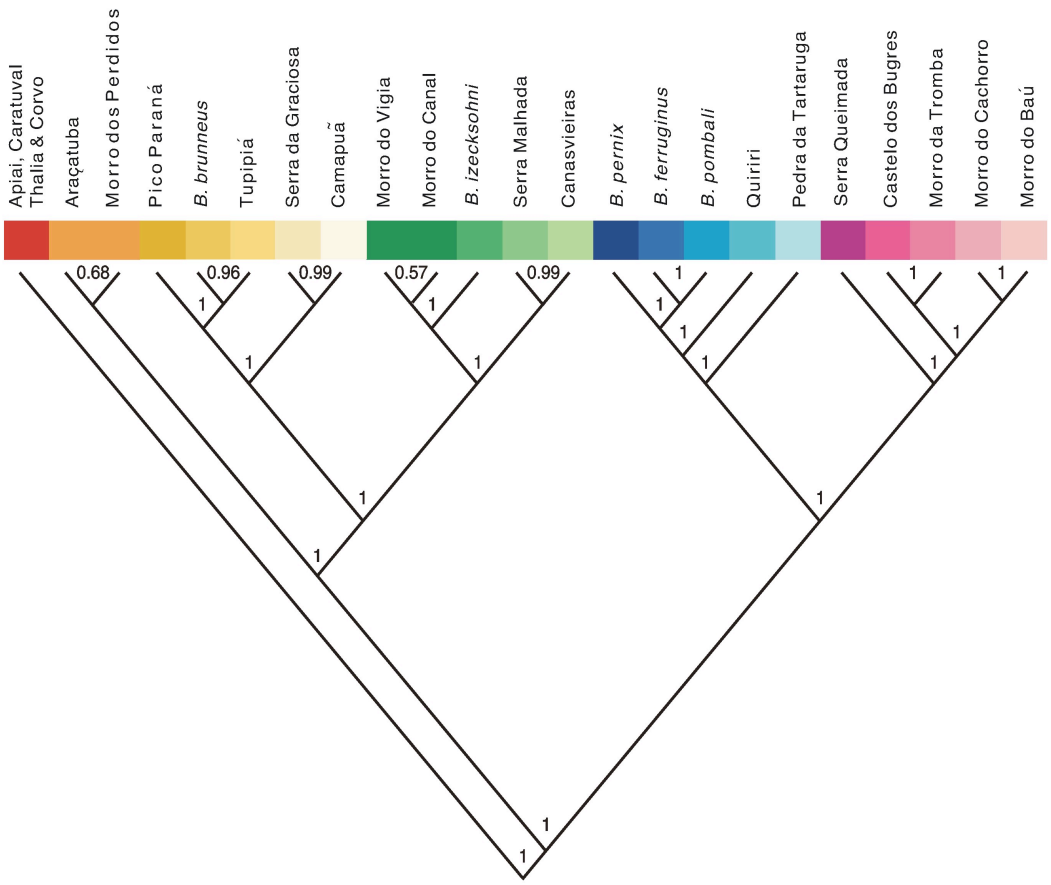

FIGURE 8. BP&P species delimitation for *Brachycephalus*. Number above nodes represents speciation probabilities values. Coloring pattern is in congruence with diagram for species delimitation.

Considering *Melanophryniscus* dataset, Quiriri – Campo Alegre, *M. alipioi*, Itapiroca and Morro do Baú showed a consistent and clear structuring pattern across the pondered *K* values. Both *M. vilavelhensis* and individuals from Morro do Boi recovered membership split into different clusters. However, no common structure pattern with the remaining populations was attained, supporting their delimitation. Joinville and Serra Queimada showed an analogous case of splitting, sharing a structure pattern across the considered *K* range. This result is supportive of their joining as a delimited species. The Apiaí population at *K*=9 and *K*=12 is split into more than one cluster, which are shared at different probabilities with Morro Boa Vista. However, a unique haplotype is shared among populations, one which is also present in many other populations. Therefore, support indicated the delimitation of Apiaí and Morro Boa Vista as single species. Although results for Caratuval (*K*=9, *K*=12-13) and Quiriri – Garuva (*K*=12-13) point to cluster splits, its assignments are not congruent when compared between different *K* values. Hence, their delineation

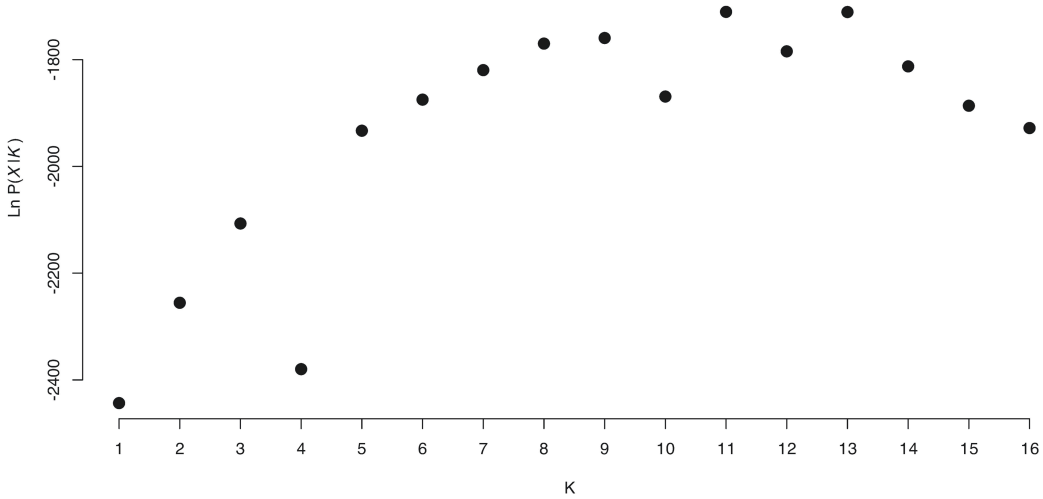

FIGURE 9. For *Melanophryniscus* data, K curve with values of LnP(*X*|*K*) for *K*=1-16.

