RAFAEL FÉLIX DE MAGALHÃES

Biogeografia e conservação das *Phyllomedusa* (Anura, Hylidae) endêmicas de ilhas de altitude do Escudo Brasileiro

BELO HORIZONTE, DEZEMBRO DE 2016

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Orientador: Fabrício Rodrigues dos Santos

Co-Orientador: Paulo Chistiano de Anchietta Garcia

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Banca examinadora:

Profa. Dra. Fernanda Pinho Werneck
Instituto Nacional de Pesquisas da Amazônia

Profa. Dra. Kátia Cristina Machado Pellegrino
Universidade Federal de São Paulo – Departamento de Ciências Biológicas

Profa. Dra. Kirstern Lica Follmann Haseyama (suplente)
Universidade Federal de Minas Gerais – Departamento de Zoologia

Prof. Dr. Adalberto José dos Santos
Universidade Federal de Minas Gerais – Departamento de Zoologia

Prof. Dr. Fernando Augusto de Oliveira e Silveira
Universidade Federal de Minas Gerais – Departamento de Botânica

Prof. Dr. Fabrício Rodrigues dos Santos (orientador)
Universidade Federal de Minas Gerais – Departamento de Biologia Geral

“Tonight I’m gonna have myself a real good time
I fell alive and the world I’ll turn it inside out – yeah!
I’m floating around in ecstasy
So don’t stop me now, don’t stop me
‘Cause I’m having a good time, having a good time”
Freddie Mercury

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RESUMO

O complexo de ilhas de altitude do Escudo Brasileiro se distribui do norte do Rio Grande do Sul à região central da Bahia e é composto por diversas cadeias de serras isoladas umas das outras. A região norte deste sistema constitui a fronteira entre os domínios Cerrado, Mata Atlântica e Caatinga. As áreas de altitude são dominadas pelos *campos rupestres*, um ecossistema rico, com alta taxa de endemismos e que se desenvolve em solos pobres e associados à afloramentos rochosos quartzíticos, areníticos ou ferríferos. Neste trabalho, nós avaliamos a influência do isolamento geográfico sobre a especiação e a estruturação genética das populações. Para tanto, nós utilizamos um complexo de espécies de pererecas endêmicas como modelo, incluindo as espécies formalmente descritas *Phyllomedusa ayeaye*, *P. centralis* e *P. oreades*. Nós encontramos evidência de especiação recente e com fluxo gênico entre estas espécies, além de uma linhagem críptica sob o nome *P. oreades*, sugerindo que, apesar das evidências geológicas apontarem que os *campos rupestres* são um ambiente antigo, as flutuações climáticas do Pleistoceno devem ter influenciado na diversificação de parte de sua biota endêmica. Além disso, nós também encontramos evidência de estruturação genética, com três unidades evolutivamente significativas associadas a diferentes cadeias serranas em *P. ayeaye*. Esta espécie é classificada como 'críticamente ameaçada' pela União Internacional para a Conservação da Natureza e nossos resultados apontam a ineficiência da rede nacional de unidades de conservação em protege-la. Estes resultados sugerem que o isolamento geográfico é um importante fator na história da diversificação dos *campos rupestres* e, sendo assim, ele deve ser considerado em pesquisas científicas e no planejamento de conservação.

Palavras-chave: genética da conservação, fluxo gênico, espécies crípticas. *campos rupestres*, isolamento geográfico.

ABSTRACT

The Brazilian Shield sky islands complex is distributed from the north of Rio Grande do Sul to the central region of Bahia, and is composed by several mountain chains mountains isolated from each other. The northern region of the system is constituting the boundary between the Cerrado, Atlantic Forest and Caatinga domains. These islands are dominated by *campos rupestres*, a rich ecosystem with a high endemism rate, and associated with quartzite, sandstone or ironstone outcrops, and poor soils. In this work, we evaluated the influence of geographic isolation on speciation and genetic structuration of populations. Therefore, we used an endemic frog species complex as model, including the formally described species *Phyllomedusa ayeaye*, *P. centralis*, and *P. oreades*. We found evidence of recent speciation with gene flow among these species, in addition to a cryptic lineage under the name *P. oreades*. This suggests that, although geological evidence points to *campos rupestres* as an ancient environment, climatic fluctuations of the Pleistocene may have influenced the diversification of part of its endemic biota. In addition, we also found genetic structuring in *P. ayeaye*, with three evolutionarily significant units associated with distinct mountain chains. This species is classified as "critically endangered" by the International Union for Conservation of Nature and our results point to the inefficacy of the national protected areas network in preserving it. These results suggest that geographic isolation is an important factor in the history of the diversification of *campos rupestres*, so it should be considered in scientific investigations and conservation planning.

Keywords: conservation genetics, gene flow, cryptic species, *campos rupestres*, geographic isolation.

INTRODUÇÃO GERAL

Ilhas de altitude (*sky islands*, em inglês) são importantes sistemas naturais para o estudo de processos evolutivos, tais como divergência populacional, adaptação e especiação (e.g. Knowles, 2000; Angert & Schemske, 2005; Salerno et al., 2015). A biogeografia desses sistemas é relativamente similar a das ilhas oceânicas: os vales funcionam como as barreiras marítimas. A área de cada montanha e a distância entre elas, por sua vez, podem estar relacionadas com os padrões metapopulacionais e os níveis de diversidade observados (Warshall, 1994, McCormack et al., 2009). Assim como em ilhas oceânicas, as comunidades biológicas das ilhas de altitude são caracterizadas pela presença de várias espécies com distribuição geográfica restrita (Fjeldså et al., 2012). Contudo, a dinâmica climática atual e/ou histórica das áreas, juntamente com as tolerâncias térmicas e capacidades de dispersão das espécies, são fatores cruciais na diferenciação entre a biogeografia das ilhas de altitude e a biogeografia de ilhas oceânicas, uma vez que mudanças climáticas sazonais, eventos geológicos e climáticos do passado e mudanças evolutivas ocorridas nas espécies podem modificar os níveis de conexão das populações entre as ilhas em curtos períodos de tempo geológico (Warshall, 1994; McCormack et al., 2009; Fjeldså et al., 2012). Por outro lado, há evidência de estabilidade climática nestes sistemas, uma vez que a velocidade das mudanças de temperatura nessas ilhas, principalmente nas regiões tropicais, tende a ser menor do que em outras partes do planeta (Sandel et al., 2011).

As ilhas de altitude das zonas tropicais possuem riqueza de espécies e taxa de endemismo altas em comparação com aquelas observadas nas zonas temperadas, sobretudo no hemisfério norte. Janzen (1967) sugeriu um modelo para explicar este padrão: devido a estabilidade climática dos sistemas tropicais há baixa ou nenhuma sobreposição entre as amplitudes térmicas anuais e altitudinais quando vales e montanhas

são comparados. Desta forma, o clima de altitude possui temperaturas médias anuais mais frias quando comparadas às dos vales adjacentes. Assim, espécies de altitude com pouca tolerância térmica estão adaptadas e se mantém restritas a estes ambientes. Em regiões temperadas, por sua vez, a variação anual de temperatura é tão grande que se sobrepõe àquela relacionada às mudanças de altitude. Desta forma, as espécies que vivem nestas zonas climáticas só conseguem se estabelecer devido a uma alta tolerância térmica, resultando em distribuições geográficas mais amplas. Sabe-se que os níveis de precipitação também influenciam na distribuição de alguns grupos de vertebrados em ilhas de altitude, especialmente quirópteros e anuros. Estes animais tendem a ter uma distribuição altitudinal mais restrita em montanhas mais áridas do que naquelas mais úmidas (McCain, 2009).

Vale ressaltar que o modelo proposto por Jansen (1967) e posteriormente testado por McCain (2009) é baseado apenas em fatores abióticos. Contudo, a capacidade de ocupação de novos ambientes pode estar relacionada a interações entre outros fatores, tais como capacidade de dispersão, competição e disponibilidade de recursos específicos (e.g. Taniguchi & Nakaro, 2000; Navas, 2006). Além disso, na zona tropical do hemisfério sul, a amplitude térmica diária em altitudes elevadas é ampla, criando ambientes com dias quentes e noites frias, o que selecionaria organismos com tolerâncias térmicas amplas (Ghalambor et al., 2006), contrariando alguns pressupostos do modelo proposto por Jansen (1967). Todavia, há fortes evidências de que a extensão da distribuição altitudinal nos trópicos é relativamente restrita para vários grupos de organismos (e.g. Navas, 2002; Alves & Kolbek, 2010; Fjeldså et al., 2012), mesmo que os processos que gerem esse isolamento não estejam claros (Ghalambor et al., 2006).

Os modelos macroecológicos também falham em explicar o padrão de diversidade das ilhas de altitude por outros aspectos. Essas ilhas possuem uma complexidade

topográfica não captável nas escalas geográficas destes modelos. Topografias complexas estão relacionadas a presença de microambientes e microclimas aos quais espécies com distribuição geográfica restrita estão adaptadas (Guarnizo & Cannatella, 2013; Rodríguez et al., 2015). Além disso, há uma miscelânea de histórias evolutivas e padrões aleatórios na ocupação do espaço que não são consideradas na escala de tempo atual (Fjeldså et al., 2012). É possível, por exemplo, que dois complexos de linhagens possuam congruência geográfica e incongruência temporal na ocupação das ilhas de altitude (Lawson, 2010). Por este motivo, o tempo e os eventos geológicos são fatores importantes na elucidação da diversidade de espécies encontrada em montanhas. Acredita-se, por exemplo, que as mudanças climáticas ocorridas desde o Plioceno, sobretudo as flutuações climáticas do Pleistoceno, possam estar relacionadas com o isolamento e especiação de várias espécies endêmicas dessas ilhas (Simpson, 1979; Baker, 2008; Fjeldså et al., 2012; Chaves et al., 2014). Outros fatores, tais como soerguimento de montanhas, transgressões marinhas e mudanças no curso de grandes rios podem ter influenciado os padrões de isolamento, dispersão e ocupação geográfica de diversas linhagens (Garda & Canatella, 2007; Ribas et al., 2007; Chaves et al., 2014). Por fim, os endemismos em montanhas podem estar relacionados não a padrões vicariantes, mas à persistência de linhagens antigas (e.g. Fjeldså et al., 2012; Chaves et al., 2014) devido a maior estabilidade climática dessas áreas durante o período Quaternário (Sandel et al., 2011; Fjeldså et al., 2012). Desta forma, para entender a riqueza das ilhas de altitude, é necessário investigar as causas históricas da diversificação das linhagens em topo de montanhas, que devem estar diretamente relacionadas à diversificação biológica destes sistemas (Fjeldså et al., 2012).

A filogeografia fornece um importante conjunto de metodologias para a investigação destes padrões históricos envolvidos na diversificação de linhagens (Avise et al., 1987). Desde sua concepção no final da década de 1980, houve grande avanço

metodológico, com o desenvolvimento de ferramentas estatísticas para o teste de hipóteses espaciais e genealógicas, estas baseadas na teoria da coalescência (Knowles & Maddison, 2002; Kuhner, 2008; Knowles, 2009). A filogeografia como foi concebida por Avise e colaboradores (1987), é focada essencialmente na história biogeográfica das espécies, com algum nível de extrapolação para a história das áreas de ocorrência das mesmas (Crisci, 2001), utilizando hipóteses *ad hoc* para a elucidação dos padrões genealógicos observados no espaço geográfico (Knowles & Maddison, 2002; Knowles, 2009). Portanto, estudos filogeográficos podem auxiliar na compreensão dos processos geradores dos padrões espaciais observados no tempo presente.

O Brasil possui um dos três complexos de ilhas de altitude da região Neotropical integralmente em seu território. Este complexo é conhecido como Complexo de Ilhas de Altitude do Escudo Brasileiro (CEB; *sensu* Warshall, 1994; Figura 1) ou Complexos Rupestres de Altitude (*sensu* Benites et al., 2003). Ele inclui os complexos serranos e chapadas do Espinhaço Meridional (=Espinhaço Mineiro), Espinhaço Setentrional (=Espinhaço Baiano), Serras do Mar e da Mantiqueira, Planalto da Canastra, Serra Geral do Sul do Brasil e Planalto Central Brasileiro. O CEB apresenta, predominantemente, paisagens campestres em altitudes elevadas, geralmente associadas à afloramentos rochosos (Safford, 1999; Alves & Kolbek, 2010; Alves et al., 2014; Silveira et al., 2016; Figuras 1 e 2) ou mosaicos de campo e floresta ombrófila mista, especialmente na Serra Geral (Behling et al., 2007; Behling & Pillar, 2007). Estas ilhas possuem habitats geograficamente subdivididos e isolados, geralmente em altitudes acima de 900m acima do nível do mar. O clima é caracterizado como subtropical úmido em todo o CEB e apresenta, nas serras Geral, do Mar e parte da Serra da Mantiqueira, clima oceânico e verão temperado, sem estação seca (Cfb – Alvares et al., 2013). Nas demais ilhas do complexo, há inverno seco e verão temperado (Cwb – Alvares et al., 2013). Os vales

adjacentes, por sua vez, possuem verão quente (ver Figura 6 em Alvares et al., 2013) e por isso os nichos Grinnellianos são distintos daqueles da matriz circundante. Por este motivo, a matriz circundante pode funcionar como barreira climática entre as linhagens de diferentes ilhas (Warshall, 1994; Baker, 2008; McCormack *et al*., 2009).

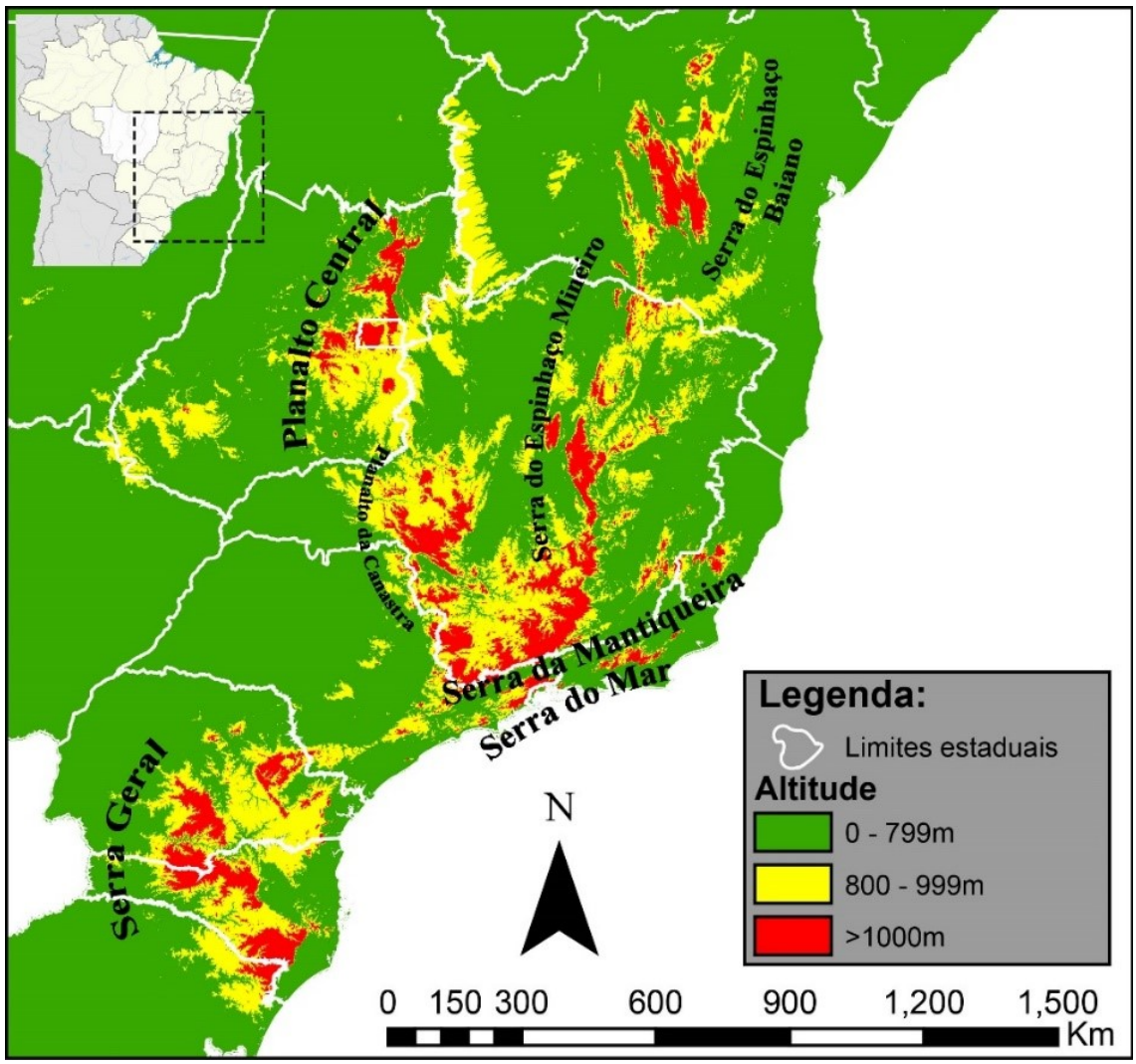

Figura 1. Complexo de Ilhas de Altitude do Escudo Brasileiro (em vermelho).

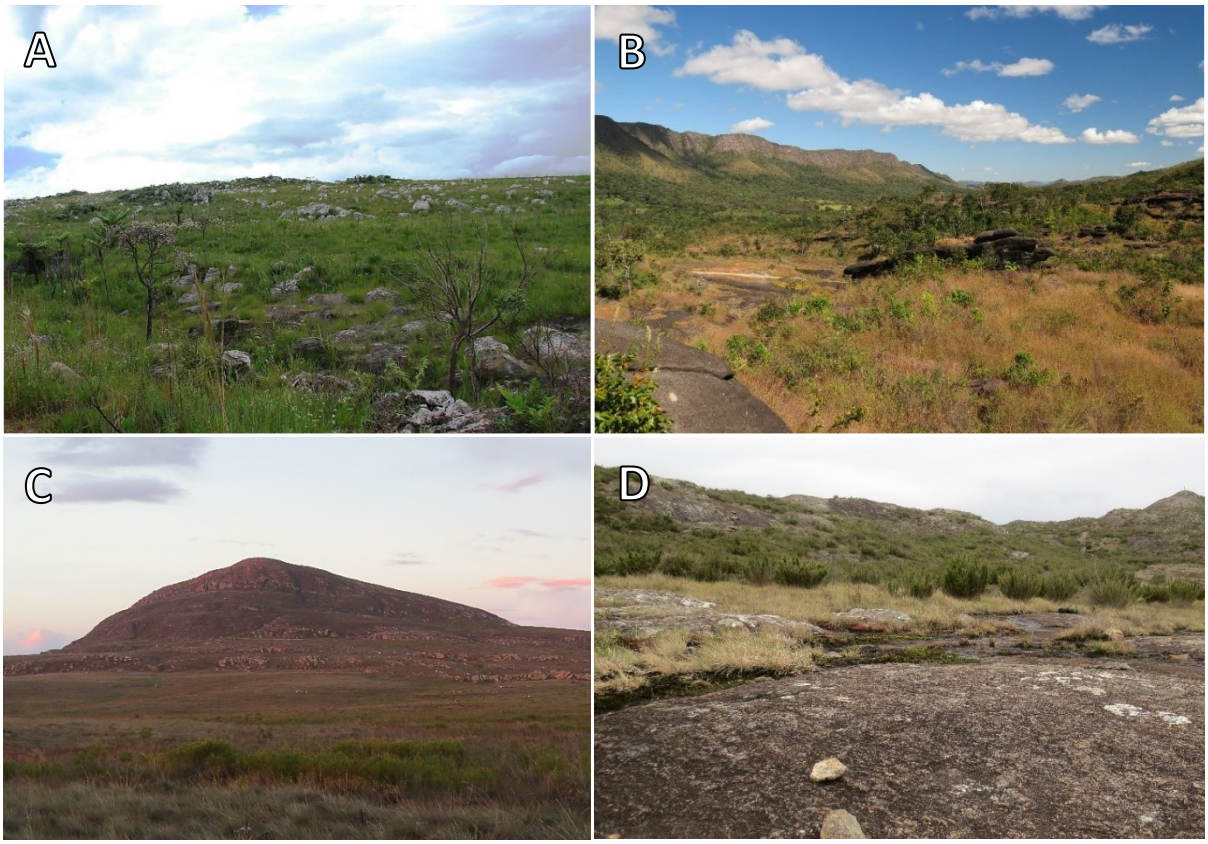

Figura 2. Alguns exemplos de paisagens observadas nas ilhas do CEB. **A.** Campos rupestres (PARNA Serra da Canastra, Planalto da Canastra, Minas Gerais), **B.** paisagem campestre-arbustiva (Cerrado) com afloramentos rochosos quartzíticos (Chapada dos Veadeiros, Planalto Central, Goiás), **C.** campos alagáveis (frente) e campos rupestres (fundo) (P.E. do Rio Preto, Espinhaço Meridional, Minas Gerais) e **D.** Campo de altitude (Caparaó, Serra da Mantiqueira, Minas Gerais). Fotos de Rafael F. Magalhães.

Nossa área focal são as ilhas de altitude inseridas no domínio Cerrado, cujo ecossistema de altitude são os campos rupestres. No sentido amplo, os campos rupestres podem ser definidos como mosaicos de vegetação montana, campestre-arbustiva, associada a afloramentos rochosos (quartzito, arenito ou canga) e propensa a queimadas. Inclui campos que crescem em áreas alagáveis, arenosas ou rochosas (Alves et al., 2014). No sentido mais restrito, estes campos são mosaicos de paisagens herbáceas associadas aos afloramentos rochosos (Silveira et al., 2016). Os campos rupestres ocorrem em áreas com ventos constantes e alta variação de temperaturas ao longo do dia, com dias quentes e noites frias. É comum a presença de espécies de plantas rupícolas (que se desenvolvem diretamente sobre as rochas) como aráceas, orquidáceas, bromeliáceas e cactáceas

(Harley, 1995; Ribeiro & Walter, 1998). Estes ambientes possuem vegetação megadiversa, são ecologicamente insubstituíveis e sofrem constantes ameaças à conservação (Fernandes et al., 2014; Silveira et al., 2016).

Os anfíbios são bons modelos para estudos filogeográficos nessas áreas, devido à alta estruturação genética associada à baixa vagilidade (Zeisset & Beebee, 2008) e as respostas ecológicas que estes organismos apresentam frente a mudanças climáticas (Sandel, 2011). Além disso, a distribuição de anuros endêmicos de áreas de altitude se ajusta às predições realizadas por Janzen (1967), com ilhas de altitude na região tropical tendendo a ter espécies endêmicas com distribuição mais restrita às porções mais altas das montanhas (McCain, 2009). Logo, os vales funcionam como barreiras efetivas entre as populações. Por este motivo, nós selecionamos um clado de pererecas endêmicas dos campos rupestres, constituído pelas espécies *Phyllomedusa ayeaye*, *P. centralis* e *P. oreades*. Um dos nossos objetivos foi investigar a influência de mudanças paleoclimáticas do Pleistoceno sobre a diversificação deste grupo. Além disso, também investigamos como a estrutura genética gerada pelo isolamento geográfico e as mudanças climáticas no futuro poderão impactar na conservação da espécie ameaçada *P. ayeaye*.