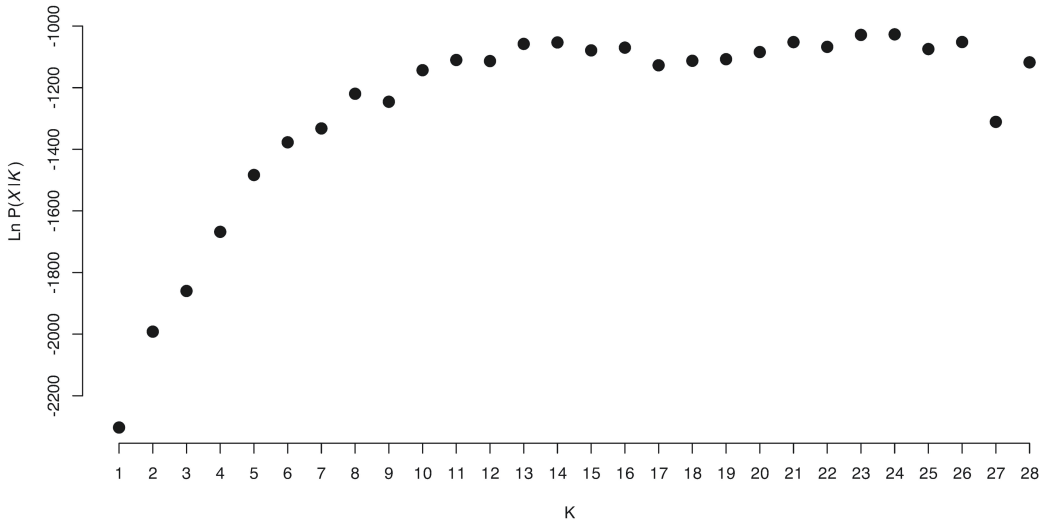

FIGURE 10. For *Brachycephalus* data, K curve with values of LnP(*X*|*K*) for *K*=1-28.

as independent evolutionary units is supported. Furthermore, one individual from Serra da Igreja was consistently classified into the Itapiroca cluster, as a response to shared haplotypes. Still, STRUCTURE results for Serra da Igreja and Serra Prata joined population as one delimited species across *K*=8-13. *Melanophryniscus* populations from Morro Azul and Morro do Cachorro are supported a unique lineage, always sharing the same varying structuring pattern from *K*=8 to *K*=13. For *Brachycephalus*, the following populations were supported as unique clusters and congruently grouped across the considered *K* range: (i) Apiaí, Caratuval, Thalia and Corvo, (ii) Araçatuba and Morro dos Perdidos and (iii) Tupipiá, *B. brunneus*, Pico Paraná, Serra da Graciosa and Camapuã. Morro do Canal and Morro do Vigia constitute short

sampled populations and showed lower values of posterior probability assignment of individuals to clusters. Nonetheless, the shared structuring pattern across K=15-19 is also supportive of their grouping in constituting a unique evolutionary lineage.

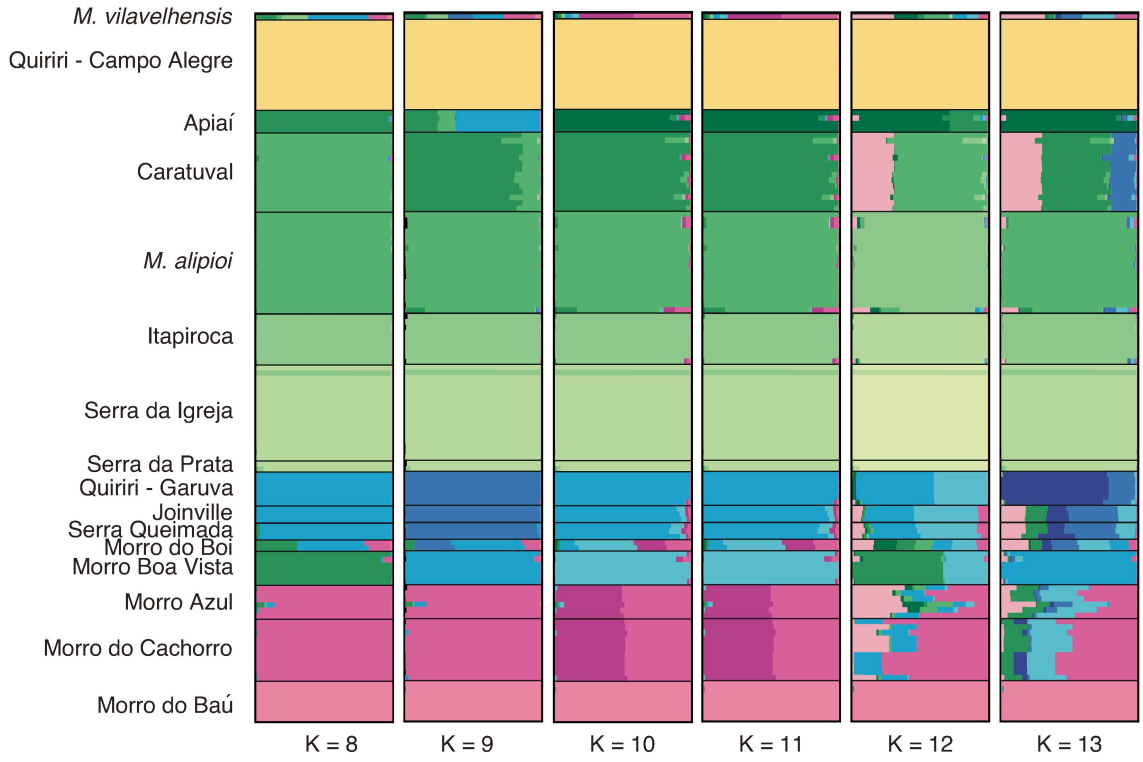

FIGURE 11. STRUCTURE barplots for *Melanophryniscus* individual membership to clusters for the range of values *K*=8-13. The y-axis represents individuals and the x-axis represents the proportion of each one derived from a specific cluster.

Canasvieiras was likewise modeled from few samples, with individual membership sharing a similar clustering pattern with Morro do Canal and Morro do Vigia. However, consistency among STRUCTURE analysis under different *K* values supports Canasvieiras delimitation as a single species. Even though a cluster split was observed in Serra Malhada (*K*=16) and *B. pernix* (*K*=18), results point to their delineation as independent units. *Brachycephalus* populations from Quiriri and Pedra da Tartaruga and *B. izecksohni* were consistently delimited as unique species. Moreover, STRUCTURE defined *B. ferruginus* as having a shared posterior probability of being clustered alone (41.2% for *K*=17) and together with *B. pombali* (58.8% for *K*=17). This result disagrees with both *BEAST reconstructed species tree topology and BP&P analysis, which yield a posterior probability of 1.00 for *B. ferruginus* and *B. pernix* as sister taxa as opposed to 0.86 posterior probability for a *B. ferruginus* and

B. pombali clade. Populations from Serra Queimada, Morro da Tromba and Castelo dos Bugres constitute another case of clusters splitting into two or more groups. Yet, no common structure pattern was attained, supporting their delimitation as isolated units. Lastly, Morro do Baú and Morro do Cachorro, with the exception of results under *K*=15, were each supported as delineated species. In relation to BP&P, STRUCTURE revealed congruent partitioning of *Melanophryniscus* populations, with the only exception of populations from Morro Azul and Morro do Cachorro. In the case of *Brachycephalus*, the two methods diverge regarding the diversification of populations that constitute the clades (i) Araçatuba and Morro dos Perdidos, and (ii) Pico Paraná, *B. brunneus*, Tupipiá, Serra da Graciosa and Camapuã.

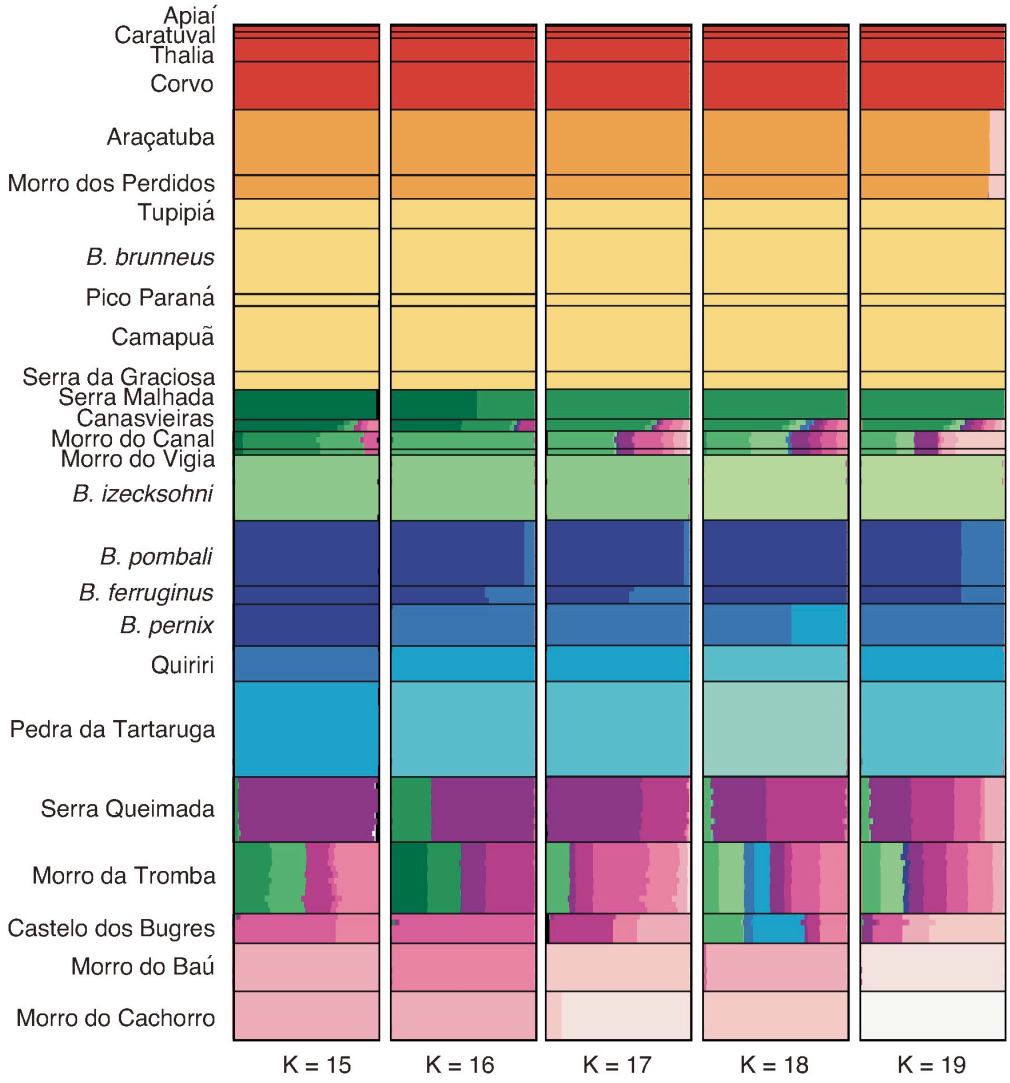

FIGURE 12. STRUCTURE barplots for *Brachycephalus* individual membership to clusters for the range of values *K*=15-19. The y-axis represents individuals and the x-axis represents the proportion of each one derived from a specific cluster.

3.3 SPECIES TREE AND DIVERSIFICATION

The species tree resulting from the *BEAST analysis had prior for species designation based on sampling locality and previous results on species delimitation analysis. Hence, grouped individuals include *Melanophryniscus* populations from (i) Serra da Igreja – Serra da Prata and (ii) Joinville – Serra Queimada and *Brachycephalus* populations from (iii) Apiaí, Caratuval, Thalia and Corvo.