CONTEXTUALIZAÇÃO TAXONÔMICA

O grupo *Phyllomedusa hypocondrialis* (Anura, Hylidae) é o mais rico do gênero, com 10 espécies formalmente descritas. Neste grupo estão incluídas *Phyllomedusa hypocondrialis* (Daudin, 1800); *Phyllomedusa azurea* (Cope, 1862); *Phyllomedusa palliata* Peters, 1873; *Phyllomedusa rohdei* Mertens, 1926; *Phyllomedusa megacephala* Miranda-Ribeiro, 1926; *Phyllomedusa centralis* Bokermann, 1965; *Phyllomedusa ayeaye* (B. Lutz, 1966); *Phyllomedusa oreades* Brandão, 2002; *Phyllomedusa nordestina* Caramaschi, 2006 e *Phyllomedusa rustica* Bruschi, Lucas, Garcia & Recco-Pimentel,

2014. Na hipótese filogenética de Faivovich e colaboradores (2010) dois clados monofiléticos bem suportados foram recuperados (Figura 3). Um destes clados inclui *P. palliata*, que se distribui na região leste da bacia amazônica mais espécies com distribuição nas áreas baixas do Escudo brasileiro, sendo elas *P. hypochondrialis*, cuja distribuição inclui os Llanos, as regiões leste e central da bacia amazônica e a região centro-norte do Pantanal brasileiro e as espécies irmãs *P. azurea* e *P. nordestina*, endêmicas da diagonal de formações vegetais secas da América do Sul (Faivovich *et al*., 2010; Bruschi *et al*., 2013) (Figura 3). O outro clado inclui *P. rohdei*, com distribuição na Mata Atlântica entre o sul da Bahia e o estado de São Paulo mais *P. ayeaye*, *P. centralis*, *P. megacephala* e *P. oreades*, sendo estas quatro últimas endêmicas de ilhas de altitude do Escudo Brasileiro (Faivovich *et al.,* 2010) (Figura 3). Dentre essas espécies, reconhecia-se um grupo fenético composto pelas pererecas de flancos reticulados, incluindo *P. ayeaye*, *P. centralis*, *P. megacephala* e *P. oreades*. Este grupo era definido com base no padrão de coloração dos adultos, na presença de um espessamento medial na parte superior do bico córneo das larvas e na distribuição geográfica restrita a áreas de altitude do Escudo Brasileiro sob influência do Cerrado (Bokermann, 1965; Cruz, 1982; Brandão, 2002). Contudo, o monofiletismo do grupo das pererecas de flancos reticulados não foi recuperado por Faivovich e colaboradores (2010). Duellman e colaboradores (2016) apresentaram uma nova hipótese filogenética com poucas diferenças topológicas em relação àquela apresentada por Faivovich e colaboradores (2010). Por outro lado, eles realizaram novas propostas nomenclaturais, incluindo a revalidação do gênero *Pithecopus* Cope, 1866, que equivale ao grupo *P. hypocondrialis*. Como Duellman e colaboradores (2016) não apresentaram nenhuma novidade relevante além da instabilidade nomenclatural, nós optamos pela adoção do gênero *Phyllomedusa* nesta tese.

A última década foi marcada por investigações sobre a taxonomia alfa das espécies de *Phyllomedusa* gr. *hypochondrialis*. No clado de áreas altas, três novas espécies foram descritas, dentre as quais duas foram subsequentemente sinonimizadas. *Phyllomedusa rustica* foi recentemente descrita com base em indivíduos proveniente de campos de altitude no município de Água Doce – SC (Bruschi *et al.,* 2014). A espécie possui padrão de coloração intermediário entre as espécies com padrão reticulado e as demais espécies do grupo de *P. hypochondrialis*. Por este motivo, na primeira vez em que *P. rustica* foi registrada, a espécie foi confundida com *P. azurea* (Lucas *et al*., 2010). Além disso, há incerteza sobre a posição filogenética da espécie (Bruschi *et al*., 2014).

Phyllomedusa itacolomi foi descrita do Quadrilatero Ferrífero – MG (localidade tipo: Lagoa Seca, P. E. do Itacolomi, Ouro Preto – MG), tendo como diagnose a seguinte combinação de caracteres, dos quais vale ressaltar: (1) presença de uma faixa reticulada no lábio superior, (2) padrão reticulado nas regiões ocultas dos flancos e membros, consistindo de manchas laranjas ou vermelhas sobre um fundo roxo escuro, (3) presença de uma faixa verde curta no terço distal da superfície dorsal das coxas, (4) padrão reticulado na borda das pálpebras, (5) ausência de faixas esbranquiçadas nas partes laterais do corpo e na superfície posterior da tíbia e (6) superfícies ventrais do corpo e dos membros possuindo coloração esbranquiçada com reticulação cinza clara (Caramaschi *et al*., 2006). Segundo Caramaschi e colaboradores (2006), a espécie se difere de *P. ayeaye* pelo padrão de reticulação mais sutil no lábio superior e na borda das pálpebras. Além disso, os autores consideraram que a reticulação ventral é mais escura em *P. ayeaye* do que em *P. itacolomi* e que o padrão reticulado dos flancos e membros é mais bem definido em *P. ayeaye.* Baêta e colaboradores (2009) reavaliaram estes caracteres em 43 indivíduos de seis localidades, incluindo as localidades tipo de ambas as espécies. Eles concluíram que a combinação de caracteres diagnósticos de *P. itacolomi* também é

encontrada em *P. ayeaye* e vice-e-versa. Com isso, *P. itacolomi* tornou-se um sinônimo júnior de *P. ayeaye*, o que foi confirmado pela hipótese filogenética de Faivovich e colaboradores (2010) (Figura 3).

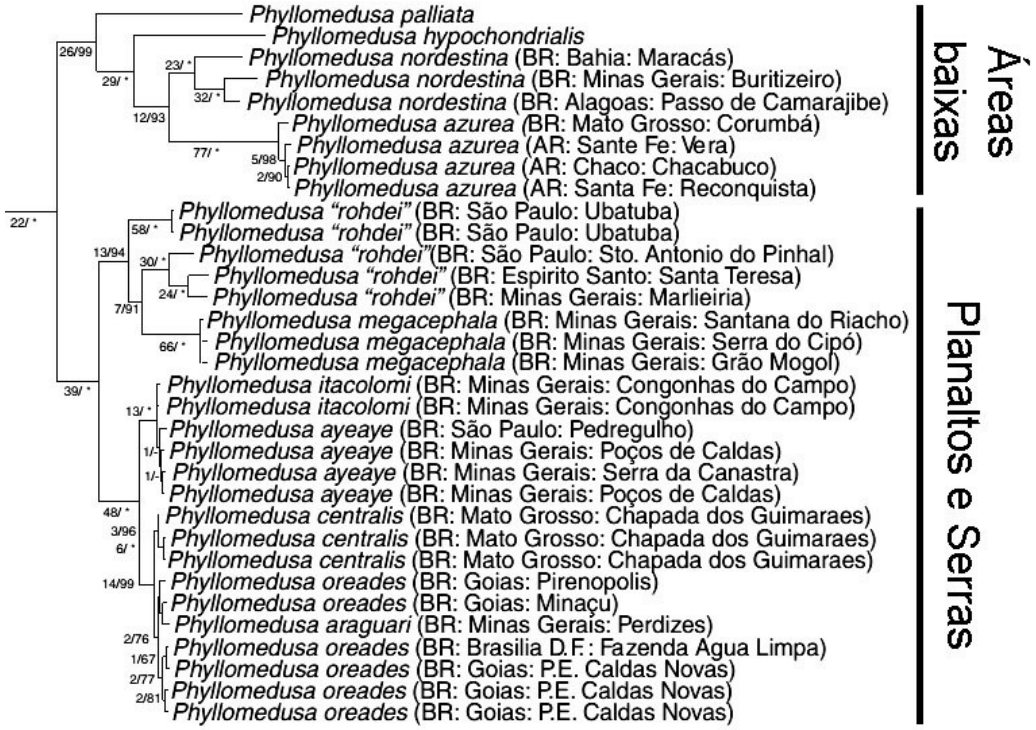

Figura 3. Hipótese de relações filogenéticas entre as espécies do grupo de *Phyllomedusa* hypochondrialis. Os números associados aos nós representam os suportes de Bremmer e as frequências absolutas de Jackknife, respectivamente. O quadrado verde representa o grupo focal das investigações desta tese. Modificado de Faivovich e colaboradores (2010).

Phyllomedusa araguari foi descrita a partir de indivíduos da região do Triângulo Mineiro – MG (localidade tipo: Estação de Pesquisa e Desenvolvimento Ambiental Galheiro, Perdizes – MG) por Giaretta e colaboradores (2007). Os autores apresentaram a diagnose da espécie como uma comparação com as outras espécies do grupo e incluíram-na no grupo fenético das *Phyllomedusa* de flancos reticulados. Neste trabalho, os autores também sugerem que o status taxonômico das populações de *P. oreades* de fora do Planalto de Veadeiros deveria ser reavaliado. A série tipo de *P. oreades* também inclui indivíduos de Brasília – DF que, segundo Giaretta e colaboradores (2007), apresentam discos digitais maiores e padrão de reticulação nos flancos mais ampla. Os estados de caracteres utilizados para diferenciar *P. araguari* de *P. oreades* da localidade

tipo foram (1) faixa reticulada mais ampla nos flancos, (2) padrão de reticulação bem definido no ventre, (3) narinas menos projetadas, (4) presença de reticulação na borda do lábio superior e em volta dos olhos (ausente em *P. oreades*), (5) superfície ventral branca em vida (rosada em *P. oreades*), (6) manchas dos flancos e membros alaranjadas (salmão em *P. oreades*) e (7) reprodução em ambiente lêntico (ambiente lótico em *P. oreades*). Desta forma, gerou-se uma confusão, uma vez que a população de *P. oreades* de Brasília possuiria características intermediárias entre a população da localidade tipo e *P. araguari.* Brandão e Álvares (2009), afim de investigarem a identidade taxonômica dos indivíduos de Brasília, reavaliaram todos os indivíduos da série tipo de *P. oreades* (Brandão, 2002) e incluíram *P. araguari* na comparação. O resultado foi que, dentre os estados de caractere utilizados por Giareta e colaboradores (2007) para diferenciar as espécies, apenas a projeção das narinas se manteve. Todos os outros estados de caracteres relacionados com coloração apresentaram grande variação intrapopulacional. Brandão e Álvares (2009) também constataram que o uso de ambiente lêntico para reprodução por *P. araguari* é resultado da degradação ambiental. Na região de ocorrência de *P. araguari*, o uso indiscriminado do solo é responsável por extensas erosões, muitas delas coincidindo com áreas onde antes haviam riachos temporários. No período de chuvas, estas erosões acumulam água e, na ausência de riachos, os indivíduos de *P. araguari* utilizam estas poças artificiais para se reproduzirem (Brandão & Álvares, 2009). Devido a todas essas evidências incluindo o parafiletismo da espécie em relação à *P. oreades* (Faivovich *et al*., 2010) (Figura 3), *P. araguari* foi considerada um sinônimo júnior de *P. oreades.*

Há um problema nitidamente perceptível na taxonomia alfa do grupo focal: a coloração dos indivíduos é extremamente variável, mesmo a nível intrapopulacional (Baêta *et al*, 2009; Brandão & Álvares, 2009; Bruschi *et al*., 2013). Em *P. megacephala*, por exemplo, este tipo de variação já foi utilizado para reconhecimento individual

(Oliveira *et al*., 2012). Sabe-se que padrões de coloração são pouco úteis na diferenciação de espécies de *Phyllomedusa* estritamente aparentadas (Bruschi *et al*., 2013; Brunes *et al*., 2014). Entretanto como são caracteres tradicionalmente utilizados na taxonomia do grupo, estes padrões continuam constituindo as principais diagnoses das espécies (e.g. Bruschi *et al.*, 2013). Bruschi e colaboradores (2013) listaram quatro combinações fenotípicas nas espécies P. *azurea* e *P. hypochondrialis*. Ao avaliar 116 indivíduos de uma ampla área geográfica, incluindo a região amazônica e áreas de Cerrado, os autores não foram capazes de atribuir com exclusividade nenhum dos padrões de coloração a quaisquer espécies. Desta forma, outros conjuntos de caracteres precisam ser avaliados para elucidar a taxonomia do grupo *P. hypochondrialis*. O uso de caracteres moleculares pode constituir uma alternativa relevante, uma vez que a obtenção de sequências está cada vez mais acessível e fornece resultados mais objetivos (Fujita *et al*., 2012).

Dentre as espécies do grupo de *Phyllomedusa hypochondrialis*, aquelas com distribuição em ilhas de altitude necessitam de uma reavaliação taxonômica. O isolamento geográfico pode ser responsável pela diferenciação de populações por deriva genética e seleção, levando à especiação (e.g. Warshall, 1994; Knowles, 2001). Dentre as espécies foco do nosso trabalho, *P. ayeaye* possui distribuição geográfica que inclui zonas de transição entre Cerrado e Mata Atlântica. A espécie é considerada como criticamente ameaçada de extinção pela IUCN (Caramaschi *et al*., 2010), já que até recentemente só era conhecida de sua localidade tipo (Morro do Ferro, Serra da Mantiqueira, Poços de Caldas – MG). Com a sinonimização de *P. itacolomi* à *P. ayeaye*, houve uma ampliação de distribuição da espécie (incluindo a região dos Campos das Vertentes e o Planalto da Canastra) (Baêta *et al*., 2009), desclassificando-a das categorias de ameaça da Lista Nacional das Espécies da Fauna Ameaçadas de Extinção (ICMBio, 2014). Acredita-se que anuros estejam sujeitos à estase morfológica, uma vez que a seleção sexual parece

afetar principalmente características não-visuais relacionadas ao sistema de reconhecimento de parceiros (como vocalizações e feromônios; Bickford *et al*., 2007). Assim é esperado que a diferenciação morfológica seja inversamente proporcional ao tempo de divergência entre as espécies, sendo dificilmente detectada através de metodologias usuais da sistemática, sobretudo em espécies com tempo de diversificação muito recente (e.g. Reid & Carstens, 2012). Desta forma, caso haja diversidade críptica em *P. ayeaye*, esta não seria diagnosticável pelos caracteres avaliados por Baêta e colaboradores (2009) nem através da filogenia de Faivovich e colaboradores (2010).

Phyllomedusa centralis é encontrada na Chapada dos Guimarães, área no oeste do Escudo Brasileiro que, apesar de estar em cotas altimétricas abaixo dos 900m, é caracterizada pela presença de afloramentos areníticos, incluindo fragmentos de campos rupestres (Brandão et al., 2009). Existem várias espécies endêmicas associadas a microhabitats rupestres nesta região, dentre as quais inclui-se o objeto de estudo (Bokermann, 1965; Brandão *et al*., 2009). Já *P. oreades* é descrita do Planalto de Veadeiros (localidade tipo: Minaçu – GO) e se distribui também nas Serras de Goiás e Distrito Federal e em planaltos do Triângulo Mineiro (Brandão, 2002; Giaretta *et al*., 2007; Brandão & Álvares, 2009). A localidade tipo de *P. centralis* (Chapada dos Guimarães – MT) está a mais de 700km de distância da população mais ocidental de *P. oreades* (Pirenópolis – GO). Entretanto, uma nova população foi amostrada em Barra do Garças – MT (localidade equidistante às supracitadas), embora sua identificação taxonômica ainda não tenha sido confirmada. Adicionalmente a esta problemática, *P. oreades* possui uma área de contato em potencial com *P. ayeaye*, entre as Serras de Goiás e o Planalto da Canastra. Esta área, onde *P. araguari* foi descrita, possui grande influência da biota da Canastra (Giaretta et al., 2007).

No primeiro capítulo da tese, intitulado “Protected areas are not effective to preserve evolutionarily significant units of the critically endangered leaf frog *Phyllomedusa ayeaye”*, nós investigamos se o isolamento em ilhas de altitude está relacionado com a presença de diversidade críptica em *P. ayeaye* através do uso de um método coalescente de delimitação de unidades evolutivamente significativas (ESUs). Além disso, nós modelamos o nicho ecológico da espécie e suas ESUs para a avaliação da efetividade das unidades de conservação de proteção integral (IUCN I-IV) na proteção de *P. ayeaye* no presente e no futuro.

No segundo capítulo, denominado “Pleistocene climate changes drove multiple speciation events in endemic frogs from Brazilian Shield sky islands”, nós avaliamos a influência dos ciclos glaciais do Pleistoceno sobre a diversificação de um clado de *Phyllomedusa* endêmico de campos rupestres. Para tanto, nós utilizamos métodos coalescentes de delimitação de espécies a partir de dados moleculares. Além disso, nós modelamos as mudanças de distribuição geográfica das espécies delimitadas no presente e sua projeção para o Holoceno Médio (6.000 anos atrás) e dois momento do Pleistoceno, o último glacial máximo (21.000 anos atrás) e o último período interglacial (~120.000-140.000 anos atrás) para verificar zonas de contato geográfico potencial entre as linhagens. Por fim, nós utilizamos técnicas de computação Bayesiana aproximada para elucidar os possíveis modos de especiação dessas espécies.

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CAPÍTULO I

Protected areas are not effective to preserve evolutionarily significant units of the critically endangered leaf frog *Phyllomedusa ayeaye*

Magalhães, R. F.; Lemes, P.; Camargo, A.; Oliveira, U.; Brandão, R. A.; Thomassen, H.; Garcia, P. C. A.; Leite, F. S. F.; Santos, F. R. Protected areas are not effective to preserve evolutionarily significant units of the critically endangered frog *Phyllomedusa ayeaye.* Manuscrito submetido ao periódico *Diversity and Distributions*, com algumas modificações de formatação para esta tese.

Protected areas are not effective to preserve evolutionarily significant units of the critically endangered leaf frog *Phyllomedusa ayeaye*

Rafael Félix de Magalhães[1,2,3,4], rafaelfelixm@gmail.com

Priscila Lemes[5], priscila.lemes.azevedo@gmail.com

Arley Camargo[4], arley.camargo@gmail.com

Ubirajara Oliveira[6], ubiologia@yahoo.com.br

Reuber Albuquerque Brandão[7], reuberbrandao@gmail.com

Hans Thomassen[2], hans.thomassen1989@gmail.com

Paulo Christiano de Anchietta Garcia[1,2], pcagarcia@gmail.com

Felipe Sá Fortes Leite[8], fsfleite@gmail.com

Fabrício Rodrigues dos Santos[1,3], fsantos.ufmg@gmail.com

1. Programa de Pós-Graduação em Zoologia, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais – UFMG, Antônio Carlos 6627, CEP 31270-901, Belo Horizonte, Minas Gerais, Brasil.
2. Laboratório de Herpetologia, Departamento de Zoologia, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais – UFMG, Antônio Carlos 6627, CEP 31270-901, Belo Horizonte, Minas Gerais, Brasil.
3. Laboratório de Biodiversidade e Evolução Molecular, Departamento de Biologia Geral, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais – UFMG, Antônio Carlos 6627, CEP 31270-901, Belo Horizonte, Minas Gerais, Brasil.
4. Programa de Desarrollo Universitario, Centro Universitario de Rivera, Universidad de la República – Udelar, Ituzaingó 667, Código Postal 40.000, Rivera, Uruguay.
5. Laboratório de Herpetologia, Departamento de Zoologia, Instituto de Biociências de Rio Claro, Universidade Estadual "Júlio Mesquita Filho" – UNESP, CEP 13506-900, Rio Claro, São Paulo, Brasil.
6. Centro de Sensoriamento Remoto, Instituto de Geociências, Universidade Federal de Minas Gerais – UFMG, Antonio Carlos 6627, CEP 31270-901, Belo Horizonte, MG, Brasil.
7. Laboratório de Fauna e Unidades de Conservação, Departamento de Engenharia Florestal, Faculdade de Tecnologia, Universidade de Brasília – UnB, Campus Universitário Darcy Ribeiro, Asa Norte, CEP 70910-900, Brasília, Distrito Federal, Brasil.
8. Laboratório Sagarana, Instituto de Ciências Biológicas e da Saúde, Universidade Federal de Viçosa – UFV, Campus Florestal, Florestal, MG, Brasil.

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Corresponding author: Rafael Félix de Magalhães
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Abstract

Aim

Protected areas (PAs) are essential to biodiversity conservation. However, the current PAs network coverage is inefficient in species preservation. Many species are also subdivided into different evolutionarily significant units (ESUs) and effectiveness of PAs in protecting them needs to be investigated. We evaluated the usefulness of Brazilian PAs network to protect ESUs of the Critically Endangered *Phyllomedusa ayeaye* in current and future climate scenarios.

Location

Campos rupestres: important endemism areas characterized by mountaintop grassland ecosystems.

Methods

We used DNA data to delimit biogeographical units (BUs) using a spatially explicit approach. These BUs were validated as ESUs through a coalescent method. We used ecological niche modelling to verify spatial changes in ESUs' potential distributions and a gap analysis for evaluate the effectiveness of Brazilian PAs network to protect *P. ayeaye* now and in future climate change scenario. We tested niche overlap between ESUs to obtain insights towards management alternatives for the species.

Results

Phyllomedusa ayeaye presents at least three ESUs, which are isolated in distinct mountain regions. There are no climatic niche differences between the units. Only 4% of the suitable potential area of the species is protected in present and future projections. One of the ESUs presents the lowest genetic diversity and is not protected in any PA.

Main conclusions

The current PAs are not effective to preserve the intraspecific diversity of *P. ayeaye* in its current and future range distributions. There are three ESUs within the species and one of them is not found in any PA. The intraspecific diversity of *P. ayeaye* could represent a typical pattern in endemic species from the Brazilian Shield sky islands. Therefore, the status of *P. ayeaye* in the Brazilian List of Endangered Species should be revised in order to preserve its current intraspecific diversity considering also its potential future expansion.

Introduction

Protected areas (PAs) are the cornerstone conservation strategy to maintain viable populations (Watson *et al*., 2014). Accordingly, the parties of the Convention on Biological Diversity (CBD) suggested expanding the global protected area network from the current 12.7% of terrestrial coverage to 17% (https://www.cbd.int/sp/targets; Françoso *et al*., 2015). Moreover, the creation and maintenance of PAs should consider not only their actual coverage, but also their effectiveness in future species survival. Consequently, intraspecific diversity must be considered in conservation planning, since adaptive potential and stress resistance are positively correlated with genetic diversity (Bálint *et al*., 2011; Pauls *et al*., 2013).

In practice, the global network of PAs is inefficient in representing biodiversity, including threatened species (Rodrigues *et al*., 2004; Nori *et al*., 2015). Most PAs' policies take into account biological diversity at the species-level or above (Gaston *et al*., 2008; Geldmann *et al*., 2013), which are related to phylogenetic and ecological dimensions of biodiversity. The intraspecific dimension, which is related to the evolutionary potential of organisms, is an essential aspect in the planning of conservation policies, especially for endangered species (Bowen & Roman, 2005). In addition, an efficient design of a PAs network should be planned considering species viability towards the future. From a long term perspective, climate changes may worsen the effectiveness of existing PAs in the future, because PAs are static while species' ranges are expected to shift spatially (Monzón *et al*., 2011; Lemes *et al*., 2014). This hypothesis is reinforced by evidence that many species' distributions have already been modified by climate changes (Parmesan & Yohe, 2003; Chen *et al*., 2011). From a population dynamics viewpoint, a species is not always a single unit for conservation, since intraspecific subunits may evolve independtly as a response to climate disruptions (Bálint *et al*., 2011; Forester *et*

al., 2013). This could mean that assessments about the future effectiveness of PAs are being overestimated (Pauls *et al*., 2013). Therefore, an effective plan for future species viability should incorporate the intraspecific levels of biodiversity. In this context, evolutionarily significant units (ESUs) represent ideal targets for conservation, because they contain the raw material for future evolutionary radiations (Fraser & Bernatchez, 2001; Bowen & Roman, 2005; Pauls *et al*., 2013). Thus, population viability evaluations should begin with the challenging task of delineating these intraspecific units (Fraser & Bernatchez, 2001).

Moritz (1994) defined ESUs as reciprocally monophyletic, mitochondrial DNA (mtDNA) groups with significant divergence in nuclear allele frequencies among them. The advantages of this criterion are its objectivity and generality (Fraser & Bernatchez, 2001), but it might not be able to distinguish ESUs with incomplete lineage sorting (ILS) or mtDNA gene flow. In face of the disadvantages of ESUs definitions, Fraser and Bernatchez (2001) proposed that ESUs are intraspecific lineages with highly restricted gene flow among each other. They advocated pluralistic criteria for ESUs delimitation that allow distinguishing among ESUs even when they are not reciprocally monophyletic, but they did not provide further guidance for distinguishing between intraspecific ESUs and species delimitation.

The conservation status of amphibians is alarming in this context because they are among the most threatened vertebrates (Pimm *et al*., 2014) and many species are suffering population declines associated to a variety of threats, including climate change (Stuart *et al*., 2004; Blaustein *et al*., 2011). Despite these serious conservation concerns, 42% of the amphibian richness is misrepresented or completely outside PAs (Nori *et al*., 2015). Amphibians generally exhibit high levels of genetic structuring usually associated with geographic isolation of demes (Allentoft & O'Brien, 2010; Rodríguez *et al.*, 2015), which

makes it difficult to assess whether the differentiated lineages are either distinct species or populations (e.g., Carnaval & Bates, 2007; Gehara *et al*., 2013). In these cases, if conservation policies do not take into account lineage differentiation, a significant loss of cryptic diversity is expected under climate change (Bálint *et al*., 2011, Pauls *et al*., 2013). For this reason, the knowledge about intraspecific ESUs and their spatial distribution and connectivity are important steps towards avoiding genetic diversity loss among isolated amphibian populations (Beebee & Griffiths, 2005, Bálint *et al*., 2011, Pauls *et al*., 2013, Weeks *et al*., 2016).

Herein, we assess cryptic lineage diversification in a hylid frog and the implications for its future conservation. The reticulated leaf frog *Phyllomedusa ayeaye* (B.Lutz, 1966) (Anura, Hylidae, Phyllomedusinae) is classified as critically endangered (CR) by the International Union for Conservation of Nature (IUCN; http://dx.doi.org/10.2305/IUCN.UK.2010-2.RLTS.T55839A11378310.en) and occurs in a threatened ecosystem (Fernandes *et al*., 2014). *Phyllomedusa ayeaye* exhibits a naturally fragmented distribution in mountaintops from southeastern Brazil at elevations higher than 900 m.a.s.l. It occurs mainly in the southern limits of 'campo rupestre' grasslands (see Silveira *et al*., 2015), but it is also found in grassland patches in the Poços de Caldas Plateau (Araújo *et al*., 2007; Baêta *et al*., 2009). These areas are part of the Brazilian Shield sky islands complex (*sensu* Warshall, 1994), which occurs at the border between Cerrado and Atlantic Forest domains. 'Campo rupestre' is a megadiverse ecosystem with high endemism rates in a variety of organisms (e.g. Jacobi *et al*., 2007; Chaves et al., 2014; Silveira *et al*., 2015), including anurans (Leite *et al*., 2008). Although there is evidence of a menaced future for this unique mountaintop ecosystem (Fernandes *et al*., 2014), it has been largely neglected in Brazilian conservation research and policies (Jacobi *et al*., 2007; Silveira *et al*., 2015). There have been few phylogeographic studies

of endemic 'campo rupestre' biota, but they all show strong genetic structuring between sky island populations (e.g. Collevatti *et al.*, 2009; Freitas *et al.,* 2012; Bonatelli *et al.*, 2014), suggesting that cryptic spatial diversification is common in this endemic biota.

We aimed to delimit ESUs within *P. ayeaye* using a broad geographic sampling and multilocus sequence data in statistical phylogeography coupled with GIS information (Forester *et al*., 2013). We analysed multiple island models to validate ESUs and used niche divergence tests to refine predictions about effectiveness of PAs in preserving diversity at the present and in the future (Warren *et al*., 2008; Beerli & Palczewski, 2010; Carstens *et al*., 2013a). In order to evaluate the future survival of distinct ESUs in the current PAs, we used ecological niche modelling (ENM) to project ESUs' ranges based on the ongoing and future scenario of climate change (Bálint *et al*., 2011).

Methods

Data collection

We obtained a sampling consisting primarily of voucher specimens from museums and collections (see Appendix S1). These were complemented with samples collected in a few locations listed in Baêta et al. (2009) to bring a total of 88 individuals from 13 sample points in nine localities (Figure 1, Appendix S1). In the case of tadpole sampling, we excluded individuals in similar developmental stages collected together in the same place or stored in the same museum lot, in order to avoid first-degree relatives and, consequently, to maximize sampling randomness. We sequenced one mtDNA and two nuclear genes (POMC and RPL3). Details towards genomic DNA extraction, sequence data generation, and edition of markers are available in Appendix S1.

ESUs discovery, diversity and relationships

For the ESUs discovery, we first tested spatial population structure using the GENELAND package in R (Guillot *et al*., 2005a; Guillot *et al.*, 2005b; R Core Team, 2016). Because our goal was to identify those lineages that minimized global gene flow (Fraser & Bernatchez, 2001), we opted for the uncorrelated allele frequencies model, which is generally unable to detect subtle structuring (Guillot, 2008). We made 10 parallel runs with $1X10^6$ iterations each, with a thinning of $1X10^3$, and K varying between 1 and 10 biogeographic units (BUs), which we consider as being putative ESUs.

To estimate the relationship among discovered BUs, we constructed a lineage tree in the STARBEAST2 (Helled & Drummond, 2010; Bouckaert *et al*., 2014). To estimate divergence times, we used an uncorrelated log-linear clock model for RPL3, and strict clock models for POMC and Cytochrome *b* (cyt *b*) (Appendix S1). In the latter case, we used a standard mtDNA substitution rate (mean of 0.01 substitutions per lineage per million years; Johns & Avise, 1998), due to the lack of *Phyllomedusa* fossils for calibration. This analysis was made with two replicates, with a pre-burn-in of $2.5X10^7$ followed by $7.5X10^7$ iterations each. Furthermore, we built statistical parsimony networks in POPART 1.7 software (Templeton & Sing, 1993; Leigh & Bryant, 2015) to visualize the relationships between the haplotypes of each gene fragment. Finally, we estimated global and BU-specific summary statistics for each locus in DNASP (Librado & Rozas, 2009).

Because STARBEAST2 analyses do not take into account potential gene flow, and consequently, it can underestimate divergence time (τ) among BU lineages (Pinho & Hey 2010), we also implemented an Approximate Bayesian Computation (ABC) approach to co-estimate demographic parameters based on the lineage tree topology and the best island model of BUs (see below). We simulated $1x10^6$ coalescent genealogies for three

loci with MS (Hudson 2002), and processed them with the MSSS.PL script (available at http://raven.iab.alaska.edu/~ntakebay/teaching/programming/ coalsim/scripts/msSS.pl) to obtain the following summary statistics: average nucleotide pairwise distances per locus (π), number of segregating sites (S), Tajima's D, π within populations, and π between populations. We used uniform prior distributions for all parameters including Θ per locus (lower bound: 0.01, upper bound: 10), divergence times in $4N_e$ units (0.0001, 0.5), and migration rates in $4N_em$ units (0, 50). The same summary statistics were calculated for the empirical data globally, and for each BU and locus (Table 1, Appendix S1). To approximate posterior distributions of parameters, we analyzed simulated and observed summary statistics with the R package ABC (Csilléry et al. 2012) using non-linear local regression (neural-network algorithm) and a tolerance of 0.0002 (to retain 200 simulations). In order to evaluate model fit and adequacy, we performed a Principal Components Analysis (PCA) of all summary statistics from the prior, the posterior, and the empirical data with the STATS package of R. Finally, we used the estimated mean substitution rates of each fragment obtained from STARBEAST2 to convert the BUs split times from coalescent units to number of generations.

ESUs validation

We estimated the historical gene flow (effective number of migrants, $M = 4N_em$,) and the genetic diversity ($\Theta = 4N_e\mu$) of the populations under a coalescent framework using MIGRATE-N (Beerli, 2006). We first implemented a Bayesian full model accounting for the BUs (Figure 2, model 1) using an empirical transition-transversion ratio ($R_{cyt\ b}$ = 4.661; R_{RPL3} = 1.154; R_{POMC} = 1.449) estimated under the K80 model in MEGA7 (Kimura, 1980; Kumar *et al*., 2016), and a scheme of relative mutation rates among loci. Individuals with missing data in haplotypes were excluded, since this may result in spurious results

in MIGRATE-N (Carstens *et al*., 2013a). Initial values for parameters were derived from F_{ST} estimates. We conducted the analysis with two parallel runs, using a static heat strategy, setting one long and 12 short chains, with the cold chain equal to one, the hottest chain equal to $5x10^5$ and values of the remaining chains growing at a cumulative exponential scale of $x^{1.4}$, starting from 1.5. We made a pre burn-in of $1.25X10^6$ generations, and $2.5X10^6$ states were visited in each run, with a thinning of 100. Exploratory analyses with the full model allowed us to determine the sampling window for Θ and M, and we assumed a normal distribution for these parameters.

Based on the GENELAND and STARBEAST2 results, we generated a set of seven models to validate the putative ESUs assignment (Figure 2, models 2-8). Fraser & Bernatchez (2001) advocated the use of criteria that "provide evidence of lineage sorting through highly reduced gene flow", but they did not propose a cutoff level of gene flow for this criterion. Therefore, we tested scenarios of BU independence (Figure 2, models 1-5) against scenarios of split between sister BUs (Figure 2, models 6 and 7), and panmixia (Figure 2, model 8). In summary, we tested models ranging between one and three ESUs, with distinct levels and routes of gene flow among them. If the gene flow is so high that two or more BUs form a panmictic population, the BUs are collapsed into a single ESUs. In order to reduce the potential set of models, they were constructed under the following assumptions: (1) when present, gene flow is always bidirectional (Figure 2, models 2,3,4 and 6), and (2) sister BUs have gene flow between them (Figure 2, models 2-4), except in the full isolation model (Figure 2, model 5), or in models where they form a panmictic population (Figure 2, models 6 and 7). The second assumption was based on the expectation that gene flow tends to decrease with longer splitting times between lineages (Pinho & Hey, 2010). We calculated the marginal likelihood of each model and compared them under a Bayes factor test using the Bezier's approximation score (Beerli

& Palczewski, 2010). Under this approach, we can select the model with the highest probability of fitting our data. We used model averaging for final estimates of Θ and M, and evaluated the performance of MCMC sampling through ESS and acceptance ratio values.

Ecological Niche Modeling (ENMs)

Using the bioclimatic data at a 2.5-minute resolution of latitude and longitude, we modeled species niche and projected distribution of *P. ayeaye* across the mountaintops of a selected region in southeastern Brazil (Appendix S1). Because we had much more collection records than sample points (Appendix S1), we used the putative population limits estimated by GENELAND to assign known records to our delimited ESUs (Appendix S1). We obtained the climate layers from Hijmans *et al.* (2005) to characterize the environmental space for ENMs using both current (1960-1990), and future climate conditions (average predicted for 2061-2080). The future climate layers are derived from three coupled Atmosphere-Ocean General Circulation Models (AOGCMs): CCSM4, CNRM-CM5, and MIROC5. We selected four variables (temperature annual range, mean temperature of warmest quarter, precipitation of wettest and driest quarter) out of 19 bioclimatic variables using a factorial analysis with a varimax rotation (implemented in PSYCH package in R; available at https://CRAN.R-project.org/package=psych) (Appendix S1). This method is based on the correlation matrix among variables to minimize collinearity problems, consequently avoiding biased predictions of the ENMs. In addition, we also included elevation (Hijmans *et al.,* 2005) to account for the topographic complexity of the region as a constraint variable in order to improve the ENMs.

A key assumption in our modeling is that ensemble forecasts based on multiple models generate more accurate or at least more conservative projections of species

distribution than single models do (Araújo & New, 2007). Therefore, four modeling methods were used to build the ENMs, including: Bioclim, Gower Distances, Maximum Entropy (MaxEnt), and Support Vector Machine (SVM) (for a review see Peterson *et al.*, 2011). All ENMs were implemented in the DISMO package in R (available at https://CRAN.R-project.org/package=dismo). In summary, models were first generated for current climate and then projected onto future conditions to predict the species geographical range for these two time periods. We assessed model performance for each decision threshold using the 'leave-one-out test' because of the small number of occurrence records for *P. ayeaye* and ESUs (Appendix S1). Hence, multiple predictions were made for *P. ayeaye* and ESUs, selecting one occurrence record for removal in each case. This approach is described as a variation to the k-fold partitioning method on which a Jackknife sampling is imposed (Pearson *et al*., 2007; Bean *et al.,* 2012; Shcheglovitova & Anderson, 2013). For each prediction, we applied the lowest presence decision threshold (LPT) to test the ability to predict the deleted occurrences. If the ENM successfully predicts both a small area and the deleted occurrence record, it is better than a random model ($p<0.05$). However, if the model predicts a large area and fails to predict the deleted occurrence record, it is not considered a good model ($p>0.05$). Therefore, the p-value is calculated from the success and failure ratio of the prediction (Pearson *et al.,* 2007).

Our modeling procedure resulted in 12 suitability maps (i.e., 4 ENMs * 3 AOGCMs) for *P. ayeaye* and ESUs, and for each climate condition. Finally, we obtained a consensus map for each combination of ENMs and AOGCM. To assess individual model variability sources, we separated and mapped uncertainties in forecast ensembles (Diniz-Filho *et al.* 2009). For this purpose, we performed a two-way ANOVA for each

grid cell using suitability as response variable and the methodological components (AOGCMs and ENMs) as explanatory variables.

Effectiveness of PAs to ESUs protection

To verify the PAs effectiveness to protect all *P. ayeaye*'s ESUs, we built future projections from ESUs analysis and overlapped them to protected areas of integral protection (IUCN Categories I to IV) already established in the region. The protected areas' layers were obtained from The World Database on Protected Areas (available at https://www.unep-wcmc.org/resources-and-data/wdpa). To obtain insights about management alternatives, we used the estimated models to test the niche overlap hypothesis between ESUs. The applied tests were Schoener's D (*D*; Schoener, 1968), I statistic (*I*; Warren *et al*., 2008) and the relative rank (*RR*; Warren & Seifert, 2001), all made in ENMTools v1.4.4 (Warren *et al*., 2010).

Results

GENELAND runs showed convergence in posterior probabilities (PP) of models, after a burn-in of $1X10^3$ replicates. The analyses returned three BUs with PP=0.47. The geographic distribution of these lineages corresponded to the following mountain ranges: (1) Canastra Plateau and surrounding mountains, (2) Poços de Caldas Plateau, southwest of Mantiqueira range, and (3) Quadrilátero Ferrífero plus Southern Minas Gerais Mountains, named hereafter Canastra, Poços and Quadrilátero, respectively (Figures 1, 3).

The lineage tree (Figure 4) showed a closer relationship between Canastra and Poços as sister BUs. The estimated mean substitution rates for POMC and RPL3 were 0.0043 (SD = 0.0021) and 0.0087 (SD = 0.0029) per lineage per million years,

respectively. Regarding ABC estimates, the evaluation of model fit done with a PCA analysis of summary statistics suggested that the simulated demographic model fits well the empirical data because the cloud of posterior data points matches the observed data point (Appendix S1). As expected, the divergence times estimated with the ABC approach (which takes gene flow among ESUs into account) were older than the STARBEAST2's estimates, especially the older splitting event, which was about 3.5 times larger on average (Table 3). Furthermore, Θ estimates had values within the 95% highest posterior density (HPD) confidence intervals from MIGRATE-N parameters, except for Canastra's Θ (Table 3). On the other hand, all migration rates were higher than those estimated in full Bayesian approach (Table 3). All cyt *b* haplotypes were reciprocally exclusive in each BU, although they did not represent distinct haplogroups in the network (Figure 5a). Despite the lack of a clear differentiation among BUs in the POMC network (Figure 5b), the RPL3 network did exhibit divergence among them (Figure 5c), further supporting each BU as an ESU.

The Canastra BU exhibited much more genetic diversity than the others (Tables 1, 3). This does not seem to be a sampling artifact because Quadrilátero BU has a similar sampling in terms of field work effort and geographic coverage (Appendix S1, Figure 3). Globally, RPL3 was the only fragment showing deviation from neutrality, but this may be attributed to the large intergroup differentiation found in this marker (Figure 5c) due to the occurrence of multiple indels, which is typical in introns. In short, RPL3 showed deep allopatric structuring, further strengthening our BUs as ESUs (Table 1, 3).

The demographic model selection confirmed each BU as a distinct ESU, but with gene flow among them. The best global island model with PP=0.67 was the one where Canastra and Quadrilátero are isolated from each other (Table 2; Figure 2, model 3). The estimated values from Θ and *M* from the best model are shown in Table 3. The model

where Poços and Quadrilátero are isolated one from another (Figure 2, model 2) had PP=0.34 (Table 2). This non-negligible probability for an alternative model may be due to incongruence among loci. While cyt *b* and RPL3 show 76 and 96% of probability associated to model 3, respectively (Figure 2), the best fit model for POMC was number 2, with 43% of probability (Figure 2). In any case, our results suggest higher gene flow between the sisters ESUs of Canastra and Poços, but limited gene flow between Quadrilátero and at least one of the other ESUs. Furthermore, models with island isolation (Figure 2, models 4, 5 and 7) and panmixia (Figure 2, model 8) had no support in either single- or multi-locus analyses (Table 2). From the genetic point of view, the three BUs are ESUs that form a historical metapopulation, with a stepping-stone island pattern among them.

We used the four ENMs to build a consensus map to projected current and future potential distribution for entire *P. ayeaye*, Canastra and Quadrilátero ESUs (Figure 6). The projections presented for *P. ayeaye* and BUs were trained using between seven to 26 localities and show high success rates in Jackknife tests (Table 4). Under current climate conditions, the potential distribution of *P. ayeaye* occurred throughout mountaintops with a relatively high suitability (>0.5), mainly in the State of Minas Gerais, but with a predicted expanded distribution in future climate conditions. The potential distributions of Canastra and Quadrilátero ESUs are both locally suitable in the current climate conditions (Figure 6). However, there are clear changes of suitability areas' patterns in future climate conditions, characterized by northward and southward expansions.

The two-way ANOVA applied shows that the median of the variation projected in future climate conditions for *P. ayeaye* and Quadrilatero ESU are due to differences in ENMs (86.4%; Table 5), with the lowest differences in the southern limit of Minas Gerais State (Appendix S1). Furthermore, the maps showing this sum of squares (Appendix S1)

indicate the largest differences among methods for *P. ayeaye*, Canastra and Quadrilatero ESUs.

If considering the ENMs, both *P. ayeaye* (all populations) and individual ESUs can potentially be found in other PAs in addition to those already known to protect the species, but less than 4% of all suitability areas (probability of occurrence > 0.5) are in protected areas for present and future projections. From an ecological perspective, Canastra e Quadrilátero populations do not present identical niches, but show great overlap (D = 0.56; I = 0.85 and RR = 0.85). Although Poços ESU was not included in niche overlap tests, it is important to note that the Quadrilátero niche model comprises Poços de Caldas plateau with high probability (Figure 6).

Discussion

Phyllomedusa ayeaye is classified as CR by IUCN based on B1ab(iii)+2ab(iii) criteria, since it was only known from two disjunct localities threatened by severe habitat loss due to mining and human-induced fires (http://dx.doi.org/10.2305/IUCN.UK.2010-2.RLTS.T55839A11378310.en). The threat sources are more numerous because the mountain grasslands where *P. ayeaye* occurs are also menaced by forestry, cattle farming, non-sustainable "ecotourism", and poorly-planned urbanization (Silveira *et al*., 2015). These threats impact direct or indirectly the highland streams where *P. ayeaye* breeds. For this reason, *P. ayeaye* was included as a priority in a Brazilian national action plan for Espinhaço Range herpetofauna (http://www.icmbio.gov.br/portal/faunabrasileira/planos-de-acao-nacional). After the discovery of new localities (Araujo *et al*., 2007; Baêta *et al*., 2009), the species no longer meets the geographic requirements to be categorized as CR. For this reason, *P. ayeaye* was recently removed from all threat categories in the Brazilian List of Endangered

Species (http://www.mma.gov.br/biodiversidade/especies-ameacadas-de-extincao/fauna-ameacada), and excluded of the national agenda of conservation priorities. However, our results show that the species displays a strong genetic structure associated to historical spatial fragmentation. Moreover, despite the reported wide range, today the species is known to occur only in three PAs: the Furnas do Bom Jesus State Park, Itacolomi State Park, and Serra da Canastra National Park (Araújo *et al.*, 2007; Baêta *et al*., 2009), which together encompass less than 4% of the entire, potential and suitable, geographic distribution of *P. ayeaye*.

To aggravate the situation, *P. ayeaye* exhibits a set of life history traits associated with vulnerability in amphibians. The species is uncommon and shows low individual density in most of its range (Araujo *et al*., 2007; Baêta *et al*., 2009). In addition, it produces egg masses with a few large eggs and the larvae generally inhabit rocky stream pools, with clear and slow-flowing water (Pezzuti *et al*., 2009; pers. obs.). Rarity, *K*-reproductive strategy, and high habitat specialization are characteristics associated with low genetic diversity, increased population structure, and/or high threat levels (Cooper *et* al., 2008; Romiguier *et al*., 2014; Toledo *et al*., 2014, Rodríguez *et al.*, 2015). As *P. ayeaye* has a fragmented distribution of populations with evolutionary independence and is endemic from Brazil, we suggest the conservation status of the species should be revised in the national list of endangered species because its long-term survival depends on local policies. For recategorization, more research on demographic size and population trends need to be conducted.

Our protocol to identify ESUs was analogous to the validation steps used in species delimitation (see Carstens *et al*., 2013b) and allowed us to validate *P. ayeaye*'s ESUs even in the lack of reciprocal monophyly (Moritz 1994). Moreover, the ESUs exhibited exclusivity in mtDNA haplotypes, meeting the requirements proposed by the

Fraser and Bernatchez's ESU definition. This idea may be applied with any coalescent sampler that implements model selection (see Carstens *et al*., 2013a), not being limited to the use of MIGRATE-N. Additionally, if there is no evidence of gene flow between ESUs, a coalescent method of species delimitation may be applied a posteriori to distinguish between ESU and distinct species with any multilocus sequence dataset (Carstens *et al*., 2013b). Geographically isolated populations may experience cyclical events of gene flow, especially in those species associated with interglacial refugia (Bonatelli *et al*., 2014). Gene flow breaks differentiation between lineages and generates mtDNA para- or polyphyly, a condition that does not meet the requirements of Moritz's (1994) definition of ESU. The lack of well-defined haplogroups in the cyt *b* network and τ estimates indicate recent diversification events between Middle and Late Pleistocene. This suggests that the distribution pattern of *P. ayeaye* may be associated with interglacial refugia, a pattern that may be common in endemic species of 'campo rupestre' (Bonatelli *et al*., 2014). Nevertheless, more detailed phylogeographical studies should be carry out to test this hypothesis.