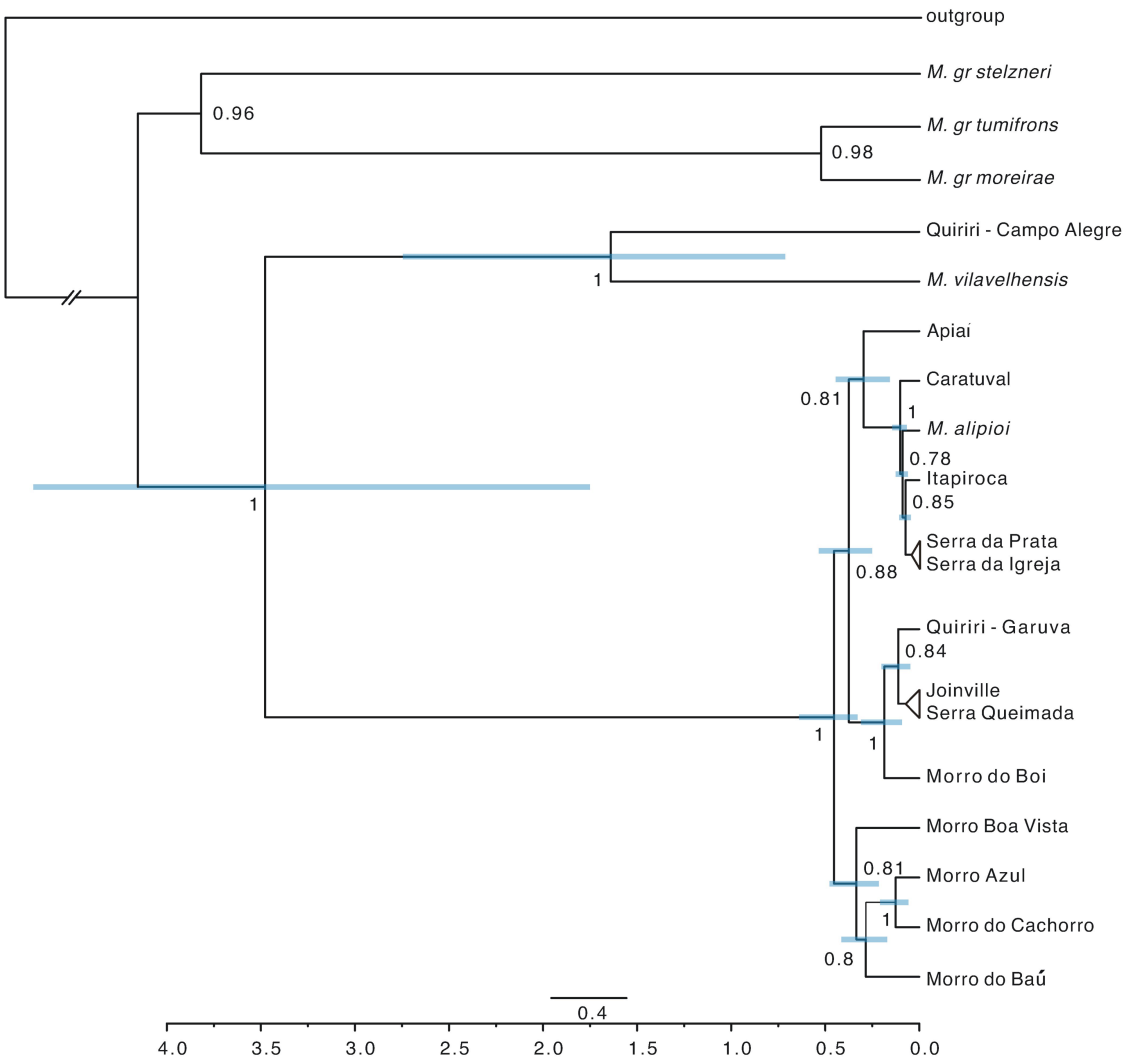

FIGURE 13. *Melanophryniscus* species tree. Triangles at tips are representative of different populations constituting a single species. Node values are Bayesian posterior probability and branch lengths are shown in substitution per site. Blue bars represent 95% HPD for dating estimates and bottom legend refers to a time scale in My.

Analysis reached stationary distribution of chains, with ESS of at least 300 for all parameters, showing convergence between runs and recovering the same topology

among replicate runs. Still, species tree showed relatively intermediate support for some nodes. The species tree is in concordance with gene trees results for major clades and their relatedness. For *Melanophryniscus* (FIGURE 13), divergence time estimates point to an initial split between *M. vilavelhensis* & Quiriri – Campo Alegre and the other montane populations to likely have occurred within the Pliocene (3.48 My, 95% HPD=1.16-5.68 My). Speciation between *M. vilavelhensis* & Quiriri – Campo Alegre was dated for 1.63 My (95% HPD=0.68-2.63) and the split between northern and southern clades was estimated in 0.46 My (95% HPD=0.23-1.2 My), both spanning the Pleistocene. Divergence time estimates point to a relatively recent process of diversification in these microendemic species, with intense speciation events occurring within each of the clades after 0.37 My (95% HPD=0.18-1.02 My).

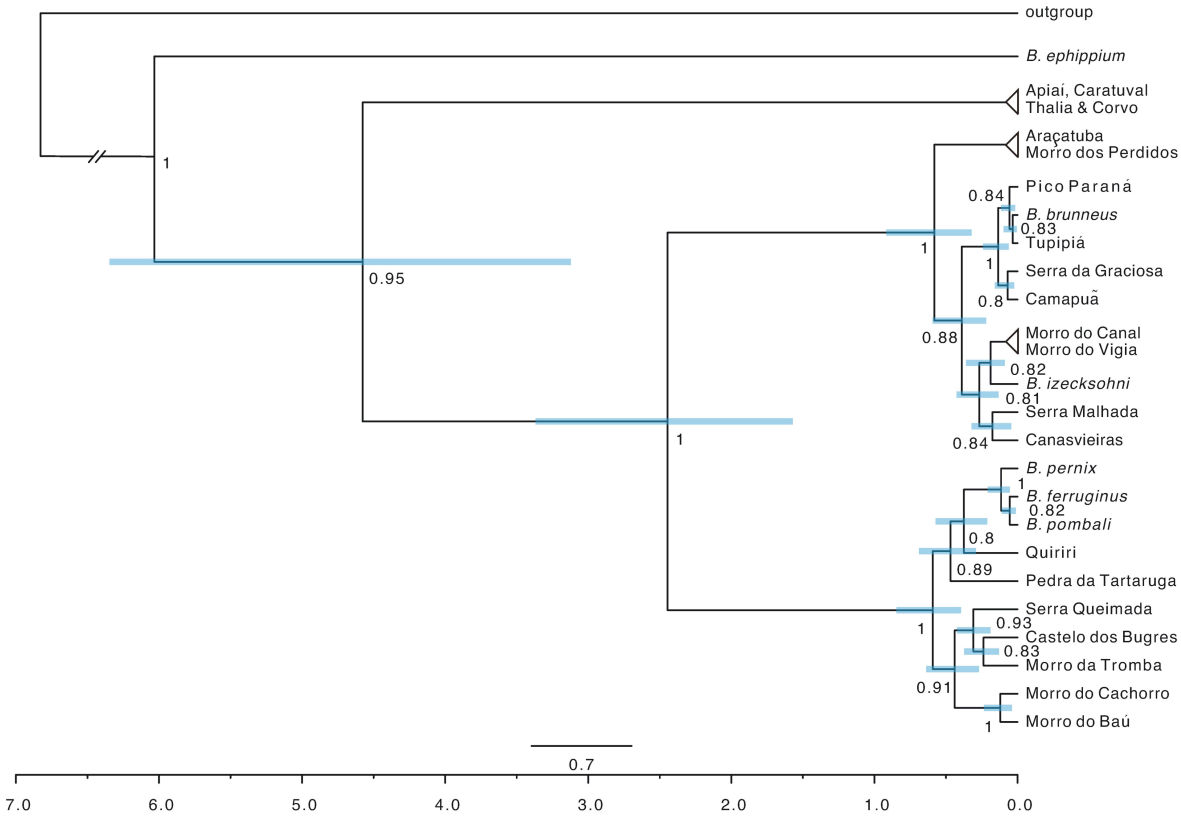

FIGURE 14. *Brachycephalus* species tree. Triangles at tips are representative of different populations constituting a single species. Node values are Bayesian posterior probability and branch lengths are shown in substitution per site. Blue bars represent 95% HPD for dating estimates and bottom legend refers to a time scale in My.