Despite 'campo rupestre' may be expected to contract in warmer climatic conditions (Bonatelli *et al.,* 2014; Fernandes *et al.*, 2014), our results show a surprising potential future expansion for the species' distribution. Moreover, niche overlap analysis indicates no climate selection between ESUs, which allows us to suggest conservation strategies that may be implemented in the present with potential positive impacts in the future. *In situ* conservation is the most feasible alternative for amphibian protection in Brazil (Haddad, 2008). Therefore, the expansion of Brazilian PAs network in mountaintops where *P. ayeaye* occurs, taking into account its intraspecific diversity, can mitigate the impacts suffered by the species, and consequently, other endemic, co-occurring organisms. Another strategy to avoid genetic diversity loss and inbreeding is the

translocation of individuals between ESUs (Weeks *et al*., 2016). Our results provide valuable information to plan this strategy, suggesting that individuals from Canastra population could eventually be translocated to Poços de Caldas Plateau, to increase the genetic diversity in Poços ESU. However, this strategy should be done carefully to avoid possible negative consequences as outbreeding depression (see Frankham *et al*., 2011).

Even though the maintenance of evolutionary potential is important for allowing amphibians to cope with environmental changes (Allentoft & O'Brien 2010), few countries take intraspecific diversity into account in their conservation policies, and Brazil is unfortunately not among them. For example, the dectection of three remarkably divergent ESUs in the Atlantic Forest sloth, *Bradypus torquatus* (Lara-Ruiz et al. 2008), was not enough to avoid downlisting the species from Endangered to Vulnerable (Brazilian List of Endangered Species and IUCN). Most *P. ayeaye* ESUs lack effective protection because areas with integral protection cover a small part of their present distribution. Although *P. ayeaye* occurs also in some protected areas of sustainable use, these categories are inefficient to avoid habitat loss in the Brazilian Cerrado (Françoso *et al.*, 2015). Additionally, many areas lack any kind of protection, as the species type-locality in Morro do Ferro in Poços de Caldas, which is the ESU with the smallest genetic diversity in our results. The creation of an integral protection PA in this area must be a priority, since another endangered species, as *Bokermannohyla vulcaniae* and *Proceratophrys palustris*, are only known to occur in this region.

Our ENM approach has caveats as the locality points were defined particularly by opportunistic sampling of literature and voucher specimens from museum and collections. Thence, our analyses based on low sample sizes with a likely sampling bias (*i.e.,* collected in shrubs near pools or rivulets) can underestimate the species occurrence with consequences to model accuracy and interpretability (see Peterson *et al*. 2011).

Many studies have shown that model accuracy using about 30 occurrences is often low, and is quite heterogeneous across species (i.e. Hernandez *et al.,* 2006; Wisz *et al.,* 2008), even though, other studies have adjusted the ENMs for fewer occurrences (i.e., Pearson *et al.,* 2007; Shcheglovitova & Anderson, 2013). Finally, Jackknife method may be a better approach for a complete model evaluation with small samples (Pearson *et al.* 2007; Bean *et al.,* 2012), but there are recent alternative strategies applicable to various standard ENM techniques and algorithms using multiple ENM algorithms (Breiner *et al.,* 2015).

Our study sheds new light for conservation practice in Brazil, since we found significant divergence among *P. ayeaye* populations that define three ESUs associated to distinct mountain regions, including one ESU found exclusively in an area without any kind of protection. This population structure reflects patterns found in many other taxa of 'campo rupestre', which suggests that common preservation strategies can be applied for many Brazilian mountaintop endemic species to ensure their future viability. Thence, intraspecific studies of Brazilian sky islands' biota are greatly needed to guide decision-makers in generating policies that considers evolutionary and ecological specificities of these ecosystems.

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Biosketch

Rafael Félix de Magalhães is a PhD candidate in Zoology interested in evolution and conservation of fauna from Brazilian sky islands. His current research includes investigations towards species limits, phylogeography and conservation genetics of endemic anurans from mountaintop grasslands.

Table 1. Global and population summary statistics for sampled loci. Parameters shown are the total number of haplotypes (N), number of unique haplotypes (h), haplotype diversity (Hd) and nucleotide diversity per site (π). Significant values of Fu's F and Tajima's D are shown for $p < 0.05$ (*), $p < 0.005$ (**) and $p < 0.001$ (***)

Gene fragment	bp	Model	ESU	N	Segregating sites	Fu's F	Tajima's D	h	Hd (SD)	π (SD)
cyt *b*	896	HKY	Total	86	26	-7.044***	-1.054	22	0.926 (0.012)	0.00393 (0.00021)
			Canastra	35	19	-3.126*	-1.037	12	0.862 (0.035)	0.00305 (0.00039)
			Poços	17	2	-1.038	-1.069	3	0.324 (0.136)	0.00038 (0.00017)
			Quadrilátero	34	8	-0.878	0.833	7	0.831 (0.036)	0.00313 (0.00019)
RPL3	518	K80 + I	Total	176	47	7.146***	2.399*	24	0.831 (0.016)	0.02907 (0.00049)
			Canastra	70	29	-0.266	-0.802	14	0.776 (0.035)	0.00120 (0.00193)
			Poços	36	24	5.95**	0.11	6	0.432 (0.099)	0.01176 (0.00323)
			Quadrilátero	70	29	6.28**	0.032	8	0.376 (0.072)	0.01199 (0.00282)
POMC	601	HKY + I	Total	176	9	-0.627	1.077	12	0.728 (0.001)	0.00382 (0.00023)
			Canastra	70	9	-0.241	1.07	10	0.793 (0.031)	0.00437 (0.00028)
			Poços	36	7	-0.306	1.224	8	0.816 (0.038)	0.00402 (0.00035)
			Quadrilátero	70	7	0.727	0.2	6	0.513 (0.005)	0.00261 (0.00045)

Table 2. Model selection with MIGRATE-N. Parameter shown are model numbers, sorted by probability, equivalent to Figure 2. PP is posterior probability.

Model	Description	Bezier's approximation score	PP	Weight
3	Canastra e Quadrilátero are isolated each other	-4266.55	0.661	1
2	Poços e Quadrilátero are isolated each other	-4267.23	0.335	0.507
1	All populations are interconnected	-4271.73	0.004	0.006
6	Poços and Canastra form a panmictic population connected with Quadrilátero	-4333.22	<0.001	<0.001
4	Poços and Canastra interconnected but isolated from Quadrilátero	-4495.11	<0.001	<0.001
8	Panmixia	-4498.78	<0.001	<0.001
5	All populations isolated each other	-4557.04	<0.001	<0.001
7	Poços and Canastra form a panmictic population isolated from Quadrilátero	-4613.54	<0.001	<0.001

Table 3. Comparison between estimated using full Bayesian (STARBEAST2 and MIGRATE-N) and ABC approaches. Parameters shown are divergence times in million years (τ, assuming one generation per year), population sizes (Θ) and effective number of migrants (M) of ESUs Canastra (C), Poços (P) and Quadrilátero (Q). τ in full Bayesian was estimated in STARBEAST2, while the remaining parameters were estimated in MIGRATE-N.

Parameter	Full Bayesian		ABC	
	Mean	**95% HPD**	**Mean**	**95% HPD**
τ_1	0.06845	0.01842 - 0.19665	0.07494	0.06894 - 0.08104
τ_2	0.1089	0.02299 - 0.32562	0.3899	0.37569 - 0.40567
Θ_C	0.00376	0.00072 - 0.00391	0.00889	0.00884 - 0.00894
Θ_P	0.00129	0.00001 - 0.00182	0.0011	0.00100 - 0.00120
Θ_Q	0.00176	0.00066 - 0.00290	0.00138	0.00106 - 0.00167
$M_{C>P}$	49.96	0.00 - 50.93	88.85	86.42 - 91.65
$M_{P>C}$	13.04	0.00 - 44.27	40.52	37.46 - 44.34
$M_{P>Q}$	9.48	0.00 - 37.61	83.41	79.86 - 87.19
$M_{Q>P}$	15.14	0.00 - 42.94	72.66	70.99 - 74.68

Table 4. Jaccknife test of distribution models for *Phyllomedusa ayeaye* (B. Lutz, 1968) and ESUs for each ENMs applied.

AOGCM	ENM	*Phyllomedusa ayeaye*			Canastra ESU			Quadrilátero ESU		
		Sample Size	Success	p-value	Sample Size	Success	p-value	Sample Size	Success	p-value
CCSM4	Bioclim	26	19	2.85E-14	7	2	0.01014	16	11	3.08E-18
	Gower Distance	26	23	6.36E-16	7	3	0.0013	16	14	1.11E-21
	Maxent	26	25	2.23E-14	7	5	0.00065	16	15	9.36E-15
	SVM	26	25	2.26E-15	7	5	0.0001	16	14	5.11E-20
CNRM-CM5	Bioclim	26	19	2.85E-14	7	2	0.01014	16	11	3.08E-18
	Gower Distance	26	23	6.36E-16	7	3	0.0013	16	14	1.11E-21
	Maxent	26	25	2.23E-14	7	5	0.00065	16	15	9.36E-15
	SVM	26	25	6.67E-15	7	5	8.06E-05	16	15	2.19E-21
MIROC5	Bioclim	26	19	2.85E-14	7	2	0.01014	16	11	3.08E-18
	Gower Distance	26	23	6.36E-16	7	3	0.0013	16	14	1.11E-21
	Maxent	26	25	2.23E-14	7	5	0.00065	16	15	9.36E-15
	SVM	26	25	3.68E-15	7	5	8.28E-05	16	15	6.03E-19

Table 5. Median proportions of the total sum of squares from the two-way ANOVA performed for each grid cell covering the Neotropics, evaluating the relative contributions of method for niche models and Atmospheric-Ocean Global Circulation Models (AOGCM) to the variability in forecasting *Phyllomedusa ayeaye* (B. Lutz, 1966) and ESUs distribution. Minimum and maximum values in the maps are also given (see also Appendix S1).

Source	***Phyllomedusa ayeaye***		**Canastra ESU**		**Quadrilátero ESU**	
	SS (%) median	**Min-max**	**SS (%) median**	**Min-max**	**SS (%) median**	**Min-max**
ENMs	0.864	0.005-0.998	0.982	0.008-1	0.864	0.005-0.998
AOGCM	0.014	0-0.543	0.003	0-0.540	0.014	0-0.543
ENMs x AOGCM	0.114	0-0.687	0.011	0-0.701	0.114	0-0.687

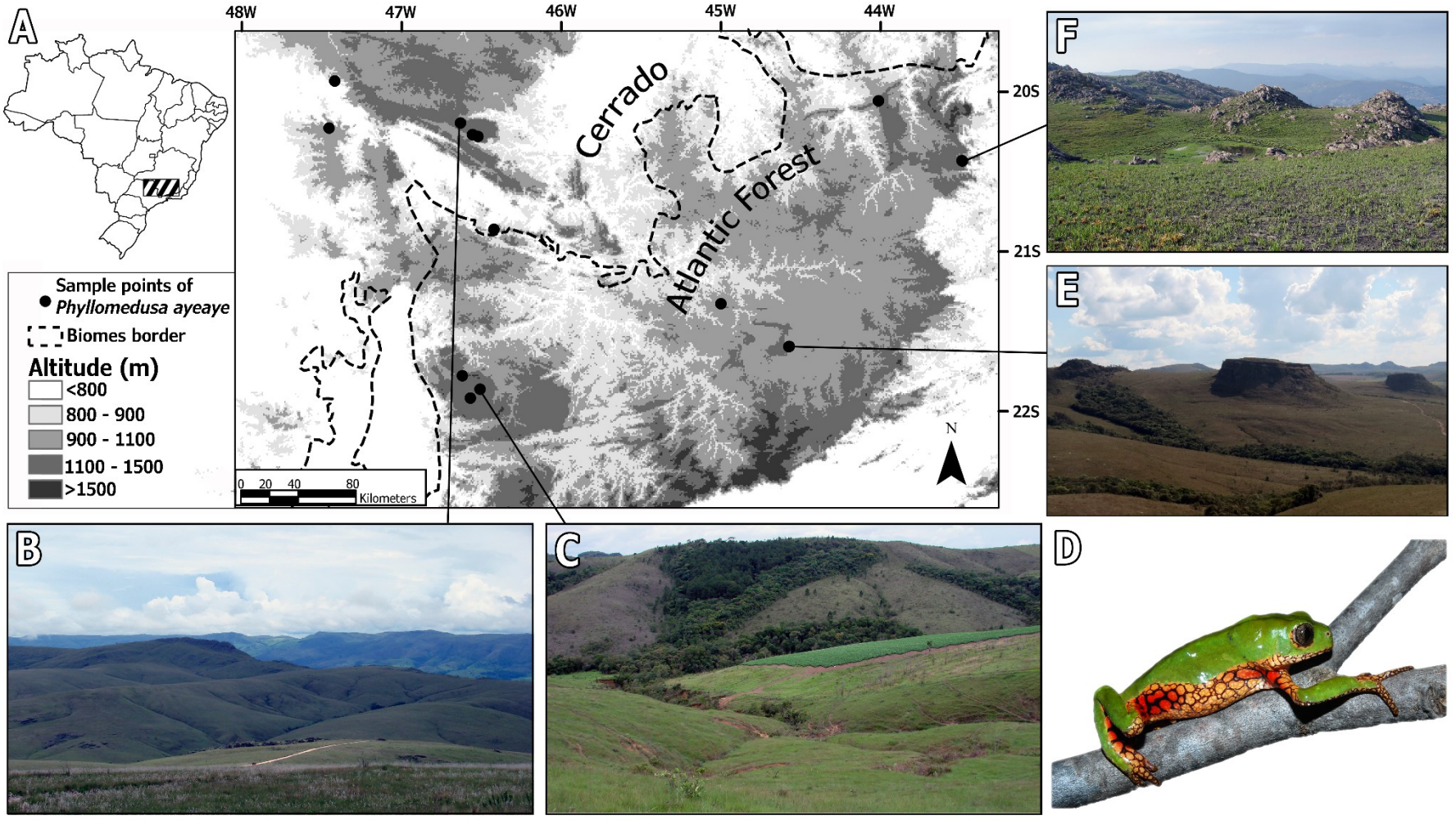
A
48W
47W
46W
45W
44W
20S
21S
22S
Cerrado
Atlantic Forest
N
0 20 40 80
Kilometers
Sample points of
Phyllomedusa ayeaye
Biomes border
Altitude (m)
<800
800 - 900
900 - 1100
1100 - 1500
>1500
B
C
D
E
F

Fig.1

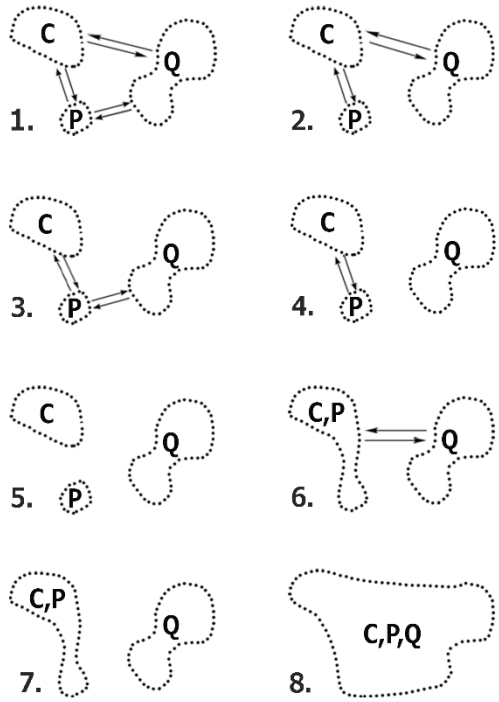
C
Q
P
1.
2.
3.
4.
5.
6.
C,P
7.
C,P,Q
8.

Fig. 2

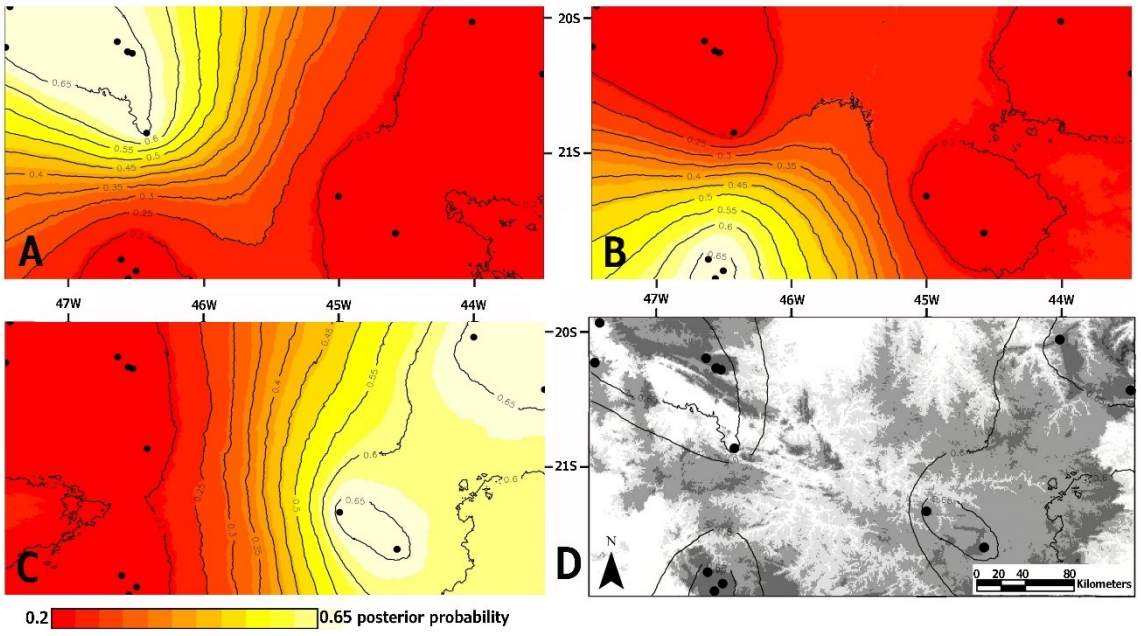
A
B
C
D
20S
21S
47W
46W
45W
44W
N
0 20 40 80
Kilometers
0.2
0.65 posterior probability

Fig. 3

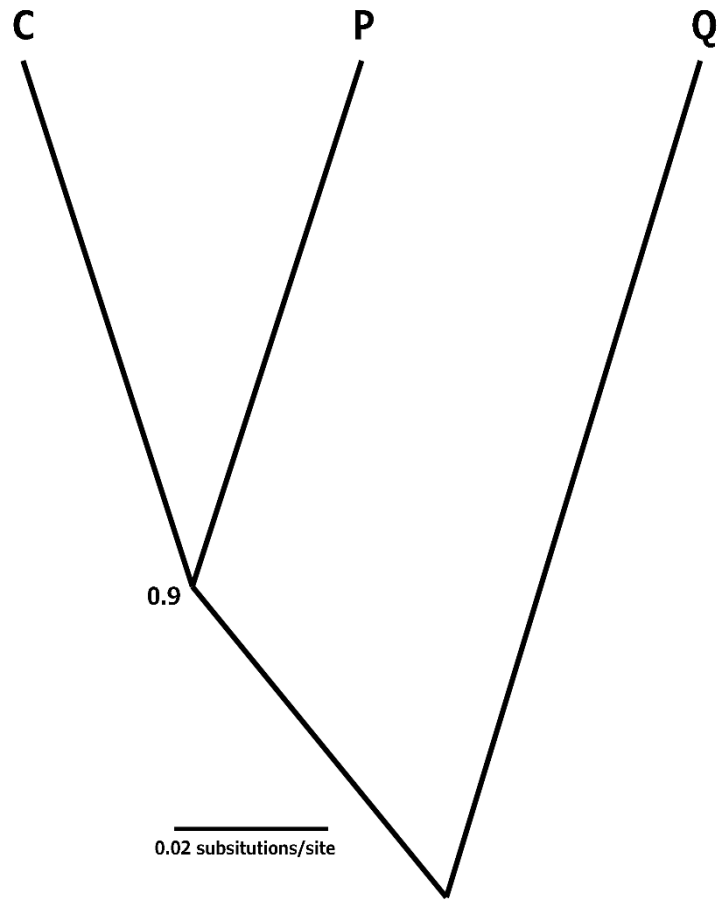

Fig. 4

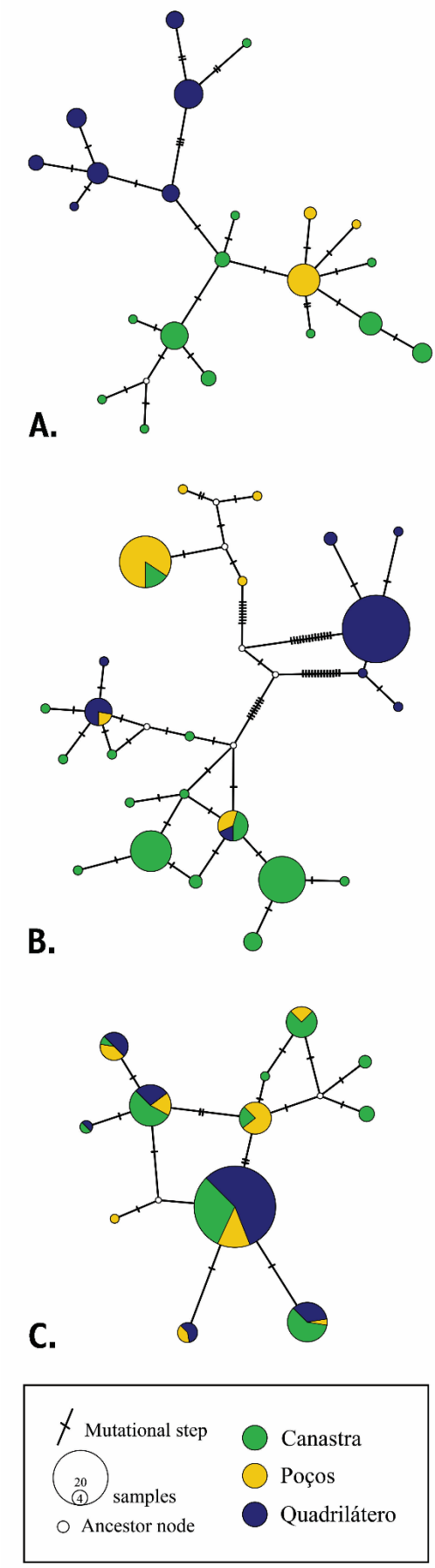
A.
B.
C.
Mutational step
20
4
samples
Ancestor node
Canastra
Poços
Quadrilátero

Fig. 5

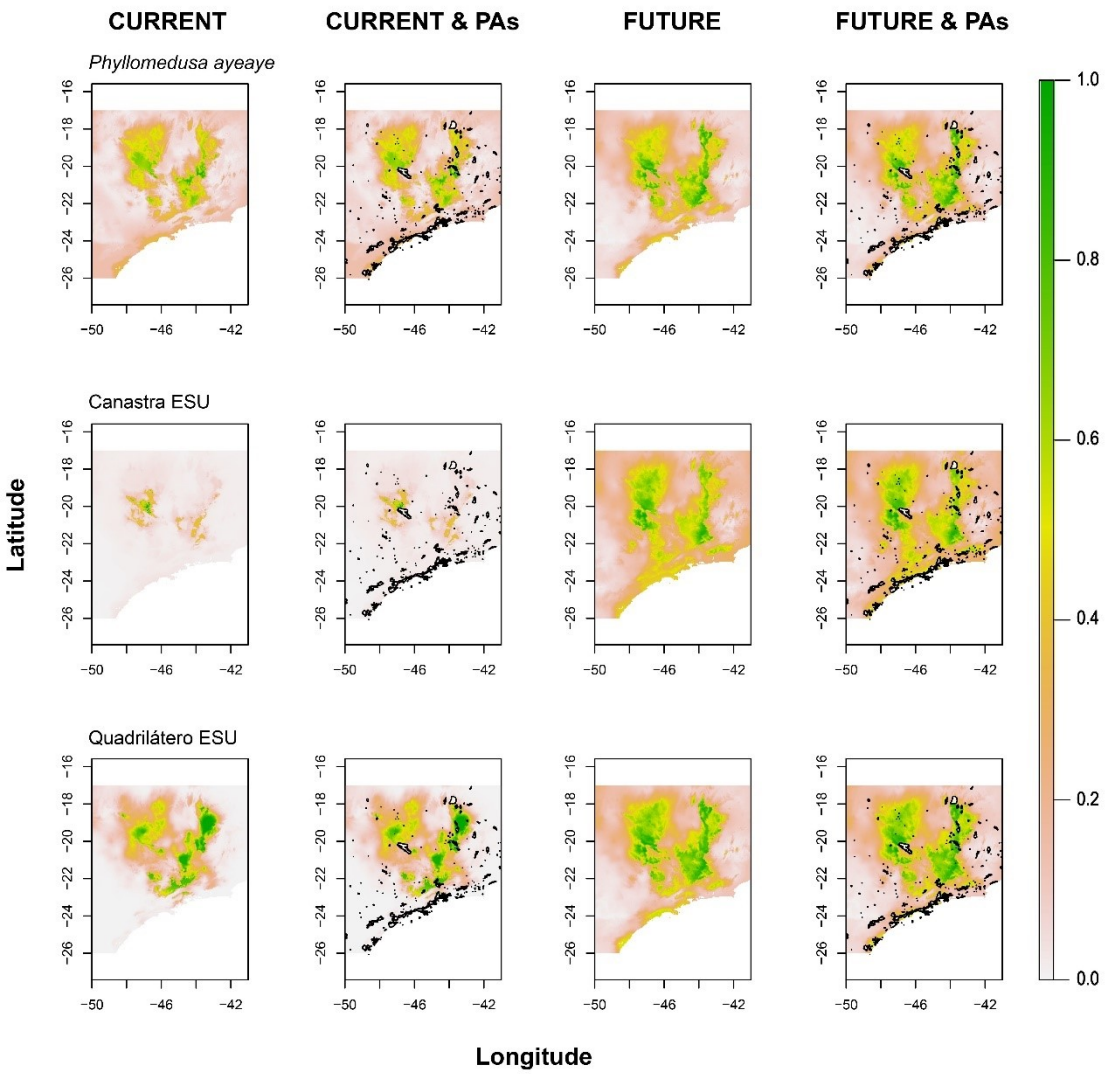
CURRENT
CURRENT & PAs
FUTURE
FUTURE & PAs
Phyllomedusa ayeaye
Canastra ESU
Quadrilátero ESU
Latitude
Longitude
−16
−18
−20
−22
−24
−26
−50
−46
−42
1.0
0.8
0.6
0.4
0.2
0.0

Fig. 6

Figure captions

Figure 1. A. Map of sampled locations of *Phyllomedusa ayeaye* (B. Lutz, 1964). Some examples of landscapes observed in **B.** National Park of Serra da Canastra, Canastra Plateau, MG, **C.** Morro do Ferro, Poços de Caldas Plateau, Mantiqueira mountain range, MG and **D.** and adult individual sampled there, **E.** Chapada das Perdizes, Southern Minas Gerais mountains, MG and **F.** Serra do Itacolomi, Quadrilátero Ferrífero, MG. Photos B and F by Tiago L. Pezutti, C and D by R.A.B and E by R.F.M.