In *Brachycephalus* (FIGURE 14), populations from Apiaí, Caratuval, Thalia and Corvo recovered a divergence date from the other montane populations from the genus of 4.58 My (95% HPD=3.01-6.46 My), covering the Pliocene. The northern

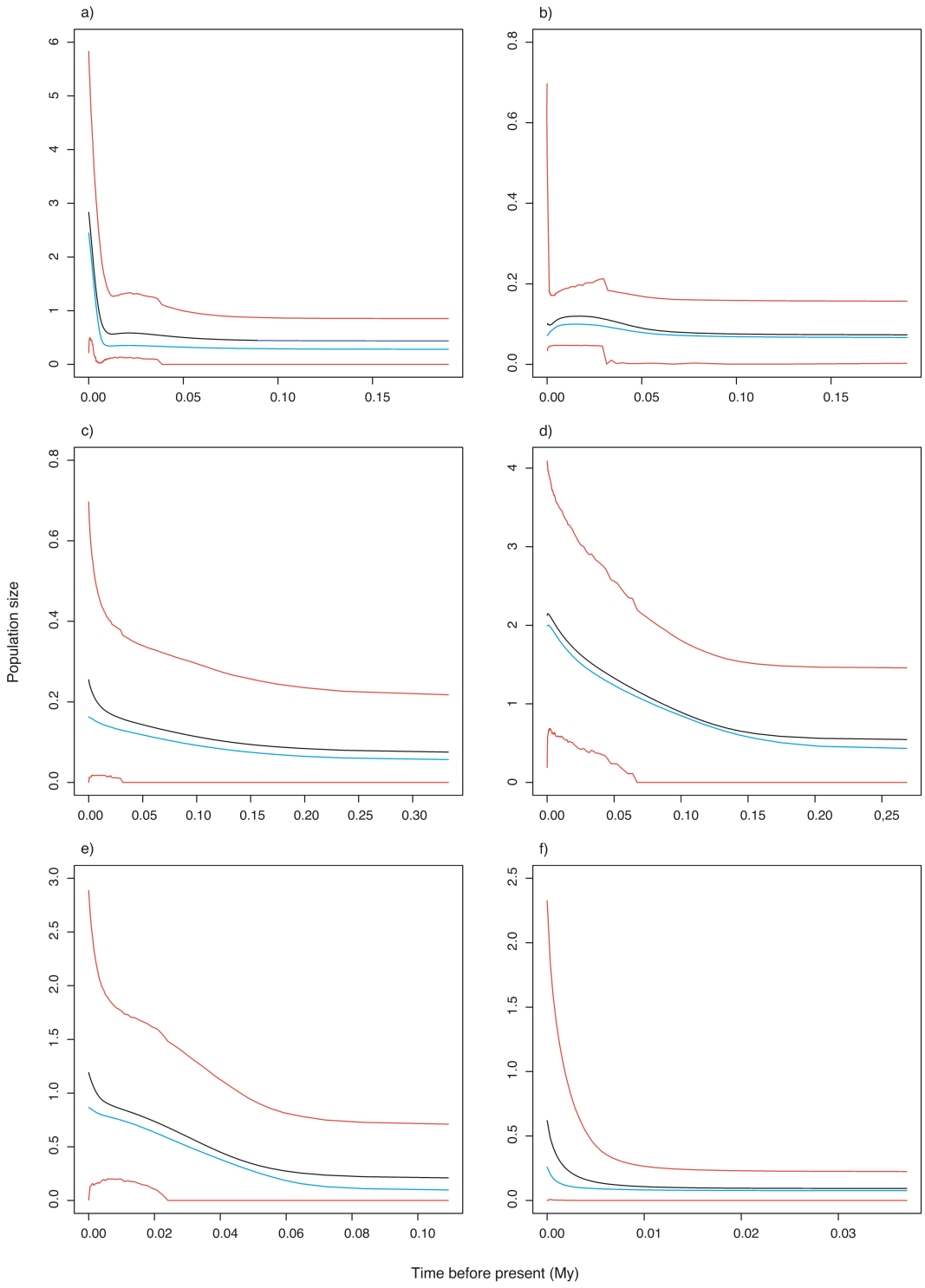

FIGURE 15. Extended Bayesian Skyline plots illustrating the demographic history pattern of six *Melanophryniscus* populations along evolutionary time. a) *M. alipioi*, b) Serra da Igreja, c) Morro do Cachorro, d) Caratuval, e) Itapiroca, and f) Quiriri. The x-axes represent time in million years and the y-axes correspond to the product between effective population size and generation time in million years. The upper and lower red lines represent the 95% HPD, black lines represents mean population size and blue lines represent median population size.

and southern clades split was dated in 2.45 My (95% HPD=1.54-3.35 My), also during the Pliocene Epoch, with diversification in each of these clades occurring in the Pleistocene, after 0.58 and 0.59 My, respectively (95% HPD=0.21-1.18 My and 95% HPD=0.26-1.19 My). For both genera, when considering more recent diversification processes, no speciation event takes places during the Holocene.

3.4 HISTORICAL DEMOGRAPHY

Coalescent-based inference of demographic history through EBSP indicated congruent responses among populations, both between and within genera.

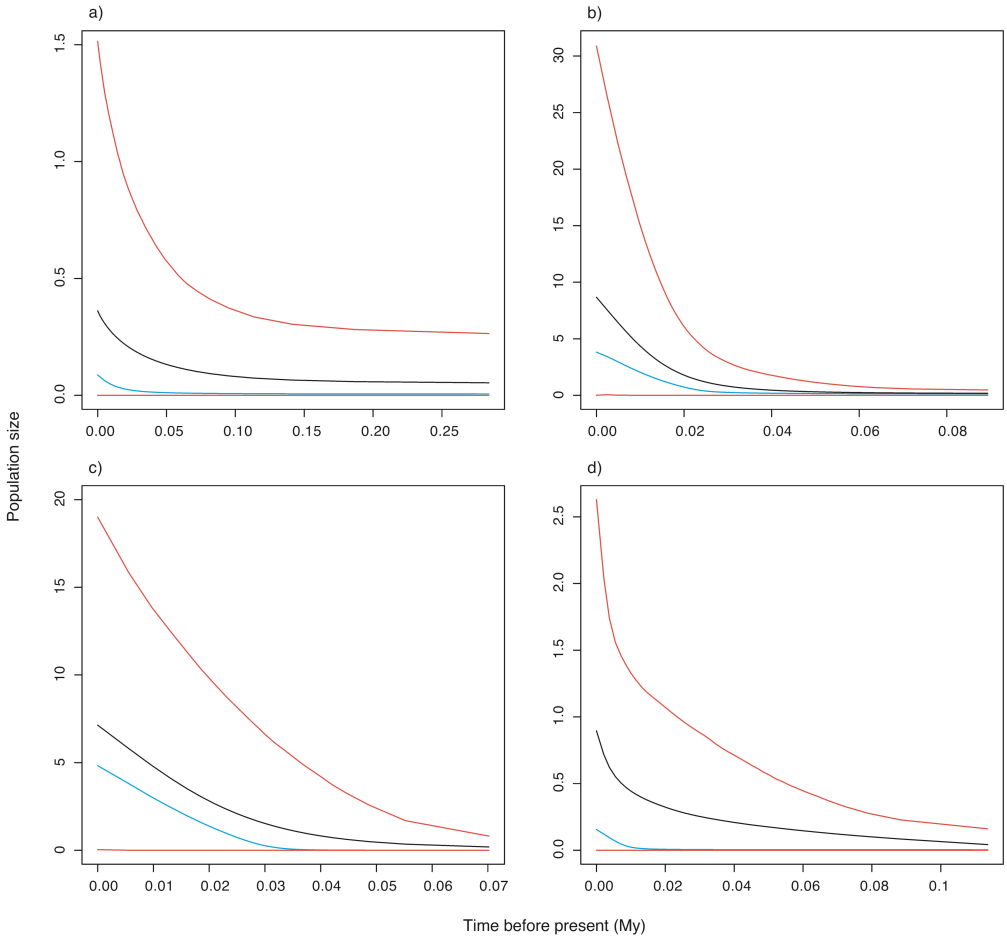

FIGURE 16. Extended Bayesian Skyline plots illustrating the demographic history pattern of four *Brachycephalus* populations along evolutionary time. a) Camapuã, b) Morro da Tromba, c) Morro do Cachorro, and d) Araçatuba. The x-axes represent time in million years and the y-axes correspond to the product between effective population size and generation time in million years. The upper and lower red lines represent the 95% HPD, black lines represents mean population size and blue lines represent median population size.

Both *Melanophryniscus* and *Brachycephalus* share a history of stability on ancestral population sizes across the evolutionary past and a recent growth around 0.05 My, (FIGURES 15 and 16). Although the pattern is supported by a change of one in the parameter for demographic size changes for all populations, this variation is likely an artifact. Presumably, only a few or no mutations have occurred during the last 0.05 My, not offering a consistent variation to support a reliable demographic size change.

4 DISCUSSION

4.1 ANALYTICAL NOTES

4.1.1 Species delimitation approaches

While incorporating differences in how evolutionary units were delimited across methods, results for species delimitation approaches defined a number of montane and isolated species: 14 for *Melanophryniscus* and 21 for *Brachycephalus*. Comparatively, species delimitation and phylogenetic reconstruction support the idea of these narrowly distributed populations representing multiple recently diverged lineages over the concept of a small number of species.

The implemented BGMYC model takes into account gene tree uncertainty, relying upon single loci and hence being subject to the potential associated errors, such as incomplete lineage sorting and selection (MADDISON 1997, PONS *et al*. 2006, LOHSE 2009, REID & CARSTENS 2012). Two other factors that could influence accuracy of the BGMYC method are population genetic structure and migration (PONS *et al.* 2006, REID & CARSTENS 2012). All of these aspects lead to the confounding of population-level structure and species boundaries, often causing the recognition of a higher number of species (LOHSE 2009, ESSELSTYN *et al.* 2012, SATLER *et al.* 2013). However, when the biological model meets the assumptions regarding migration and gene flow, BGMYC shows congruent results

when compared to phylogenetic analysis and other species delimitation methods (HAMILTON *et al.* 2011, KHAN *et al.* 2014). In our model system, no structure or migration is observed and analysis showed lower sensibility in delimiting species when compared to both other methods. BGMYC was unable to distinguish relationships between individual populations, being consistent only with the existence of major clades, as a response to the low resolution in gene trees. Although mitochondrial and nuclear gene trees differed in power for resolving relationships among tips, results were not conflicting in regard to the phylogenetic relatedness of populations. While BGMYC did not aggregate further information and delineation for species delimitation, it contributed to the results incorporating uncertainty in gene tree estimation and supporting the estimated relationships at a large scale.

For STRUCTURE, in order to overcome potential bias in delimiting species based on a single value of *K*, we choose to base this analysis results in the consistency between individual memberships to clusters at a defined range of *K* values. STRUCTURE is known to overestimate the number of clusters from data (PRITCHARD *et al.* 2000, HUBISZ *et al.* 2009). The splitting of clusters into two or more groups, with every individual being admixed from each cluster, is an effect of populations being consisted of largely invariant individuals. Such results include *Melanophryniscus* from Apiaí, Morro Boa Vista, Morro do Cachorro and Morro Azul, *Brachycephalus* populations from Serra Queimada, Morro da Tromba and Castelo dos Bugres and *B. pombali*, *B. ferruginus* and *B. pernix*. As a consequence of low genetic variation, little change in Hardy-Weinberg equilibrium occurs when splitting individuals into two or more clusters, and STRUCTURE chooses these least costly places to introduce additional clusters (PRITCHARD *et al.* 2000). It is likewise argued that clustering algorithms such as STRUCTURE, do not alone offer compelling proof that the delimited units have a history of phylogenetic divergence. This argument is raised on the basis that the population structure is inferred without consideration of historical patterns of diversification. As a consequence, there is not always a clear correspondence between a given level of clustering and the branching pattern of a species tree (CARSTENS *et al.* 2013). Nonetheless, with the exception of the relatedness between *B. ferruginus*, *B. pombali* and *B. pernix,* STRUCTURE assigned individuals to populations with clear correspondence to the reconstructed phylogenetic relationships.