Figure 2. Set of eight models tested in MIGRATE-N model selection.

Figure 3. Maps of **A.** Canastra, **B.** Poços and **C.** Quadrilátero biogeographical units (BUs) membership posterior probabilities (PP). The geographical distribution of each BU is shown in **D.**, where the internal and external curves indicates geographical limits with PP = 0.65 and 0.6, respectively. The color scale of probabilities refers to A, B and C. Grey scale in D refers to altitude, as in Fig. 1.

Figure 4. Maximum credibility lineage tree showing genealogical relationship between biogeographical units. Number in node is Bayesian posterior probability. C = Canastra, P = Poços and Q = Quadrilátero.

Figure 5. Haplotype networks from (A) cytochrome b, (B) intron 5 of ribosomal protein L3 and (C) proopiomelanocortin.

Figure 6. Potential geographic distribution for *Phyllomedusa ayeaye* (B. Lutz, 1964), Canastra and Quadrilátero ESUs for both current and future climate conditions. Protected areas are shown in black.

Appendix S1 – Detailed methodology and additional results

Sampling

Individuals sampled and geographical data

We sampled 88 individuals from nine counties (Table S1). These samples were obtained from field sampling and vouchered individuals from Coleção Herpetológica "Alfred Russel Wallace", tissues – UFLA (CHARW-T), Coleção Herpetológica "Ariovaldo Antônio Giaretta" (AAG), Coleção Herpetológica "Célio Fernando Baptista Haddad", tissues (CFBH-T), Coleção Herpetológica da Universidade de Brasília (CHUNB) and Coleção Herpetológica of the Centro de Coleções Taxonômicas of the Universidade Federal de Minas Gerais (UFMG), consisting of adult amphibians and tadpoles (UFMG-A and UFMG-G, respectively). Because we had much more occurrence records than sampled points (Table S2), we used the putative geographical limits modelled by GENELAND to assign unsampled points to evolutionarily significant units (Figure S1). These sample points were obtained by R.A. Brandão (R.A.B.), and from previously cited collections plus Museu de Zoologia da Universidade Federal de Viçosa (MZUFV).

Table S1: Sampled locations of vouchered individuals of *Phyllomedusa ayeaye* (B. Lutz, 1966) used in this study. N = number of individuals, ESU = evolutionarily significant units.

Locality	N	ESU	Latitude	Longitude	Source
Alpinópolis - MG	1	Canastra	-20.8597	-46.4203	AAG
Lavras - MG	1	Quadrilátero	-21.2013	-44.9413	CHRWT
Minduri - MG	18	Quadrilátero	-21.5934	-44.5719	UFMG
Nova Lima - MG	2	Quadrilátero	-20.0552	-44.0135	UFMG
Ouro Preto - MG	14	Quadrilátero	-20.4309	-43.4904	UFMG
Pedregulho - MG	2	Canastra	-20.2256	-47.4539	CFBH
Poços de Caldas - MG	18	Poços	-21.9172	-46.5686	UFMG
			-21.8612	-46.5086	UFMG
			-21.7772	-46.6192	UFMG
Sacramento - MG	12	Canastra	-19.9316	-47.4183	CFBH
São Roque de Minas - MG	20	Canastra	-20.2669	-46.5553	UFMG
			-20.1939	-46.6306	UFMG
			-20.2792	-46.5208	UFMG

Table S2: Additional occurrence points of *Phyllomedusa ayeaye* (B. Lutz, 1966) used in ecological niche modelling. ESU = evolutionarily significant units.

Locality	ESU	Latitude	Longitude	Source
Arantina - MG	Quadrilátero	-21.8609	-44.2165	R.A.B.
Brumadinho - MG	Quadrilátero	-20.1014	-43.9888	UFMG
	Quadrilátero	-20.0940	-44.0210	UFMG
	Quadrilátero	-20.0837	-43.9951	UFMG
	Quadrilátero	-20.1016	-43.9884	R.A.B.
Carrancas - MG	Quadrilátero	-21.5939	-44.6272	R.A.B.
Congonhas do Campo - MG	Quadrilátero	-20.4536	-43.8742	UFMG
	Quadrilátero	-20.1278	-43.9835	UFMG
	Quadrilátero	-20.4381	-43.9398	UFMG
	Quadrilátero	-20.4867	-43.9431	UFMG
	Quadrilátero	-20.4334	-43.8740	R.A.B.
	Quadrilátero	-20.4827	-43.9368	R.A.B.
Itabirito - MG	Quadrilátero	-20.2309	-43.8524	UFMG
	Quadrilátero	-20.2934	-43.9306	R.A.B.
Lavras - MG	Quadrilátero	-21.3277	-44.9797	UFMG
Luminárias - MG	Quadrilátero	-21.5535	-44.8187	R.A.B.
Minduri - MG	Quadrilátero	-21.5941	-44.5739	UFMG
Nova Lima - MG	Quadrilátero	-20.1015	-43.9886	UFMG
	Quadrilátero	-20.0051	-43.9281	UFMG
	Quadrilátero	-20.0083	-43.9309	R.A.B.
Ouro Branco - MG	Quadrilátero	-20.5143	-43.6197	UFMG
	Quadrilátero	-20.5089	-43.6155	UFMG
	Quadrilátero	-20.4797	-43.5931	MZUFV
	Quadrilátero	-20.5086	-43.6131	MZUFV
	Quadrilátero	-20.5149	-43.6262	R.A.B.
Ouro Preto - MG	Quadrilátero	-20.4323	-43.4917	UFMG
	Quadrilátero	-20.4315	-43.4870	UFMG
	Quadrilátero	-20.4321	-43.4877	UFMG
	Quadrilátero	-20.2777	-43.5257	MZUFV
	Quadrilátero	-20.3333	-43.4833	R.A.B.
	Quadrilátero	-20.4777	-43.6874	R.A.B.
Poços de Caldas - MG	Poços	-21.7766	-46.6178	CFBH
	Poços	-21.9088	-46.5467	R.A.B.
	Poços	-21.8977	-46.5470	R.A.B.
Pedregulho - SP	Canastra	-20.2148	-47.4264	R.A.B.
Sacramento - MG	Canastra	-20.2197	-47.1062	CFBH
São Roque de Minas - MG	Canastra	-20.2682	-46.5549	UFMG
	Canastra	-20.2283	-46.4564	R.A.B.
	Canastra	-20.2778	-46.5221	R.A.B.
	Canastra	-20.2977	-46.5244	R.A.B.
	Canastra	-20.2682	-46.5549	R.A.B.

The voucher numbers were ***ESU Canastra:*** Alpinópolis – MG: AAG 510; Pedregulho – SP: CFBH-T 4388-89; Sacramento – MG: CFBH-T 1890-91, 11889-92, 12548-52, 19783-85; São Roque de Minas – MG: AAG 502, 588, 1265; CFBH-T 89, 153; CHUNB 51420-21, 56876; UFMG-A 16828-32, 17117; UFMG-G 1608 (two individuals), 1610, 1613 (two individuals), 1840. ***ESU Poços***: Poços de Caldas – MG: AAG 486; Délio Baêta field series 557 (to be deposited in CFBH-T); CHUNB 51410, 51412-18; UFMG-A 17115-16; UFMG-G 1837-40, 1844. ***ESU Quadrilátero:*** Lavras – MG: CHARW-T 1463; Minduri – MG: UFMG-A 16414-28, 16436-38; Nova Lima – MG: UFMG-A 14920, 18736; Ouro Preto – MG: UFMG-A 14925, 14978-80, 15420-24, 15426-29.

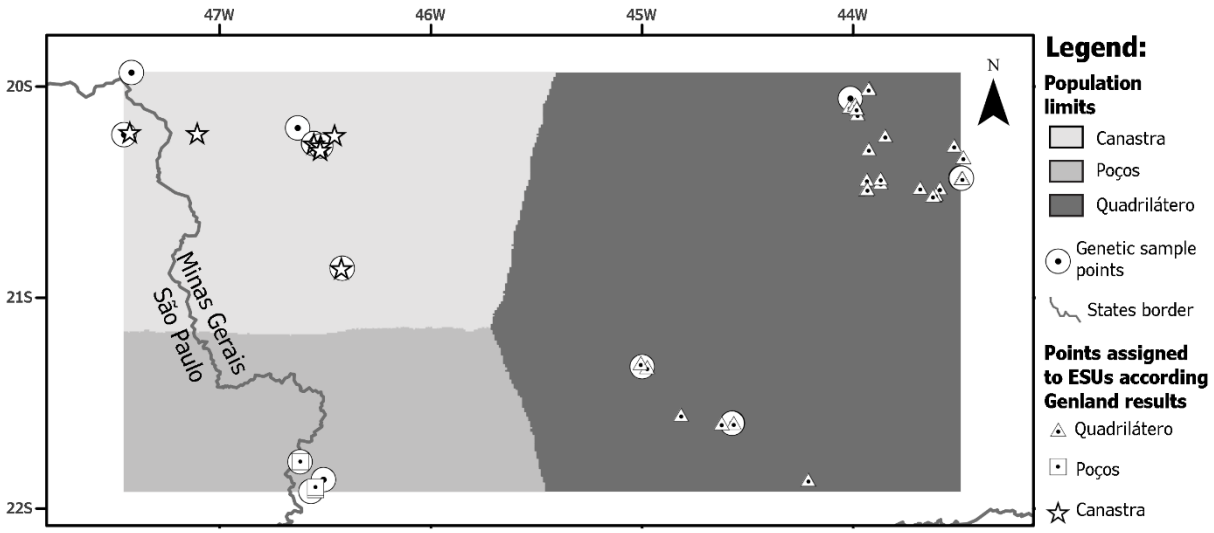

Figure S1: Putative borders between biogeographical units (population limits) and sets of occurrence points used in ecological niche modelling.

Molecular data obtaining and characterization

DNA amplification and sequencing

We extracted genomic DNA from liver or muscle samples using the phenol-chloroform protocol (Sambrook & Russel, 2001). For all specimens, we amplified and sequenced an 896-bp fragment of the mitochondrial Cytochrome b (Cyt-b), a nuclear 601-pb exon fragment of proopiomelanocortin (POMC), and a nuclear 518-pb intron 5 fragment of ribosomal protein L3 (RPL3) (Table S1). Polymerase chain reactions (PCR) were

performed in a 15 µL reaction volume containing: 20 ng of genomic DNA, 1X buffer, 2.5 mM $MgCl_2$, 1.25µM each primer, 3mM dNTPs, 0.72 µg bovine serum albumin, and 0.625 U Platinum™ *Taq* DNA polymerase (Thermo Fisher Scientific). Amplifications were performed as one initial denaturation for 95 ºC during 5min followed by 35 cycles [denaturation at 95 ºC for 30 secs, variable melting temperatures and times between fragments (Table S1), extension at 72 ºC for 1min/1000 bp] and a final extension at 72 ºC for 7min. We reduced temperature melting, increased time melting and/or increased up to 3.5 mM $MgCl_2$ for samples difficult to amplify. All single PCR products were purified using a Sambrook and Russel's (2001) polyethylene glycol 20% protocol, with some modifications (Santos Júnior *et al* 2015).

Purified amplicons were fluorescence-marked through BigDye® Terminator v3.1 cycle sequencing kit (Thermo Fisher Scientific) following the manufacturer's instructions. The primers used in this reaction were the same to those used in PCRs, except for RPL3-intF (Pinho *et al* 2010), which was replaced with RPL3-P3 (5'-WCTGGCCTGCTCTGGTTAT-3') designed by us. The marked amplicons were analyzed in an automatized ABI 3130xl DNA sequencer (Thermo Fisher Scientific) in both directions.

Table S3: Primers, temperature (Tm) and time (tm) melting used for each fragment and the references for them.

Fragment	Primer Name	Primer Sequence 5'-3'	Tm (ºC)	tm (sec)	Source
cyt-*b*	MVZ15 (F)	GAACTAATGGCCCACACWWTACGNAA	54	40	Moritz *et al.* 1992
	Cytb-ARH (R)	TAWAAGGGTCTTCTACTGGTTG			Goebel *et al.* 1999
POMC	POMC-1	GAATGTATYAAAGMMTGCAAGATGGWCCT	60	50	Wiens *et al.* 2005
	POMC-2	TAYTGRCCCTTYTTGTGGGCRTT			
RPL3	RPL3intF	AGTCTTTGGCCAGGATGAAATG	62	40	Pinho *et al.* 2010
	RPL3intR	TCACACCTAGGAGGGATAATG			

Sequence edition and data characterization

Sequence chromatograms were interpreted, assembled, pre-aligned and edited in SEQSCAPE™ 2.6 (Thermo Fisher Scientific). Edited fragments were aligned with the CLUSTALW module of the MEGA7 software (Larkin *et al.,* 2007; Kumar *et al.,* 2016), with gap opening penalized 10 times more than extension. The gametic phases of nuclear markers were reconstructed through the algorithm implemented in PHASE 2.1.1 software (Stephens *et al*. 2001), which were interconverted to fast format using SEQPHASE web tool (Flot, 2010). In cases where more than one haplotype pair was reconstructed, we selected the more probable pair for subsequent analyzes, except those based in genealogies reconstruction. In these cases, haplotype phases not fully resolved were checked by eye and the uncertain nucleotide positions were kept. Because there were some heterozygous individuals for insertions and deletions in RPL3, we estimated their haplotypic phases via the codification of superimposed traces in INDELLIGENT 1.2 web-based software (Dmitriev & Rakitov, 2008). After these steps, we selected the best fit model of molecular evolution among 88 substitution schemes using the BIC criterion in JMODELTEST 2.1.10 software (Darriba *et al.,* 2012) for each fragment.

We did a Maximum Likelihood (ML) test of the strict molecular clock for each gene fragment under the same substitution model selected in the previous step as implemented in MEGA7 (Kumar *et al*., 2016). Because an input tree is required for this test, we generated gene trees of unique haplotypes in RAXML 8.2.4 software (Stamatakis, 2014), choosing the 'best scoring ML-tree with rapid bootstrap' option under the GTRCAT model. The null hypothesis of equal evolutionary rate along the tree was not rejected for both Cyt-b ($p=0.85$) and POMC ($p=0.47$), but it was rejected for RPL3 ($p<0.001$). We applied this test to build simpler and less parametric models in subsequent analyses, aiming to achieve a balance between bias and variance in results (Kelchner & Thomas 2006).

DNA divergence between biogeographical units (π between populations) was calculated as the average number of nucleotide substitutions per site between populations (Nei 1987) for each locus in DNASP (Librado & Rozas, 2009) (Table S4).

Table S4: DNA divergence between biogeographical units for all markers. C. Canastra, P. Poços and Q. Quadrilátero

Marker	π_{CP}	π_{CQ}	π_{PQ}
Cyt-b	2.231	4.874	4.328
POMC	2.542	2.212	1.983
RPL3	16.778	21.300	21.974

Additional results

Approximate Bayesian Computation

The principal component analysis made to evaluate the performance of ABC results shows adequate model fit and adequacy regarding observed data (Figure S2).

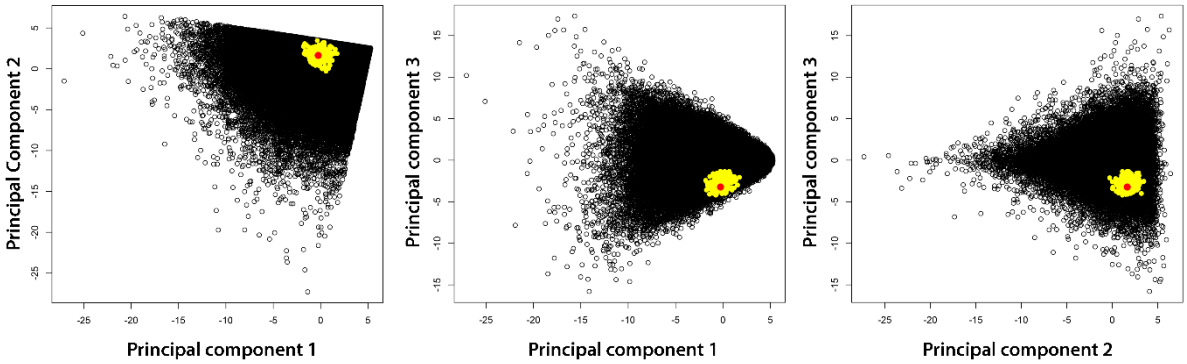

Figure S2: Principal component analysis showing prior (black), posterior (yellow), and observed data (red).

Ecological Niche Models

From the 19 bioclimatic variables, four were selected to minimize collinearity in data (Table S5).

Table S5: Loadings of the bioclimatic variables in the first five axes of Varimax Rotated Factor Analysis, based on current climate. Numbers in bold highlight the highest loading, and based on this highest value one variable per factor was selected.

Bioclimatic variables	I	II	IV	III	V
1	0.945	0.282	-0.127		
2	0.597	0.751	0.25		
3	0.186	0.823	0.171	0.462	
4	-0.351	-0.747	0.264	-0.468	
5	0.93	0.19	-0.226	0.179	
6	0.891	0.147	-0.144	-0.389	
7	-0.105	0.976	-0.103		
8	0.969	-0.171			
9	0.883	0.385	0.221		
10	0.973	-0.195			
11	0.900	0.38	-0.101	0.157	
12	-0.209	-0.416	0.858	-0.132	0.101
13	-0.138	0.381	0.879		
14	-0.192	-0.968	-0.112		
15	0.167	0.929	0.235	0.120	
16	-0.135	0.3	0.937		
17	-0.208	-0.972			
18	-0.32	-0.166	0.727		
19	-0.172	-0.958			
	I	II	IV	III	V
SS loadings	6.524	6.122	3.169	1.868	0.633
Proportion Var	0.343	0.322	0.167	0.098	0.033
Cumulative Var	0.343	0.666	0.832	0.931	0.964

1.Annual Mean Temperature; 2.Mean Diurnal Range (Mean of monthly (max temp - min temp)); 3.Isothermality; 4.Temperature Seasonality (standard deviation *100); 5.Max Temperature of Warmest Month; 6.Min Temperature of Coldest Month; 7.Temperature Annual Range; 8.Mean Temperature of Wettest Quarter; 9.Mean Temperature of Driest Quarter; 10.Mean Temperature of Warmest Quarter; 11.Mean Temperature of Coldest Quarter; 12.Annual Precipitation; 13.Precipitation of Wettest Month; 14.Precipitation of Driest Month; 15.Precipitation Seasonality (Coefficient of Variation); 16.Precipitation of Wettest Quarter; 17.Precipitation of Driest Quarter; 18.Precipitation of Warmest Quarter; 19.Precipitation of Coldest Quarter.

The uncertainties of forecast ensembles were mapped to make easy the visualization of the main sources of variability (Figure S3).