BP&P integrates over the uncertainty in gene tree space, accounting more information when compared to STRUCTURE and BGMYC. Potential shortcomings of Bayesian species delimitation with BP&P may arise due to inaccuracies in the guide tree, leading to the delimitation of every putative lineage. This is a response to an artificial increase in the genetic divergence between sister populations (LEACHÉ & FUJITA 2010, CARSTENS *et al.* 2013) and will result in a strong support for models containing more species than sustained by data (FUJITA *et al.* 2012). However, when the proper topology is assigned as guide tree, BP&P has shown better performance and efficiency when compared to other species delimitation methods (YANG & RANNALA 2010, ZHANG *et al.* 2011, CAMARGO *et al.* 2012). This drawback of BP&P arising from improper assignment of guide tree for species delimitation was mitigated by directly estimating the topology of the guide tree through a species tree analysis. Moreover, different prior distributions on θ_s and τ_0 did not affect the output and we recovered consistent results among runs. The higher sensibility of BP&P in delimiting species when compared to both other methods relies on its premise of no gene flow between populations (YANG & RANNALA 2010, LEACHÉ & FUJITA 2010, BURBRINK *et al.* 2011, SETIADI *et al.* 2011, FUJITA *et al.* 2012). The absence of support for the nodes of the remaining non-delimited populations might have been an error due to recent speciation, which is an accounted insufficiency of BP&P related to the number of used loci (YANG & RANNALA 2010, CAMARGO *et al.* 2012). Given an accurate guide tree, simulations and real data experiments have demonstrated the efficacy of BP&P under scenarios of limited or absent gene flow, no population structure, constant size, no hybridization or admixture after speciation event (YANG & RANNALA 2010, ZHANG *et al.* 2011, CAMARGO *et al.* 2012). Studies dealing with more complex scenarios, involving gene flow, migration, introgression, population structure, isolation by distance, have chosen the more conservative delimitation of species under the righteous concern of analyses assumptions violation (e.g. LEACHÉ & FUJITA 2010, SETIADI *et al.* 2011, SATLER *et al.* 2013). Our study model of endemic and isolated populations of anurans, with absence of gene flow and population structure, meets a scenario that supports optimum performance of BP&P for species delimitation.

4.1.2 Species tree

Species tree inference under *BEAST reached stationary distribution of chains and ESS of at least 300 for all parameter, showing convergence between runs and recovering always the same topology. Yet, *BEAST species tree shows relatively low support for some nodes. This recurring issue amongst phylogenetic analyses has grounding in three main discussed arguments: (i) differences of few base pairs in the alignment may lead to uncertainty on the placement of individual sequences, leading to lower posterior probability of nodes (DRUMMOND & RAMBAUT 2007, DRUMMOND *et al.* 2012); (ii) the existence of multiple optimum peaks along the chain, isolated by valleys of extremes low likelihood, yield low posterior probabilities for nodes (LARGET & SIMON 1999, DRUMMOND & RAMBAUT 2007); and (iii) even though the final topology is that of highest likelihood, given the massive topology space MCMC chain samples from, the posterior values for each branch and node are diluted over all other possibilities and low posterior values are generated (BERGSTEN *et al.* 2013). The observed posterior probability values for node support recovered in BEAST analysis was likely due to a combining effect of these underlying aspects. Congruence between runs and estimated topology reflects the support for the estimated species tree.

4.2 OVERVIEW OF THE DIVERSIFICATION PROCESS

The results herein highlight a remarkable number of highly endemic montane *Melanophryniscus* and *Brachycephalus* species over a geographically restricted area of the southern BAF. The observed elevated endemism degree has its basis on the influence of (i) the BAF topographic complexity, (ii) species' restriction to mountain tops, (iii) highland climatic refugia and (iv) an altitudinal migration strategy.

The BAF topographic complexity is featured more extremely in southeastern and southern Brazil, with ridges comprising groups of mountainous reliefs disconnected by lowlands. Such configuration supports numerous and isolated montane sites over relatively short geographic distances, with high altitude areas

constituting suitable surviving areas and acting in the isolation of low dispersal species (CRUZ & FEIO 2007, MATA *et al.* 2009). This scenario promotes diversification, contributing to the high biological diversity. In extension, *Melanophryniscus* and *Brachycephalus* extreme small geographical ranges and species' restriction to montane cloud forests or altitude grasslands, is a magnifier effect of the isolation levels. Such species' constraints additionally contribute to the observed elevated degree of microendemism.

The results of our study indicate a high degree of climatic stability in southern BAF montane sites during the middle and late Pleistocene, despite climatic fluctuations that have take place over the region during the period. This setting supports the concept of highland refugia, buffering species from the shifts in vegetation and environmental conditions stemming from the impact of climatic oscillations. Previous studies elsewhere in the BAF have proved a severe influence of climatic shifts during the late Quaternary on species persistence (FITZPATRICK *et al.* 2009, CARNAVAL *et al.* 2009, AMARO *et al.* 2012, THOMÉ *et al.* 2010). Counter intuitively, Pleistocene biota was adapted to glacial conditions, with the interglacial warm peaks constituting disturbances. Over the cyclical and fluctuating climatic process, a number of species likely became extinct, whereas others managed to survive. After the interglacial epoch at approximately 0.5 My, climate during the glacial and interglacial cycles experienced a shift to greater temperature extremes, with longer glacial periods and warm phases showing higher temperatures than subsequent interglacials (STAUFFER 2009). Glacial low temperatures and the enhanced warmer conditions during interglacials would have driven populations currently restricted to mountain tops to their extinction. However, spatial reorganization through altitudinal migration as a response to climate change would have driven previously widespread ancestral lineages to higher altitudes, where environmental conditions were stable and suitable for their survival. Provided a stable environmental envelope obtained through altitudinal migration, their persistence in suitable microrefugia would have protected specimens from unfavorable regional environmental conditions (BUSH *et al*. 2003, BUSH 2002). The altitudinal migration strategy and existence of suitable survival envelopes assured in highland refugia, equally contributed to the observed elevated endemism degree and diversity. This assessment is supported by the influence of glacial and interglacial cycles on ancestral populations, eventually leading to their isolation by valleys of unfavorable

climatic envelopes and multiple nearly simultaneous events of allopatric speciation. Such historical configuration incites the expectation of a strong spatial component shaping phylogenetic relationships between *Melanophryniscus* and *Brachycephalus* species, leading to a cluster of closely related species in a phylogenetic and geographic perspective. The current genus distribution is uniquely consistent with this scenario, exhibiting a north-south diversification pattern that follows the above-mentioned expectations. The dynamics of diversification have also been subjected to stochastic processes, which have shaped variation of evolutionary histories and defined phylogenetic relatedness within clusters for closely distributed species. Divergence time estimates and historical demography also conform to this scenario: dating results point to a relatively recent process intense speciation occurring after 0.5 My, coincidental with the shift in climate occurred for glacial and interglacial cycles; and the recovered stability on ancestral population sizes across the evolutionary past is in agreement to the recent warming of the climate. Moreover, although interglacial epochs and Holocene climatic conditions could have led to range expansions into lower altitudes and enable sympatry among closely related species, our results and fieldwork records do not support this scenario in any of the studied populations.

Evolution in high altitudes, shaping diversity and distribution of species, incorporates historic and contemporary effects. Our results consistently indicate that there are multiple lineages within *Melanophryniscus* and *Brachycephalus* montane populations, being supportive of lineage differentiation, lineage maintenance through time, and range restriction. These underlying evolutionary drivers of endemism indicate an extreme degree of spatial restriction and specificity of different species over a mosaic of isolated small ranged areas. As a response to their low dispersal ability and restriction to mountains tops in areas surrounded by inhospitable habitat, these montane species constitute isolation and lineage differentiation prone evolutionary units. The suitable stable microclimates attained by altitudinal migration provided protection from the unfavorable regional environmental conditions, subsiding the lineages maintenance through time. At the present scenario, contemporary climatic heterogeneity must not be overtaken, given current climatic conditions may constitute a potential driver of lineage range restriction, preventing the secondary contact of isolated populations.

4.3 SPECIES DELIMITATION

We propose the recognition of 14 microendemic species of *Melanophryniscus* and 21 for *Brachycephalus*. We base this delimitation on (i) the bGMYC recognition of the major clades identified in the gene trees, (ii) the existence of a single incongruence between BP&P and STRUCTURE results, (iii) the consistency of BP&P results across runs and different prior settings, (iv) the BP&P better performance and efficiency when compared to the other species delimitation methods, (v) the phenotypic diversity of the studied populations and (vi) the conservation implications of such configuration.

In addition to the 2 *Melanophryniscus* and 6 *Brachycephalus* species previously recognized to occur in the present region, our results have uncovered 14 and 21 new occurrence records, respectively. When one considers the genera entire geographic distribution, this data aggregates an impressive value to the present knowledge. For *Melanophryniscus*, our results are supportive of the following populations as species: Quiriri – Campo Alegre; *M. vilavelhensis*; Apiaí; Caratuval; *M. alipioi*; Serra da Prata & Serra da Igreja, Quiriri – Garuva; Joinville & Serra Queimada; Morro do Boi; Morro Boa Vista; Morro Azul; Morro do Cachorro and Morro do Baú. This constitutes 12 new species over 250 km, in addition to the 26 currently know to occur over the extent of 1,000,000 km^2. For *Brachycephalus*, recommendation for species delimitation is as follows: Apiaí, Caratuval, Thalia & Corvo; Araçatuba & Morro dos Perdidos; Pico Paraná; *B. brunneus*; Tupipiá; Serra da Graciosa; Camapuã; Morro do Vigia & Morro do Canal; *B. izecksohni*; Serra Malhada; Canasvieiras; *B. pernix*; *B. ferruginus*; *B. pombali*; Quiriri; Pedra da Tartaruga; Serra Queimada; Castelo dos Bugres; Morro da Tromba; Morro do Cachorro; and Morro do Baú. Regarding the genus endemic distribution to the BAF across 2.000 km along the biome, this result uncovers 18 new species. A more conservative estimate would differ solely in being supportive of joining *Melanophrynicus* populations from Morro Azul & Morro do Cachorro and *B. brunneus* with *Brachycephalus* populations from Pico Paraná, Tupipiá, Graciosa & Camapuã. A less stringent estimate would consider *Brachycephalus* populations from Araçatuba, Morro dos Perdidos, Morro do Vigia and Morro do Canal as four distinct species.

Apart from species delimitation results and molecular differentiation, these species feature striking phenotypic diversity supporting their acknowledgement. The *Melanophryniscus* montane populations are characterized by coloration patterns that follow different combinations of the subsequent traits: presence of yellow patches along the forearms; presence of white spots along the forearms, mouth, abdomen and cloaca; pattern and disposition of warts; and presence and number of corneous spines. The varying coloration patterns, morphological differences in body size and specific traits, differentiations in advertisement calls and ecology constitute diagnosis for species support (PIE *et al.* in prep). When considering *Brachycephalus*, montane populations are mostly brightly colored, with evidence that such vivid colors might be aposematic. The distinct variation in coloration between species ranges from yellow to orange and may include spots in shades of green, blue and brown. Some species lack aposematic coloration pattern, exhibiting dark brown body colors and featuring yellow spots along the ventral surface. In addition, it is important to emphasize that there is intraspecific variation in coloration in most species (PIE *et al.* in prep).

Adding to these species' isolated geographic distributions, species delimitation has strengthened the evidence that these species constitute distinct genetic units. One has to take into consideration the elevated diversity of these microendemic populations, which have evolved under such pressures and managed to overcome climatic extremes, surviving in microendemic sites and constituting a genetic history and richness of immeasurable value. The contemporary scenario supports this delineation and offers the opportunity of optimism for the conservation of each of these unique evolutionary units and the entire ecological community they are part of.

4.4 IMPLICATIONS FOR CONSERVATION

Our results underscore the exceptionality of this south BAF region of high diversity and urgent need for prioritizing conservation efforts. The concept of evolutionary significant units should be considered in order to provide a rational basis for conservation actions, ensuring that an evolutionary heritage is recognized, protected, and the evolutionary potential inherent across the set of lineages is maintained. A set of historically isolated populations features distinct potentials, and

as a strategy to preserve adaptation to previous climatic conditions, one should seek their recognition as independent units and protect the full array of geographic variants (MORITZ 1994). The idea of stable climatic refugia safeguarding species from unsuitable environmental condition is currently widely acknowledged. However, such defined and potential areas must be preserved in order to serve as refugia in the near evolutionary future. The notion of microrefugia is mainly important under the context of biodiversity conservation and ongoing climate change, being especially beneficial for mid to high-altitude endemic species. In this particular case, the risk of habitat loss by upward vegetation displacement as a response to climate change is considerable, and the establishment of conservation strategies could assure suitable microhabitat conditions for threatened species.

5 CONCLUSION

When considering *Melanophryniscus* and *Brachycephalus* species as a model system to understand the diversification and endemism evolutionary dynamics, our results uncovered the distinctiveness of montane species and their evolutionary history in the southern BAF. The observed common demographic history and divergence time estimates are both in concordance with the proposed hypotheses of altitudinal migration and common mechanisms underlying their diversification. Compared to data for the remaining distribution of the biome, the considered region harbors a remarkable high number of microendemic species that have endured the Pleistocene climatic fluctuations in highland climatic refugia. The biological strategy for survival through altitudinal migration comprehends a unique feature in the dynamics of persistence and diversification across the BAF, contributing to the observed high level of endemism.

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