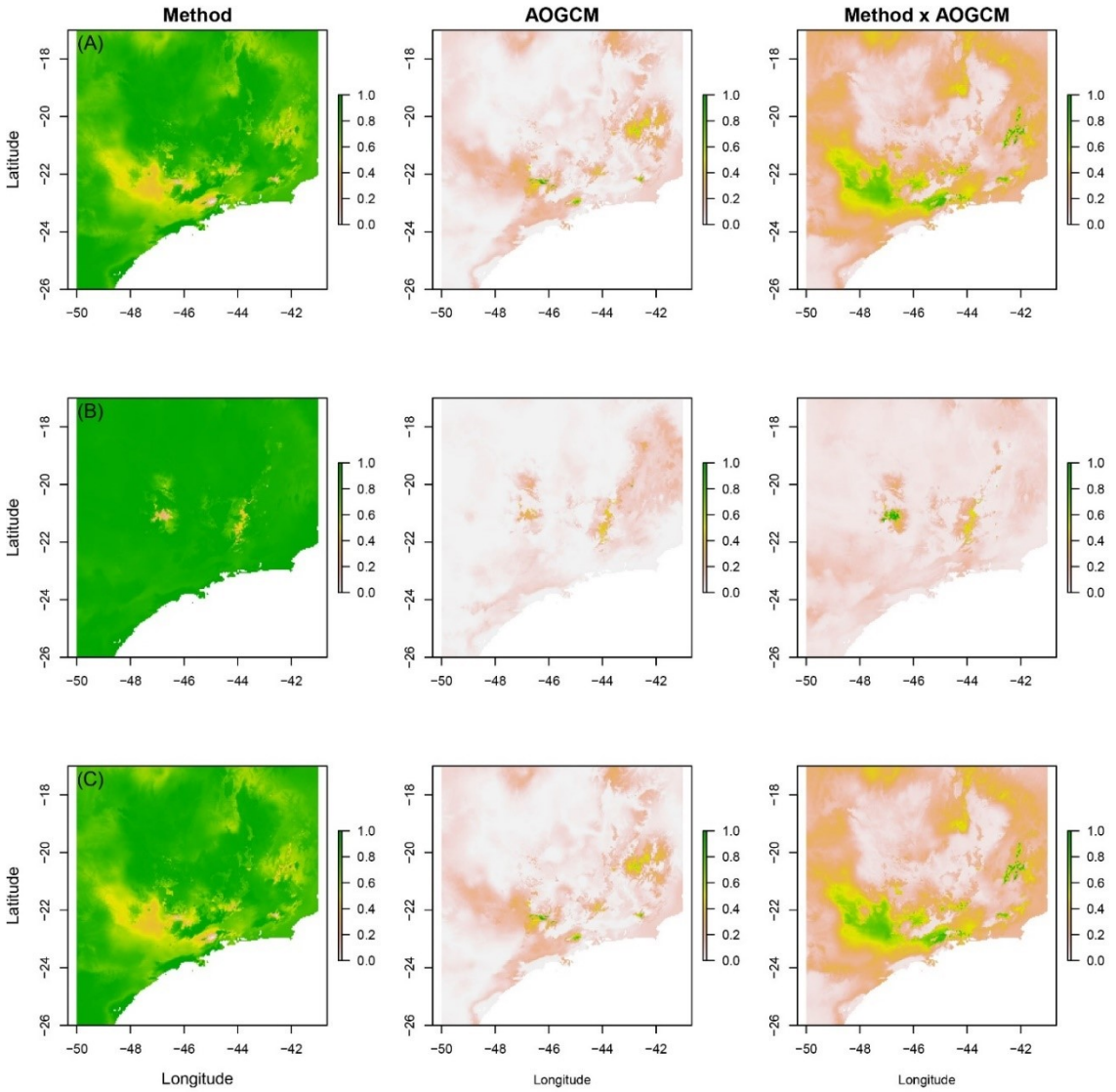

Figure S3: Maps of variance (relative sum of squares) for the effect of ecological niche modelling (ENMs), Atmosphere-Ocean Global Circulation Models (AOGCMs), and the interaction between both sources of uncertainty for (A) *Phyllomedusa ayeaye*, and ESUs (B) Canastra and (C) Quadrilátero.

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CAPÍTULO II

Pleistocene climate changes drove multiple speciation events in endemic frogs from the Brazilian Shield sky islands

Magalhães, R. F.; Camargo, A.; Oliveira, U.; Brandão, R. A.; Garcia, P. C. A.; Santos, F. R. Pleistocene climate changes drove multiple speciation events in endemic frogs from the Brazilian Shield sky islands. Manuscrito em preparação para submissão no periódico *Evolution*, com algumas modificações de formatação para esta tese.

PLEISTOCENE CLIMATE CHANGE DROVE MULTIPLE SPECIATION EVENTS IN ENDEMIC FROGS FROM THE BRAZILIAN SHIELD SKY ISLANDS

Short title: Speciation in *Phyllomedusa* from Brazilian sky islands

Authors: Rafael F. Magalhães[1,2], Arley Camargo[2], Ubirajara Oliveira[3], Reuber A. Brandão[4], Paulo C. A. Garcia[1], Fabrício R. Santos[1]

Affiliations:

[1]Programa de Pós-Graduação em Zoologia, Departamento de Zoologia, Universidade Federal de Minas Gerais, Belo Horizonte, Brasil, rafaelfelixm@gmail.com, pcagarcia@gmail.com, fsantos.ufmg@gmail.com

[2]Programa de Desarrollo Universitario, Centro Universitario Rivera, Universidad de la República, Rivera 40000, Uruguay, arley.camargo@gmail.com

[3]Centro de Sensoriamento Remoto, Instituto de Geociências, Universidade Federal de Minas Gerais, Belo Horizonte, Brasil, ubiologia@yahoo.com.br

[4]Laboratório de Fauna e Unidades de Conservação, Departamento de Engenharia Florestal, Universidade de Brasília (campus Darcy Ribeiro), Brasília, Brasil, reuberbrandao@gmail.com

Correspondence to: Rafael F. Magalhães and Fabrício R. Santos, Programa de Pós-Graduação em Zoologia, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais – UFMG, Antônio Carlos 6627, CEP 31270-901, Belo Horizonte, Minas Gerais, Brasil, rafaelfelixm@gmail.com, fsantos.ufmg@gmail.com

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DNA data will be archived upon manuscript acceptance in GenBank

Abstract:

Little is known about the spatial and temporal factors related to high endemism rates in Neotropical sky islands. 'Campo rupestre' is a mountaintop ecosystem found in Brazilian Shield sky island complex, whose high richness and endemism contribute significantly to the high diversity levels related to Cerrado domain. We investigated the influence of Pleistocene climate change in diversification of a 'campo rupestre's' biota using an endemic clade of monkey-frogs as model. Therefore, we used multispecies coalescent approaches to delimit species, generate genealogical relationship hypothesis and date the divergence between them. Additionally, we used ecological niche modelling (ENM) with projections back to Upper Pleistocene plus approximate Bayesian computation (ABC) techniques to link tempo to possible processes of diversification. Our results point to four species that appeared in Middle Pleistocene. *Phyllomedusa ayeaye* and *P. centralis* were speciated allopatrically. On the other hand, discordance between nuclear and mitochondrial loci plus ENM and ABC results show evidence for allopatric speciation with secondary contact between *P. oreades* and *P. "araguari"*. Results show the influence of Pleistocene changes on complex geographic dynamics and speciation modes of endemic sky island frogs, but how this is a general pattern in 'campo rupestre' biota needs further investigation.

Introduction

Sky islands are continental environments characterized by mountain chains isolated by valleys. Their high-altitude ecosystems are distinct from those in surrounding areas

leading to isolation and island-like diversification (Rull 2014). In allusion to oceanic islands, the lowland matrices act as sea barriers, preventing dispersal among mountaintops (Warshall 1994; McCormack et al. 2009). Thence, sky islands are characterized by the presence of endemic species with restricted distributions (Fjeldså et al. 2012; Kok et al. 2012). The diversification patterns observed in these environments are the result of multiple ecological and geographical factors such as: ecosystem age, persistence of ecological conditions over time, island size, island connectivity, topographic complexity, and degrees of climatic overlap between mountains and adjacent valleys (Ghalambor et al. 2009; Graham et al. 2014; Rodríguez et al. 2015). For these reasons, these high-elevation habitats are natural laboratories for evolutionary investigations, including tempo and mode of speciation (e.g. Salerno et al. 2012; Kok et al. 2012; Madriñán et al. 2013; Kok et al. in press). Twenty sky island complexes were identified worldwide, of which the Andes, Pantepuis and Brazilian Shield's mountains and mesas occur in the Neotropical region (Simpson 1979; Warshall 1994; McCormack et al. 2009).

The Brazilian Shield sky island complex (BSSI; *sensu* Warshall 1994) comprises mountainous regions from southern, southeastern and central Brazil. These hilltop ecosystems are mainly formed by grasslands usually associated with infertile soils and rocky outcrops (Safford 1999, Behling and Pillar 2007, Alves et al. 2014, Silveira et al. 2016). 'Campo rupestre' (used here in *sensu lato*; Alves et al. 2014) is a grassy-shrubby ecosystem found in some mountain regions of BSSI, generally higher than 900 m a.s.l, consisting of quartzitic, arenitic, or ironstone soils. This ecosystem is found mainly within Cerrado and Caatinga dominions (Alves et al. 2014; Silveira et al. 2016). Despite being in the tropical region, the climate in 'campo rupestre' regions are classified as humid subtropical with dry winter and temperate summer (Cwb), and humid subtropical with

dry winter and hot summer (Cwa) in areas with highest and intermediate altitudes, respectively (Alvares et al. 2013). A significant part of the high species richness and endemism reported for the Cerrado (Brazilian savanna) is contained within 'campo rupestre' areas (Silveira et al. 2016). Besides, Azevedo et al. (in press) have identified endemism areas for herpetofauna in the Cerrado hotspot, many of which are BSSI regions. Nonetheless, few studies have investigated the origin and evolution of BSSI's endemic species (e.g. Collevatti et al. 2009; Antonelli et al. 2010; Gustafsson et al. 2010; Freitas et al., 2012; Franco and Manfin 2013; Trovó et al. 2013; Bonatelli et al. 2014; Chaves et al. 2014).

There has been a heated debate on the temporal origin of the Neotropical biota in the last decades (Rull 2011). The most supported hypothesis indicates that most clades resulted from diversification events that begun in the Tertiary period, mainly in the Neogene, and continued during the Quaternary, governed by geological and climatic changes (Rull 2011; Turchetto-Zolet et al. 2013). Ancient origin (i.e., Tertiary) with subsequent Pleistocene radiation has been suggested in some studies of 'campo rupestre' endemic clades (e.g. Gustafsson et al., 2010; Bonatelli et al., 2014). Essentially, the climate-driven hypothesis of diversification is based on influence of Pleistocene's palaeoclimatic changes on speciation. This hypothesis suggests that organisms lack the phenotypic plasticity necessary for acclimatization in the face of climatic oscillations, and consequently, these species change their distributions, evolve or become extinct in unfavorable conditions (Madriñán et al. 2013; Rull 2014). This set of premises of the climate-driven hypothesis must be assessed separately in the BSSI scenario. First, the BSSI should not have experienced continental climatic extremes over the Pleistocene, with glacial periods characterized by drier and cooler paleoclimates, and the lack of ice sheets (Vuilleumier 1971). Furthermore, the 'campo rupestre' region consists of arid

environments endowed with species adapted to colder climates (Simpson 1979; Bonatelli et al. 2014). Taking this into account, the climate-driven hypothesis predicts that cold-adapted mountain biota should have experienced vertical migrations, retracting to high altitudes in interglacial periods, and expanding to lower areas during the arid and cold glacial conditions (Vuilleumier 1971; Rull 2014). Thence, the genetic divergence between populations of distinct sky islands may be lower than expected in a long-term isolation model (Kok et al. 2012). Additionally, topographic complexity and habitat heterogeneity provides micro-habitats that promotes adaptation and creates the opportunity for individuals to remain in micro-refugia during unfavorable periods (Rull 2009; Rodríguez et al. 2015).

Neotropical mountaintops could act as interglacial refugia, where endemic species are confined during warm climates (Peterson and Ammann 2013; Bonatelli et al. 2014). This dynamic pattern of isolation has consequences in the understanding of speciation modes of montane species. Glacial periods were longer than interglacial ones, especially in the Late Pleistocene (Hewitt 2011; Rull 2015). For this reason, today's distinct sister lineages may have spent more time in contact than isolated, making speciation a difficult issue to address due to recurrent gene flow. However, if diversification rates are high enough and reproductive isolation is completed in any given interglacial cycle, separate populations may evolve into distinct species (Baker 2008; McCormack et al. 2009; Rull 2009).

On the other hand, if reproductive isolation is incomplete, the formation of hybrid zones can be common (Vuilleumier 1971; Hewitt 2011), since incomplete premating and postmating barriers can result in interspecific introgression events (Toews and Brelsford 2012). These hybrid zones appear because the divergence accumulated during an interglacial phase may be lost if subsequent geographical expansion with secondary contact takes place between previously diverging lineages (McComarck et al. 2009;

Hewitt 2011). Molecular and geographical signatures can be used to identify intermixed individuals in these hybrid zones or to detect strong discordance between nuclear (nDNA) and mitochondrial (mtDNA) markers (Funk and Omland 2003; Toews and Brelsford 2012; Brunes et al. 2014).

Phenotypic traits (e.g., morphology) may also indicate a hybrid zone when there is evidence of individuals showing mixed phenotypes between putative hybridizing lineages (Bonatelli et al. 2014; Brunes et al. 2014). These diversification and mixing processes may result in sibling species' complexes, but the lack of time for differentiation and imperfect taxonomy may also result in apparent morphological clines (De Queiroz 2007, Toews and Brelsford 2012). Herein, we address a clade of reticulated leaf frogs as a model to test if, and how, Pleistocene climatic changes influenced the diversification of 'campo rupestre' endemics. *Phyllomedusa ayeaye* (B. Lutz, 1966), *P. centralis* Bokermann, 1965 and *P. oreades* Brandão, 2002 constitute an endemic clade from the BSSI in the 'campo rupestre' region (Faivovich et al. 2010). *Phyllomedusa centralis* is endemic from Chapada dos Guimarães plateau (Mato Grosso State, Brazil), which despite being only 600-700m a.s.l. on average, have patches of 'campo rupestre' where the species breeds (Brandão et al. 2009). The geographic limits between *P. ayeaye* and *P. oreades* are unknown, but populations from Poços de Caldas Plateau, Quadrilátero Ferrífero, Southern Minas Gerais Mountains, and Canastra Plateau are assigned to *P. ayeaye* (Baêta et al. 2009; Magalhães et al. submitted.), while populations from Central Brazilian and Veadeiros plateaus are attributed to *P. oreades* (Brandão 2002; Brandão and Álvares 2009) (Fig. 1). These *Phyllomedusa* species are distinguished from each other mainly by color patterns such as density and shape of reticulations in limbs, flanks, venter, lips, and eyelids (Bokermann 1965; Lutz 1966; Brandão 2002). However, these traits are not discrete (Baêta et al. 2009) and interspecific overlap is common in species complexes of

Phyllomedusa (Bruschi et al. 2013; Brunes et al. 2014). For instance, individuals from Perdizes (Minas Gerais State, Fig. 1, no. 12) are identified as *P. oreades,* but have the color pattern like *P. ayeaye* from Canastra Plateau (Fig. 1, no. 13-15) (A. A. Giaretta, pers. comm.). Consequently, *P. ayeaye* and *P. oreades* entities have been experiencing taxonomic instability (Caramaschi et al. 2006; Giaretta et al. 2007; Baêta et al. 2009; Brandão and Álvarez 2009). Moreover, this species complex endemic from 'campo rupestre' shows the lowest intragroup genetic distances recorded among all *Phyllomedusa* clades (Faivovich et al. 2010), generating doubts about the validity of these species. On the other hand, there is evidence of genetic structuring coinciding with mountain ranges in *Phyllomedusa ayeaye* (Magalhães et al. submitted.).

Based on these patterns of distribution and genetic differentiation, we tested the climate-driven diversification hypothesis in this clade following the rationale proposed by Rull (2014), which consists in using an integrative approach to link observed patterns to processes. We assume a Pleistocene origin for our focal group due to the shallow divergence between them (Faivovich et al. 2010), and genetic evidence suggesting that the most recent common ancestor of *P. ayeaye* dates back to the Middle Pleistocene (Magalhães et al. submitted.). However, given the taxonomic uncertainty in our focal group, we first carried out a coalescent species delimitation following the approach following the protocol of Carstens and collaborators (2013). Therefore, (i) we applied independent discovery methods with mitochondrial and nuclear data to verify the congruence between these two data sources (Toews and Brelsford 2012); and (ii) we used a validation method to rigorously test the putative species discovered (Carstens et al. 2013). The previous results guided us to (iii) construct a dated species tree to confirm our assumption about tempo of diversification, and (iv) verify the fit of our data to climate-driven distribution changes in sky islands. For this, we generated ecological niche models

(ENM) for the delimited species and projected them to past glacial and interglacial models. (v) We finally used all previous results to test alternative hypotheses about modes of diversification in our focal species complex (Rull 2014).

Material and Methods

Data sampling and characterization

Since the species boundaries are unclear, we attempted to sample as many locations as possible with records in museums, herpetological collections, and the literature. Therefore, we sampled 99 individuals from 21 localities (Table S1, Fig. 1), totaling most of the known species records.

Extraction, polymerase chain reactions and Sanger's sequencing protocols were made following Magalhães et al. (submitted). We screened 17 DNA loci, eight of which (one mitochondrial – mtDNA – and seven nuclear – nDNA) were successful in amplification and sequencing tests (Table 1). All eight loci were sequenced in both directions; temperature and melting times are given (Table 1). The amplification rate of the anonymous nuclear loci with the primers designed by Bell et al. (2011) was low. Thence, we redesigned a specific internal pair for them taking our successfully sequenced samples as a reference (Table 1).

The resulting sequence chromatograms were assembled and edited in SeqScape™ 2.6 (Thermo Fisher Scientific). The fragments were aligned in the MUSCLE module of MEGA7 software (Edgar 2004; Kumar et al. 2016). We reconstructed the gametic phases of nuclear loci in PHASE 2.1.1 (Stephens et al. 2001) under default parameters. We conducted multiple independent runs with distinct initial seeds to verify the consistency of the reconstructions. Inputs and outputs were interconverted through SeqPHASE web tool (Flot 2010). In case of haplotype phases with posterior probability (PP) < 0.9, we

selected the most probable pair for allele frequency-based analyses. For genealogy-based analyses, we checked all phased pairs by eye and kept the unsolved uncertainties. For the introns, some individuals showed heterozygous insertions/deletions. In these cases, the gametic phases were reconstructed in the Indelligent 1.2 web tool (Dmitriev and Rakitov 2008). Thereafter, we selected the best fit model of molecular evolution between 88 substitution schemes for each fragment in jModelTest 2.1.10 (Darriba et al. 2012) using the BIC criterion.

Lineages discovery and validation

We tested the validity of current taxonomic assignments by using coalescent species delimitation. As described by Carstens and collaborators (2013), we applied a two-step protocol including a discovery stage, where there is no a priori assumptions about assignment (except by the range of K assumed lineages in some methods), and a validation stage, where the putative species discovered in the previous step are evaluated in a speciation test under an explicit genealogical model.

For the lineage discovery, we applied independent tests to nDNA and mtDNA data. For nDNA, we codified the phased nuclear haplotypes as alleles and proceeded to a Bayesian clustering analyses in STRUCTURE 2.3.4 (Pritchard et al 2000). The analysis was conducted under the independent allelic frequencies model with admixture. We set a range of K between 1 and 8, which corresponds with the number of mountain ranges or plateaus where the samples were collected. This assumption was made because the isolation in sky islands is probably relevant for diversification in the species complex. We conducted 10 independent runs, with 100,000 generations each, and 25,000 generations discarded as burn-in. The most suitable K value was chosen by plotting the average log probability of the data [ln Pr(X/K)] and estimating the relative rate of change in

successive log probability K values (ΔK) in STRUCTURE HARVESTER web tool (Evanno et al 2005; Earl and vonHoldt 2013). The results of replicates were summarized in CLUMPP 1.2.2 (Jakobsson and Rosenberg 2007) and the *Q-plot* was generated in DISTRUCT 1.1 software (Rosenberg 2004).

Collapsing haplotypes into alleles for Bayesian clustering analysis results in the loss of genealogical information. Thence, we constructed a nuclear Neighbor-Net in SplitsTree4 software (Bryant and Mouton 2004; Huson and Bryant 2006) to check the STRUCTURE result. For this, we generated pairwise haplotype distance matrices for each nuclear fragment in MEGA7 (Kumar et al. 2016) under K80 model (Kimura 1980). These matrices were combined in a single multilocus distance matrix among individuals using the Phylogenetic Bray-Curtis (PBC) algorithm (Göker and Grimm 2008) implemented in POFAD 1.07 software (Joly et al. 2015). We choose PBC because of its good performance in hybridization detection (Joly et al. 2015).

We applied a Generalized Mixed Yule Coalescent model (GMYC; Pons et al. 2006) for discovering mtDNA gene tree lineages. We first constructed an ultrametric gene tree for unique cyt-*b* haplotypes using a Yule model of diversification in BEAST 2.4.3 software (Bouckaert et al. 2014). This was made under an uncorrelated log-normal molecular clock without accounting for absolute divergence times between lineages. We made 10,000,000 Markov Chain Monte Carlo (MCMC) generations with a pre-burn of 1,000,000 and thinning of 10,000. The analysis was repeated twice and we checked for convergence, stationarity and minimum adequate effective sampling size (ESS > 200) of analyses in Tracer v1.6 (Rambaut et al. 2014). A target maximum credibility tree was annotated and used in a maximum likelihood (ML) GMYC test based on the single threshold method in the R package *splits* (Fujisawa and Barraclough 2013; R Development Core Team 2016). In the same package, we also performed a log-likelihood ratio test between the best fitted

model and the null hypothesis of no distinct clusters. To evaluate topology and branch length uncertainties of the target tree used in ML-GMYC, we performed aBayesian implementation of the GMYC method in R package *bGMYC* (Reid and Carstens 2012), sampling 1,000 random trees from the posterior distribution of BEAST analysis.

Finally, the discovered putative species were validated with a Bayesian species delimitation approach (Yang and Rannala 2010). We choose the BPP method due its accuracy under many situations, including gene flow between lineages (Camargo et al. 2012). As the previous discovery tests showed distinct but nested results (see below), we chose the most split scenario of putative species to test. The analysis was implemented in BPP 3.2 (Yang and Rannala 2010) using an unguided tree, species delimitation algorithm (analysis A11; Yang and Rannala 2014). The analysis was made with four distinct gamma prior combinations for theta (Θ) and tau (τ) parameters, including: (1) large ancestral population sizes and deep divergence ($\Theta \sim G(2, 100)$, $\tau \sim G(2, 200)$), (2) small ancestral population size and shallow divergences ($\Theta \sim G(2, 1000)$, $\tau \sim G(2, 2000)$), (3) small ancestral population sizes and deep divergences ($\Theta \sim G(2, 1000)$, $\tau \sim G(2, 200)$), and (4) large ancestral population sizes and shallow divergences ($\Theta \sim G(2, 100)$, $\tau \sim G(2, 2000)$). Other divergence time parameters were assigned the Dirichlet prior (Yang and Rannala 2010: eq. 2). All analyses were made under the 'uniform SLH' species model prior (Yang and Rannala 2014). We chose the random mutation rates model (Burgess and Yang 2008) and automatic adjustment in MCMC steps length. We did all analyses with 200,000 generations with a thinning of 10 and a burn-in of 20,000 generations. Each analysis was run twice with distinct initial trees and seeds to confirm consistency between runs.

Species tree estimation and dating

Even though we generated a species tree hypothesis in the previous step, the BPP software uses an oversimplistic J69 substitution model (Yang and Rannala 2010). Because of this, it is desirable to reconstruct an independent species tree using algorithms that accommodate more complex substitution models for comparison with BPP results (Caviedes-Solís et al 2015). Despite the accuracy of multi-species coalescent (MSC) methods in reconstructing a dated species trees, even in the presence of incomplete lineage sorting (ILS) (Angelis and Reis 2015), the absence of gene flow is an assumption of most of them (Liu et al 2015). For this reason, we estimated a dated species tree in *BEAST with STACEY operators (Heled and Drummond 2010, Jones 2016). Based on STRUCTURE and Neighbor-Net results, we excluded all potential migrants or hybrid individuals found with the nDNA genome. *Phyllomedusa megacephala* is a sister species of our focal group and another endemic from 'campo rupestre' (Faivovich et al. 2010). Including this species as outgroup allowed us to test the monophyletism of our ingroup and to date the 'campo rupestre' crown group diversification (see Rull 2011). Next, we used the Yule diversification model with a linear population function to construct the species tree. Due to the lack of fossils for a robust calibration, we used a standard 1% divergence rate per lineage per million years for mtDNA (Johns and Avise 1998) under an uncorrelated log-linear clock model. This standard rate is similar to the ND2 rate (Crawford 2003), a widely mtDNA marker used for dating diversification in anurans (e.g. Carnaval and Bates 2007; Thomé et al 2010; Brunes et al 2014). The divergence rates of the other other nDNA markers were estimated as relative to the mtDNA one. The analysis was made in BEAST 2.4.3 (Bouckaert et al. 2014), with two independent replicates of 250,000,000 MCMC generations each, a burn-in of 6% initial generations and thinning 5,000. The stationarity, convergence, and ESS were accessed as described above.

Pleistocene range shifts

To verify potential range shifts in the past, we implemented seven modeling algorithms for each delimited species: Bioclim, Euclidean distances, Malahanobis distances, Ecological Niche Factor Analysis (ENFA), Genetic Algorithm for Rule-set Prediction (GARP), Support Vector Machine (SVM), and Maximum Entropy (MAXENT) (see reviews in Peterson et al. 2011 and Alvarado-Serrano and Knowles 2014). We used multiple procedures to accommodate distinct premises and uncertainties of each separate algorithm (Araújo and New 2007). MAXENT was implemented in the proper program (Phillips et al. 2006) while the remaining methods were implemented in Openmodeller v. 1.5 (Muñoz et al., 2011). The occurrence dataset contained the sample points from the individuals used in genetic analysis (Table S1). We used all occurrence points as training due to the scarcity of records for all species. The present-time ENMs were estimated using current climatic layers available in the WorldClim repository (Hijmans et al. 2005). To avoid collinearity problems, we made a linear correlation analysis between variables and selected only those with correlation coefficient lower than 0.7 (i.e., annual mean temperature, mean diurnal range, isothermallity, annual precipitation, precipitation seasonality and precipitation of warmest quarter). We generated 10,000 pseudo-absence points and evaluated the predictive performance of each algorithm through areas under the curves (AUC) of ROC plots. All algorithms that showed AUC < 0.7 were discarded. From the remaining algorithms, we projected the current distribution to mid-Holocene (6 thousand years before present – Ky BP), Last Glacial Maximum (LGM, 21 Ky BP based on CCSM and MIROC to accommodate uncertainties about past climates), and Last Interglacial (LIG, ~120-140 Ky BP; Otto-Bliesner et al. 2006). Our final scenario for each time projection's ENM was obtained through a conservative consensus between all modelling algorithms that showed AUC > 0.9 for all species. Finally, we built these maps

for all species together, showing lowest presence threshold to see potential contact areas between them.

Selection of diversification models

Based on species delimitation, dating and ENM, we constructed a set of credible diversification models following Rull's (2014) suggestion. From the previous results, we tested for allopatric (null model), parapatric, and alloparapatric modes of speciation (Fig. 2). As there is uncertainty in the species tree's topology (see above), we assumed an unsolved polytomy among species. We simulated 250,000 coalescent genealogies for each model with MS (Hudson 2002). We used uniform prior distributions for all parameters including Θ per locus (lower bound: 2, upper bound: 7), divergence times in $4N_e$ units (0.001, 1), and migration rates in $4N_em$ units (0, 50). We obtained the average nucleotide pairwise distances (π), number of segregating sites (ss), Tajima's neutrality test (D), π within and between populations through MSSS script (Takebayashi 2011). These summary statistics were also estimated from the observed data (Table S2) in DnaSP 5 (Librado and Rozas 2009). To approximate the posterior probability of models, we implemented model selection with Approximate Bayesian computation using multinomial logistic regression (mnlogistic) and non-linear local regression (neuralnet) algorithms in the R package *abc* (Csilléry et al. 2012) with a tolerance of 0.00033 (to retain ~250 simulations). We evaluated model adequacy and fit through a principal component analysis (PCA) of prior, posterior, and observed summary statistics in the *stats* package of R.

Results

Lineages discovery and mitonuclear discordance

Regarding nuDNA evidence, the ΔK calculations showed a peak at K=4 (Fig. S1), which is congruent with isolated mountain ranges. Two of the clusters were concordant with the taxonomic units known as *P. ayeaye* and *P. centralis* (Fig. 3a). Hereafter, all numbers associated to regions or localities refers to figure 1. *Phyllomedusa oreades* was split into two groups, one including all localities from Veadeiros plateau (no. 3-5) and another including Central Brazilian plateau (no.7-11), southernmost of Serra Geral plateau (no. 6) and northern of Canastra Plateau (no. 12) (see Azevedo et al. in press for mapping of these areas). This last location is the type-locality of *Phyllomedusa araguari*, a junior synonym of *P. oreades*. We named these clusters as *P. "araguari"*, *P. ayeaye*, *P. centralis* and *P. oreades* (Fig. 3a). The PBC neighbor-net recovered the same general structure of the Bayesian assignment analysis (Fig. 3). In both, an individual from the *P. oreades* distribution (PHY108, no. 5) was assigned to *P. "araguari"*. Nonetheless, another individual from Alto Paraíso de Goiás (PHY106, no. 5) was clustered with *P. ayeaye* individuals only in PBC. *Phyllomedusa centralis* showed the largest genetic distance between all pairs of clusters (Fig. 3b), indicating early divergence in nDNA.

In comparison with nDNA, evidence from mtDNA data showed a distinct pattern, which is identical to current taxonomy for the complex. The ML-GMYC returned three putative species (Fig. 4), with a confidence interval from two to eleven. The null hypothesis of no distinct species was rejected (LR test = 9.513; p = 0.009). *Phyllomedusa "araguari"* and *P. oreades* were collapsed into a single putative species, which corresponds to the recognized *P. oreades* entity. There is some uncertainty in mtDNA delimitation, especially against *P. oreades*, because the *b*GMYC support for this cluster is moderate, PP < 0.9 (Fig. 4, Fig S2). Therefore, the main discrepancy between nDNA and mtDNA discoveries is related to *P. oreades*, with the first data set supporting two species while the second supports only one. Both results are nested and the mtDNA gene tree shows

paraphyletism between the two nDNA lineages of *P. oreades*, with haplotypes from them merged into two well supported clades by *b*GMYC (Fig. 4). The only mtDNA haplotype shared between *P. "araguari"* and *P. oreades* was H2 (Fig. 4), sampled in Alto Paraíso de Goiás (no. 5, PHY01) and Cristalina (no. 10, PHY345). All the other haplotypes were exclusive from each lineage.

Species validation

We evaluated the nDNA's putative species with a BPP test. All eight replicates returned four species with 100% posterior probability, independent of priors and starting point of analysis. This evidence supports mtDNA bidirectional introgression between *P. "araguari"* and *P. oreades*. However, there was great uncertainty about the species tree topology (Table 2). There were 15 topologies visited, with a prior probability of ~ 6.67% for each. Using the criteria of posterior probability < prior probability for discarding unfit hypothesis, there are two or five possible species topologies, depending mainly on gamma prior distribution for Θ. When we assumed small ancestral populations, two topologies were recovered with moderate probabilities (PP = 34.5 – 47.6%; Table 2). In these topologies, *P. centralis* was always the sister species of the other species, and there was uncertainty about the position of *P. ayeaye* and *P. oreades* (Table 2). Three additional topologies could not be ruled out when large ancestral population size was assumed, but none of them showed PP > 12% (Table 2). Distinct prior distributions of divergence times did not have much effect on the topologies recovered.

Species tree and dating

The species tree constructed from more complex substitution models resulted in the same best supported topology recovered by BPP analysis (Fig. 3, Table 2). However, we still

found topological uncertainty on the clade that includes *P. "araguari"*, *P. ayeaye* and *P. oreades*, with a support of PP < 0.9 (Fig. 5). The divergence between the *P. megacephala* and the ingroup occurs in the Calabrian stage of Pleistocene (see Cohen et al., 2013 for chronostratigraphic dating and nomenclature), with 95% highest posterior density (HPD) interval between the beginning of Gelasian stage (2.437 million years before present – My BP) and beginning of Middle Pleistocene (0.639 My BP). *Phyllomedusa centralis* diverged from the remaining species in the ingroup in Middle Pleistocene with a 95% HPD between 0.696-0.247 My BP, both in MP. Assuming that the topology (*P. oreades*,(*P. "araguari*", *P. ayeaye*)) is correct, the two remaining divergence events occurs in Middle Pleistocene (Fig. 5), with 95% HPD for the older event between 0.406-0.200 My BP and for the younger event between 0.338-0.161 My BP.

Upper Pleistocene range shifts

The ENMs indicated past distribution changes for both species (Fig. 6). *Phyllomedusa centralis* was not included in analysis due to the scarcity of occurrence points to construct reliable models. Bioclim, Malahanobis distances and ENFA algorithms showed low performance (AUC < 0.7) and their results were disregarded. GARP and SVM methods showed the best results in relation to AUC values for all species (Table 3), thence only they were used in consensual maps construction (Fig. 6). The results of single algorithms had discordant results, with GARP and SVM showing more conservative results while Euclidean distances and Maxent presented clear commission errors, mainly for *P. "araguari"* (Fig. S3). All models showed omission errors for *P. "araguari"* because they did not predict the species distribution in Perdizes (no. 12).

Regarding the consensus maps, *P. "araguari*" showed northwestern expansion in LGM both in CCSM and MIROC circulation models (Fig. 6a-d). In LIG, this species showed a

potential distribution change to the southeast, and contraction in relation to other periods, occupying the Canastra Plateau (Fig. 6e). Concerning *P. oreades*, the predicted distribution in current climate was restricted to northern Goiás State in all algorithms, but MAXENT predicted a wider distribution in north-central Goiás (Fig. S3). The LGM circulation models were discordant in *P. ayeaye* as well, with expansion and contraction predicted in CCSM and MIROC, respectively (Fig. 6c, d). In LIG, *P. oreades* has no potential occurrence predicted to any area (Fig. 6e). The consensus maps showed potential geographic overlap between *P. "araguari"* and *P. oreades* since the LIG to the current time (Fig. 6).

The results from *P. ayeaye*'s current distribution model were concordant with that found by Magalhães and collaborators (submitted) (Fig. 6a). CCSM and MIROC were discordant regarding this species range shift in LGM. While in CCSM *P. ayeaye* showed a contraction in easternmost distribution (Fig. 6c), MIROC predict distribution expansion in nearby valleys (Fig. 6d). As in *P. "araguari"*, *P. ayeaye* exhibited potential southeastern contraction in LIG. All algorithms appear to have provided similar current distribution models to *P. ayeaye* (Fig. S3).

Speciation modes

The neuralnet model selection showed ~53.3, 35.7 and 11.1% of PP for allopatry, alloparapatry and parapatry models, respectively. The PP for alloparapatry model in mnlogistic algorithm was 99.9%. The prior probability for each model was ~33%. Thence, discarding hypothesis with PP < prior probability, the parapatry model was unsupported in both algorithms. Despite the models showed adequacy regarding prior distribution of simulated genealogies, they were all unfit in relation to the posterior

distributions (Fig. S4). Because of this, we did not proceed with parameter estimation, evaluation of misclassification error nor posterior predictive analysis.

Discussion

Our species delimitation analysis showed evidence of imperfect taxonomy of the focal group. The taxonomical entity *P. oreades* was divided into two well supported lineages in BPP analysis. Apparently, today these lineages are geographically segregated in distinct plateau regions with a contact zone, which we could not locate with precision due to our limited geographic sampling. A landscape genetics approach with a dense sampling is necessary to assess a potential HZ between *P. "araguari"* and *P. oreades,* which appear to be, together with zones of character displacement, common in *Phyllomedusa* (Bruschi et al. 2013, Brunes et al. 2014). Despite the availability of the name *Phyllomedusa araguari* Giaretta et al. 2007, we opted not to revalidate the species. Hence we named *P. "araguari"* with quotation marks because its type-locality in northern Canastra Plateau is a mountain region where *P. ayeaye* occurs. As pointed out above, our ENM results showed omission errors exactly for this location (Fig. 6). For this reason, we cannot rule out the existence of a HZ between *P. "araguari"* and *P. ayeaye*. This should be investigated and, if the holotype is a hybrid or a migrant, *Phyllomedusa araguari* would become a 'nomem nudum' and the species should be described from an unequivocal locality. Our results do not indicate signs of hybridization or migration between these species, however, we have just two samples from Perdizes (Fig 1., no. 12). As we could not examine the holotype of *P. araguari* to confirm its identity, we chose to be conservative in our taxonomic decisions.

The taxonomic units *Phyllomedusa ayeaye* and *P. centralis* remain as they are currently known. Additionally, a new sampled locality was assigned to *P. centralis* (Fig. 1, no. 2),

which has important implications for the species conservation. This species was only known from its type-locality in Chapada dos Guimarães (no. 1) and former areas now inundated by the Manso's hydroelectric lake (Strüssmann, 2002). Due to the scarcity of information concerning its geographical distribution and biology, *P. centralis* was categorized as Data Deficient (DD) by the International Union for Conservation of Nature (IUCN) (Caramaschi et al., 2004). Brandão and collaborators (2009) suggested the allocation of the species to the Vulnerable (VU) category of IUCN Red List, based on its restricted distribution, rarity, habitat specificity, and human impacts on its known localities. Our results show a wider distribution with the new occurrence point ~400 km away from Chapada dos Guimarães (Fig. 1, no. 1 and 2). Nevertheless, more expeditions to the Guimarães plateau and research on *P. centralis*'s population structure, demography and threats need to be conducted to re-evaluate the species categorization.

Our phylogenetic study of the focal group resulted in a species tree different from the Faivovich and collaborators' (2010) hypothesis. Contrasting with our results (Fig. 5), their topology was (*P. ayeaye*, (*P. oreades*+*P. "araguari"*,(*P. centralis*))), which matches the topology of our mtDNA gene tree (Fig. 4). The phylogenetic hypothesis by Faivovich and collaborators (2010) was generated through the concatenation of seven unlinked DNA fragments from 17 individuals. The generation of a "super-gene" in concatenation approaches implicitly gives more weight to the fragments with higher mutation rates (Liu et al. 2015). In other words, the resulting "species-tree" cannot be anything different than the most variable fragment's gene tree topology. Additionally, 66% of nDNA sequence data were missing in Faivovich and collaborators' (2010), which represent 57% of the total data, reinforcing our hypothesis that their topology reflects the mtDNA gene tree. Furthermore, our focal group shows shallow divergences that imply a high ILS expectation for nuclear sequences (Funk and Omland 2003). Therefore, the multispecies

coalescent is the most appropriate approach for the phylogenetic reconstruction of these species relationships in order to accommodate the uncertainties due to ILS in each gene tree independently (Angelis and Reis. 2015; Liu et al. 2015).

The divergence between our ingroup and the sister species *Phyllomedusa megacephala* occurred in the Early Pleistocene, while the split times between our ingroup species must have occurred in Middle Pleistocene as expected (Faivovich et al. 2010; Magalhães et al. submitted). This timing of events is congruent with the predictions of the climate-driven hypothesis of diversification. There is an alternative hypothesis related to 'old climatically-buffered infertile landscapes' theory (OCBIL; Hopper 2009). Due to the age of these landscapes and climate stability compared with adjacent ecosystems, it is expected high local endemism, low vagility, rarity of endemics and long-term persistence of species, resulting in old crown groups (Hopper 2009; Hopper et al. 2016). 'Campo rupestre' region is an OCBIL (Hopper et al. 2016; Silveira et al. 2016) and, even though its endemic *Phyllomedusa* are geographically restricted and rare (Baêta et al. 2009, Brandão et al. 2009, Magalhães et al. submitted), our dating results do not meet the expectations of old lineages. The same pattern was observed for most species of *Pilososcereus* cacti (Bonatelli et al. 2014), several endemic cactophilic *Drosophila* (Franco and Manfrin 2013) and the ovenbirds sister species *Cinclodes espinhacensis* and *C. pabsti* (Freitas et al. 2012). The OCBIL's expectation of old lineages is related to slow generation times coupled with unique ecosystem characteristics, like phosphorus-depleted soils, that lead to long term adaptation in plants and associated animals (Hopper et al. 2009; Hopper et al. 2016). In contrast, the "species pump" model of diversification in sky islands asserts that recent palaeoclimatic dynamics (i.e., Pleistocene) induced distribution changes that resulted in speciation during the isolation cycles due to local adaptation and drift (Vuilleumeier 1971; Baker 2008; McCormack et al. 2009). Because

of the evidence given above regarding the evolution of many endemic clades from 'campo rupestre', we believe most do not fit a OCBIL theory, and reflects more recent climate-driven diversification events in sky islands.

Pleistocene speciation is not necessarily related to refuge speciation (Rull 2015), which is intrinsically related to the "species pump" model. However, our ENM results support geographical isolation of species during LIG, suggesting that these species became cyclically isolated in mountaintops during Middle Pleistocene interglacial periods. *Phyllomedusa oreades* showed no potential occurrence areas in LIG (Fig. 6) that may be the result of either low reliability of models, or interglacial microrefugia undetectable at the ENM scale (Rull 2009; Bonatelli et al. 2014). Assuming the first alternative is true, due to scarcity of occurrence points, we believe that another predictive model test, like Jackknife, can be more appropriate for evaluation of our results (Pearson et al. 2007).

Concerning the diversification models, genetic data and known geographic distributions supported *P. centralis* speciation in allopatric mode. The same datatype was observed for *P. ayeaye*, also indicating allopatric speciation with additional support provided by ENMs (Fig. 6). On the other hand, mtDNA evidence plus ENM indicated introgression and putative HZ between *P. "araguari"* and *P. oreades*. These results indicate feasibility in the proposed diversification models (Fig. 2). Our results support two alternative modes of speciation between *P. "araguari"* and *P. oreades*. The null hypothesis (i.e., allopatric mode) was not rejected, and it is even the more supported by neuralnet. This result implies shallow divergence and lack of time for morphological differentiation. In this case, ENM results would have little meaning to our conclusions. However, we would expect greater divergence signs in mtDNA than in nDNA for this model (Funk and Omland 2003), disagreeing with our results. The alternative hypothesis (i.e., alloparapatric mode) was supported by both neuralnet and mnlogistic algorithms, and agree with other evidence

sources. It agrees with evidences of contact zones from ENMs, suggesting isolation in interglacial cycles followed by geographic expansion and secondary contact in glacial ones. Additional support for alloparapatric model is given by the mitonuclear discordance found in our results. There are multiple causes for this pattern, which we cannot rule out such as adaptive introgression of mtDNA, demographic disparities, sex-biased asymmetries and hybrid zone movements (Toews and Brelsford 2012). Considering our ENM results, the hybrid zone movements is the most plausible, while to consider the other explanations to mitonuclear discordance, we would have to assume a set of premises, like sex-biased dispersal and positive selection on mitochondrial variants, over which we have no information. All those conclusions should be taken carefully because, although the models were suitable, our simulations did not fit well to the observed data indicating the need for adjustment, including more informative prior distribution of parameters (we assumed all uniform) and the choice of more adequate summary statistics (e.g., we do not used pairwise F_{ST}, which is a good metric of gene flow inference) (Bertorelle et al. 2010; Csilléry et al. 2010).

Our conclusions are not free of caveats because of predictions errors in ENMs and poorly fit coalescent models or low performance of ABC-based, model selection. Further work will be directed to refinement of models and analytical techniques to provide more resolution to the complex evolutionary history of these frogs. Nevertheless, our study provides important insights about the evolutionary history of 'campo rupestre' biota. We found evidence of speciation related to mountain ranges as expected in sky islands endemics. Furthermore, our evidence points to the importance of Pleistocene climate changes in current diversity patterns. Cryptic diversity may be common due to gene flow in interglacial cycles that constrained morphological differentiation.

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Figure legends:

Fig.1 (A) Distribution of sampled regions for *Phyllomedusa*. 1. Chapada dos Guimarães, Mato Grosso State (MT) (type-locality of *P. centralis*); 2. Barra do Garças, MT; 3. Minaçu, Goiás State (GO) (type-locality of *P. oreades*); 4. Cavalcante, GO; 5. Alto Paraíso de Goiás, GO; 6. Chapada Gaúcha, Minas Gerais State (MG); 7. Brasília, Distrito Federal; 8. Pirenópolis, GO; 9. Poços de Caldas, GO; 10. Cristalina, GO; 11. Brasilândia de Minas, MG; 12. Perdizes, MG (type-locality of *P. araguari*, a junior synonym of *P. oreades*); 13. Sacramento, MG; 14. São Roque de Minas, MG; 15. Perdizes, São Paulo State; 16. Alpinópolis, MG; 17. Poços de Caldas, MG; 18. Minduri, MG; 19. Lavras, MG; 20. Ouro Preto, MG; 21. Nova Lima, MG. Some examples of shrub-grassland landscapes found in sample points: (B) View of valley from top of the Stone City, Guimarães Plateau (1); (C) Morro da Baleia view, on top of the Veadeiros Plateau (5); (D) Orographic rainfall on Canastra Plateau (14) and (E) grasslands and mountains from Quadrilátero Ferrífero (20). Photos B by RAB, C by RFM, D and E by Tiago L. Pezzuti.

Fig. 2 Alternative models for the diversification history of *Phyllomedusa "araguari"*, *P. ayeaye* and *P. oreades* tested with multilocus ABC. The models were named allopatry,

parapatry and alloparapatry after results of species delimitation and ecological niche modelling.

Fig. 3 (A) STRUCTURE results for K = 4. Individuals are represented by vertical bars and colors are the proportion of individual assignment to each cluster. Four highlighted blocks are thereabout related to mountain regions (see Fig. 4). (B) Neigbor-net of PBC distances. Arrows show introgressed individual. Scale bar represents genetic distance in the network.

Fig. 4 Putative species identified by ML-GMYC analysis (below) and geographical distribution of putative species (above) from STRUCTURE analysis (Fig. 3) for comparison purposes. Color bars indicate the discovered species. The colors are the same from Fig. 3. Green: *Phyllomedusa ayeaye*, Yellow: *P. centralis* and Blue+Red: *P. oreades*. Circles in nodes denote posterior probabilities (PP) in gene tree estimation. Numbers below nodes are the PP for each species from *b*GMYC analysis. Numbers in parenthesis refer to the locations in Fig. 1.

Fig. 5 Species tree from *BEAST analysis. Numbers below nodes are the posterior probabilities for each node. Above nodes are shown the mean of split times in million years before past. Blue bars represent the 95% highest posterior density for divergence dates (see text).

Fig. 6 Consensus maps between GARP and SVM algorithms. (A) Current time, (B) mid-Holocene, (C) Last Glacial Maximum (CCSM), (D) Last Glacial Maximum (MIROC) and (E) Last Interglacial period. Weak colors indicate lowest presence threshold (LPT) for species only in one algorithm while strong colors are LPT in a quadrat for both. Purple tones are zones with potential co-occurrence for *P. "araguari"* and *P. oreades.*

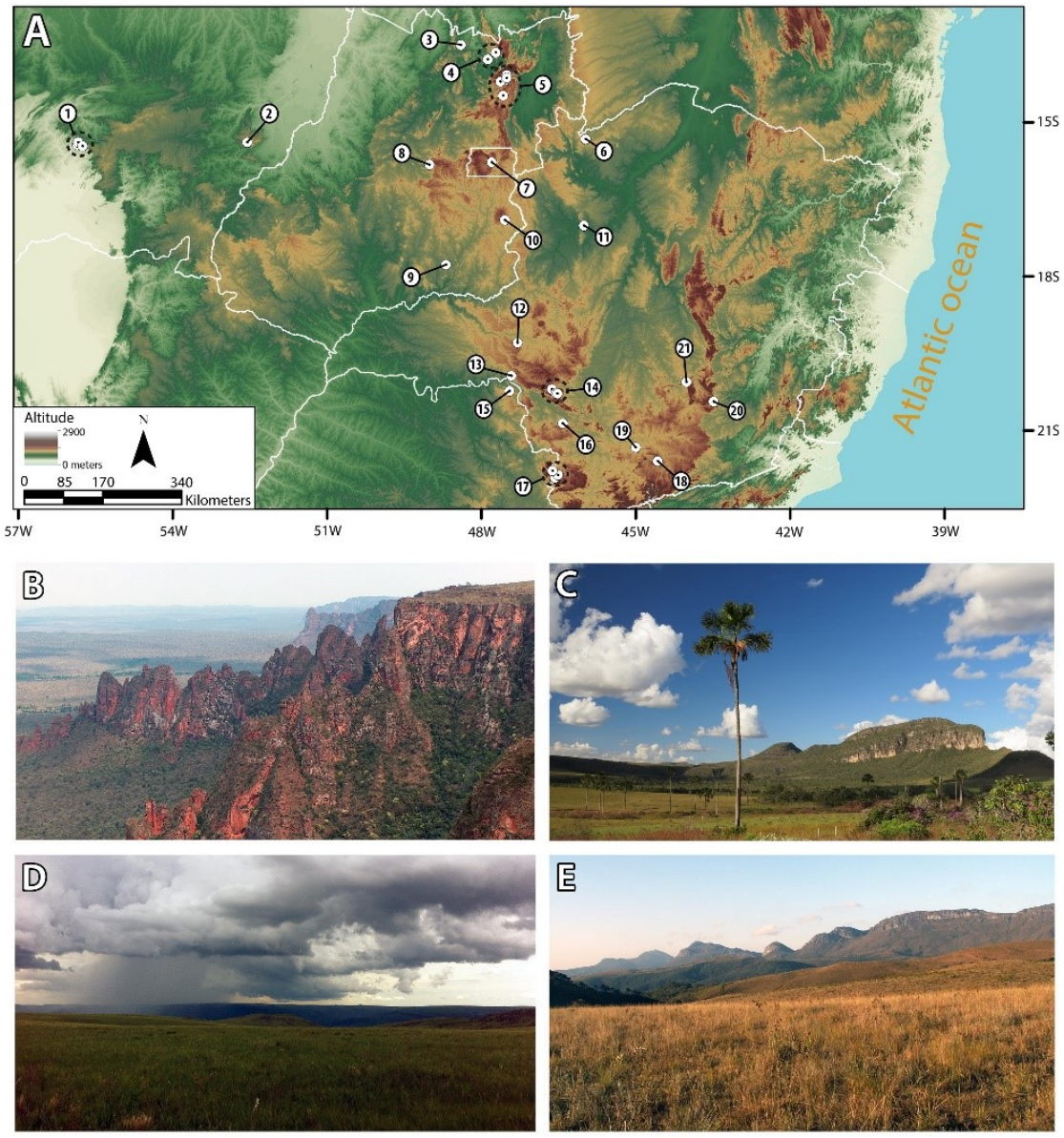
A
Altitude
2900
0 meters
N
0 85 170 340
Kilometers
57W
54W
51W
48W
45W
42W
39W
15S
18S
21S
Atlantic ocean
B
C
D
E

Fig. 1

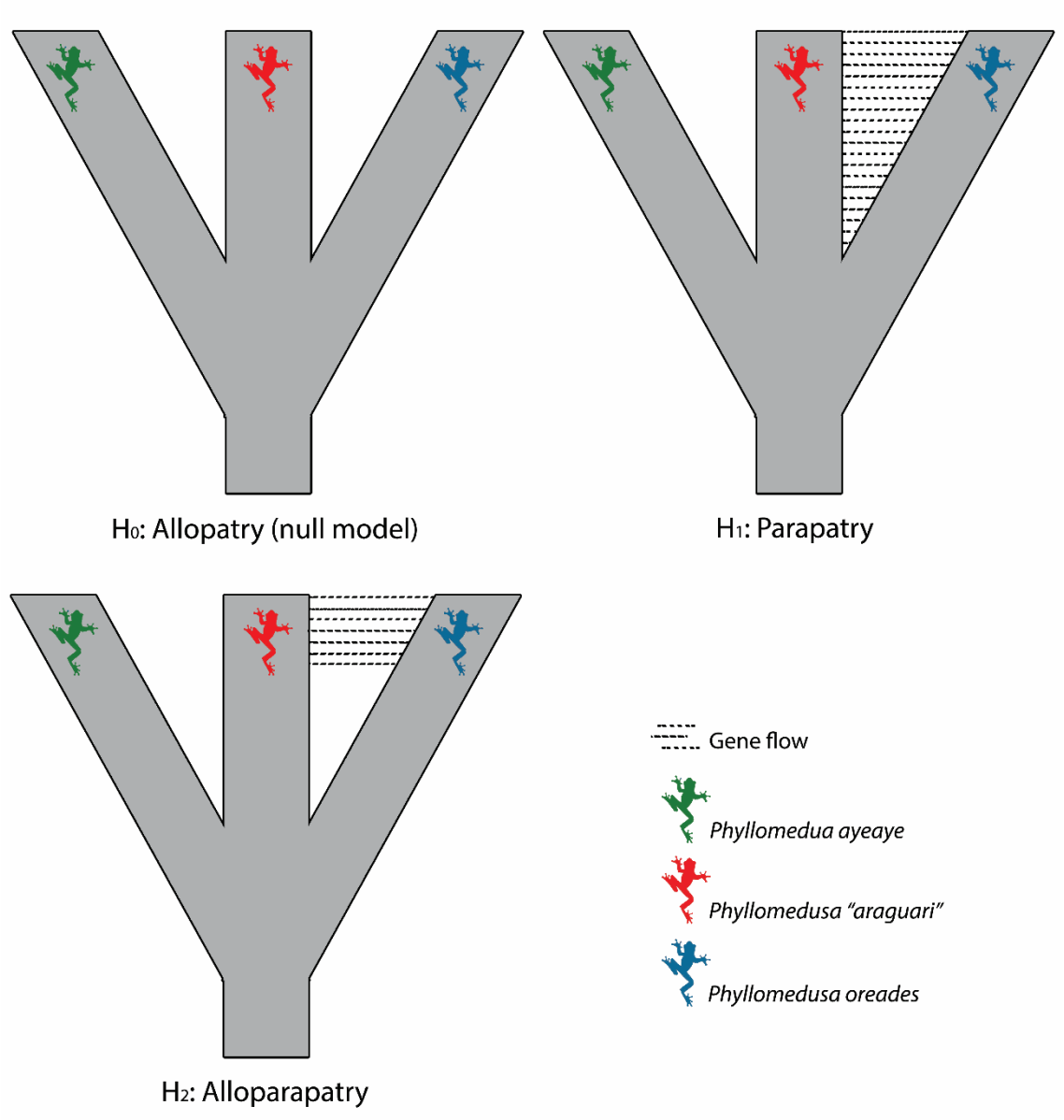
H0: Allopatry (null model)
H1: Parapatry
H2: Alloparapatry
Gene flow
Phyllomedua ayeaye
Phyllomedusa "araguari"
Phyllomedusa oreades

Fig. 2

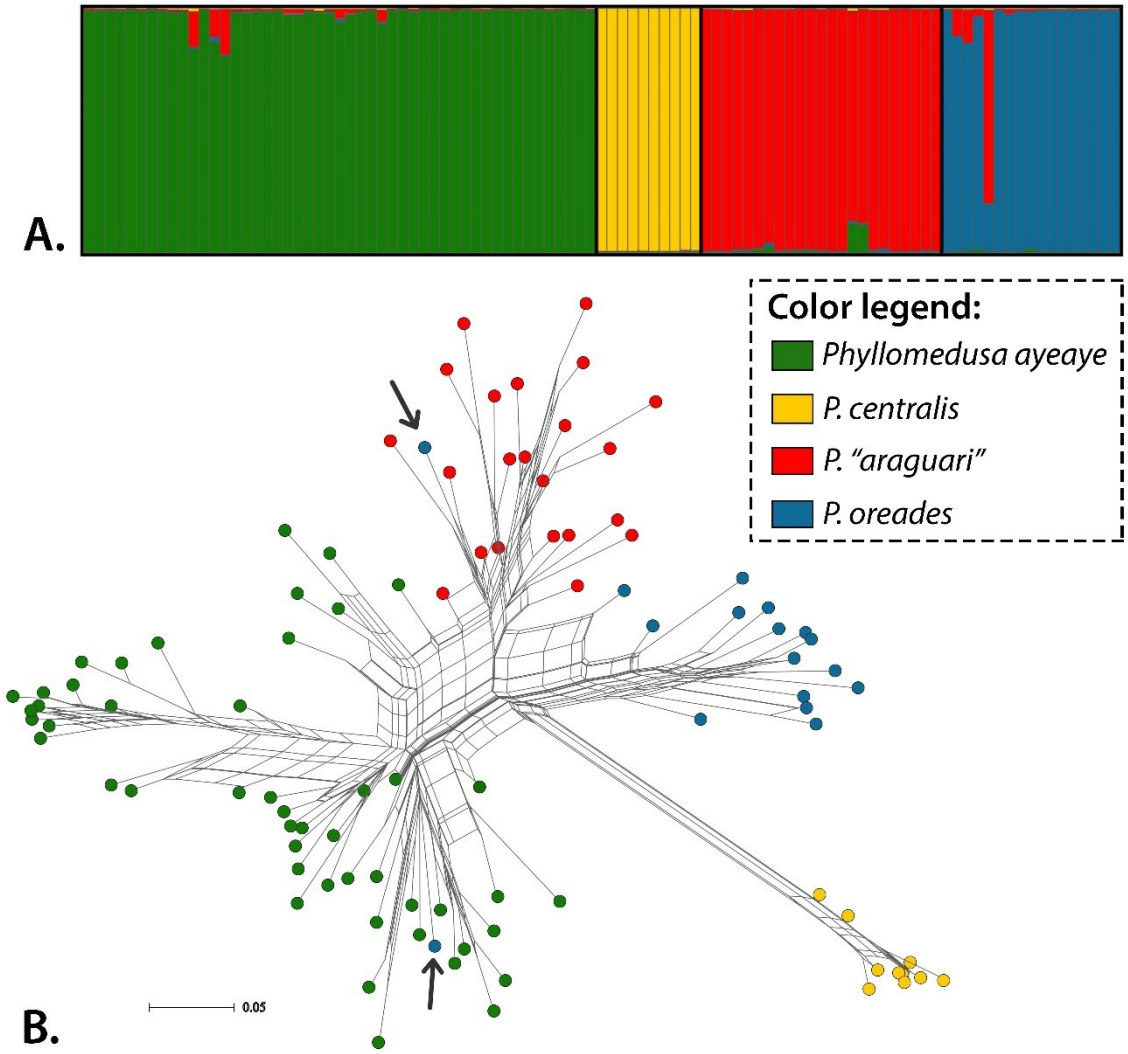
A.
Color legend:
Phyllomedusa ayeaye
P. centralis
P. "araguari"
P. oreades
B.
0.05

Fig. 3

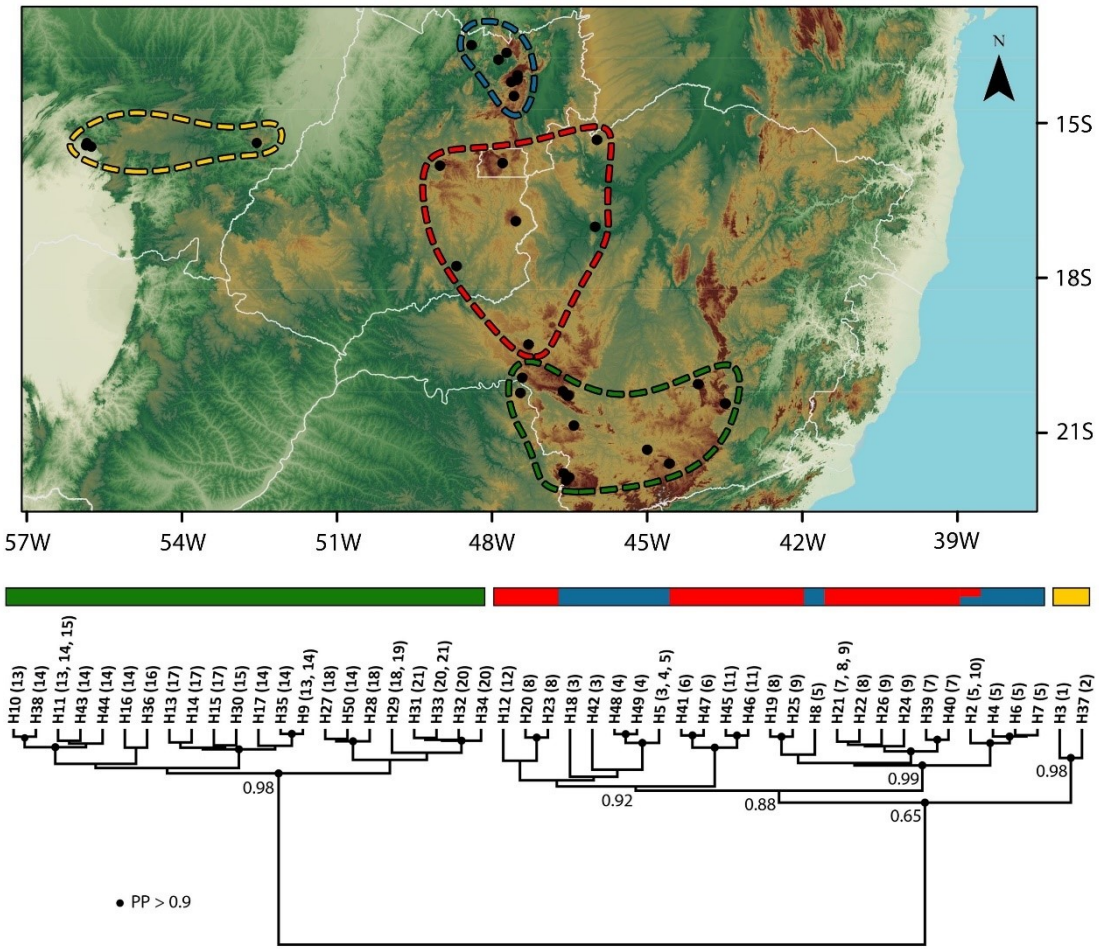
N
15S
18S
21S
57W
54W
51W
48W
45W
42W
39W
H10 (13)
H38 (14)
H11 (13, 14, 15)
H43 (14)
H44 (14)
H16 (14)
H36 (16)
H13 (17)
H14 (17)
H15 (17)
H30 (15)
H17 (14)
H35 (14)
H9 (13, 14)
H27 (18)
H50 (14)
H28 (18)
H29 (18, 19)
H31 (21)
H33 (20, 21)
H32 (20)
H34 (20)
H12 (12)
H20 (8)
H23 (8)
H18 (3)
H42 (3)
H48 (4)
H49 (4)
H5 (3, 4, 5)
H41 (6)
H47 (6)
H45 (11)
H46 (11)
H19 (8)
H25 (9)
H8 (5)
H21 (7, 8, 9)
H22 (8)
H26 (9)
H24 (9)
H39 (7)
H40 (7)
H2 (5, 10)
H4 (5)
H6 (5)
H7 (5)
H3 (1)
H37 (2)
0.98
0.92
0.88
0.99
0.65
0.98
• PP > 0.9

Fig 4.

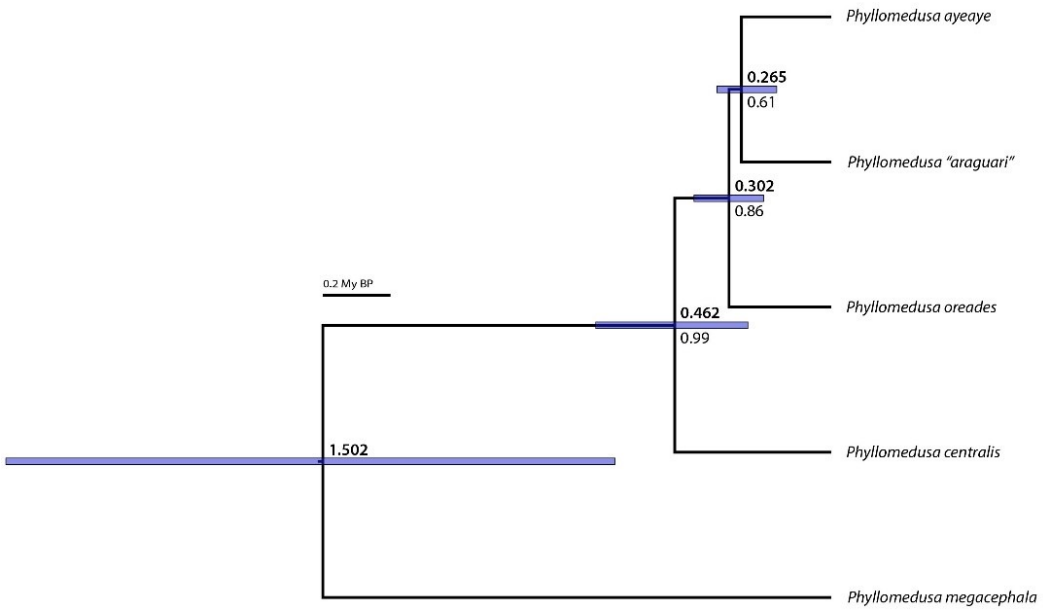
Phyllomedusa ayeaye
0.265
0.61
Phyllomedusa "araguari"
0.302
0.86
0.2 My BP
Phyllomedusa oreades
0.462
0.99
1.502
Phyllomedusa centralis
Phyllomedusa megacephala

Fig. 5

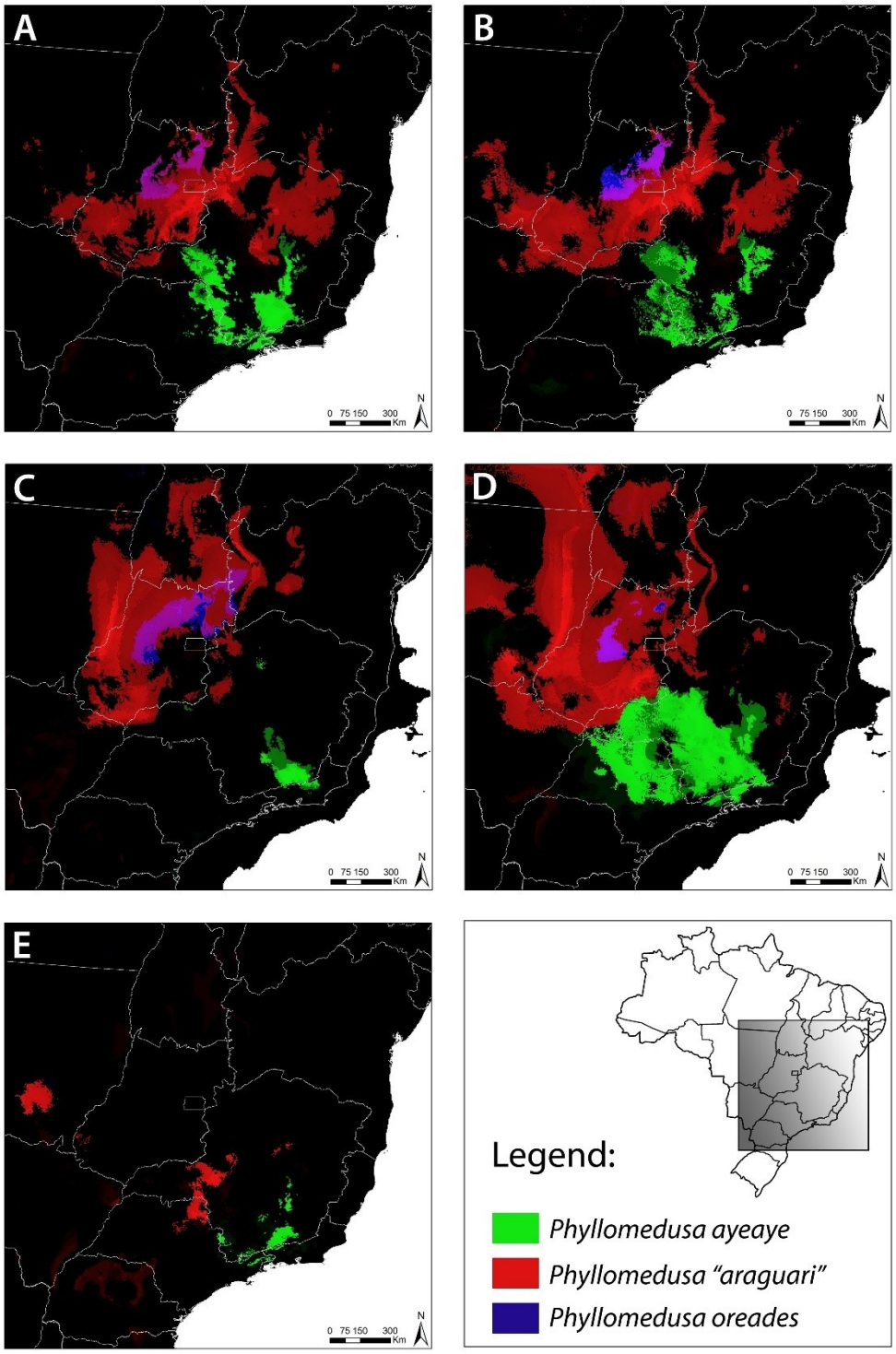
A
B
C
D
E
0 75 150 300 Km
N
Legend:
Phyllomedusa ayeaye
Phyllomedusa "araguari"
Phyllomedusa oreades

Fig. 6

Table 1: Primers, temperature (Tm) and time (tm) melting used for each fragment and the references for them.

Fragment	Primer	Sequence (5'-3')	Tm (ºC)	tm (sec)	Reference
Cytochrome b	MVZ15	GAACTAATGGCCCACACWWTACGNAA	62	30	Moritz et al. 1992
	CytbAR-H	TAWAAGGGTCTTCTACTGGTTG			Goebel et al. 1999
Fibrinogen, A α-polypeptide, intron 1	MVZ47	AGTGAAAGATACAGTCACAGTGCTAGG	55	60	Bell et al. 2011
	MVZ48	GGAGGATATCAGCACAGTCTAAAAAG			
β-fibrinogen, intron 7	FIB-B17U	GGAGAAAACAGGACAATGACAATTCAC	56	60	Prychitko and Moore 1997
	FIB-B17L	TCCCCAGTAGTATCTGCCATTAGGGTT			
Ribosomal protein L3, intron 5	RPL3intF	AGTCTTTGGCCAGGATGAAATG	62	40	Pinho et al. 2010
	RPL3intR	TCACACCTAGGAGGGATAATG			
	RPL3-P3*	WCTGGCCTGCTCTGGTTAT			Magalhães et al. submitted.
Anonymous nuclear loci (former 'anonymous nuclear intron')	ANIF1	YSTGAMGTTTTRSAATGGCTG	60	60	This work
	ANIR2	KGTTTTYCAATGGCTGYGTA			
Proopiomelanocortin	POMC1	GAATGTATYAAAGMMTGCAAGATGGWCCT	60	60	Wiens et al. 2005
	POMC2	TAYTGRCCCTTYTTGTGGGCRTT			
Cellular myelocytomatosis	cmyc1U	GAGGACATCTGGAARAARTT	54	60	Crawford 2003
	cmyc-ex2R	TCATTCAATGGGTAAGGGAAGACC			Wiens et al. 2005
Tensin 3	WL421	CAGTGTTGGAGAAGATGGTATGTC	56	60	Smith et al. 2007
	WL423	CAGCATAGGTACTTTATCATCATCAG			

*Primer used in place of RPL3intF in sequencing reactions.

Table 2: Posterior probabilities for each topology (represented in newick format) for each gamma prior combinations for theta (Θ) and tau (τ) parameters. First and second values refers to each two-independent analysis. Values lower than priori probabilities (~6.67%) are not showed. ar: *Phyllomedusa "araguari"*, ay: *P. ayeaye*, ce: *P. centralis* and or: *P. oreades.*

Prior	(ce,(or,(ay,ar)))	(ce,(ay,(or,ar)))	(or,(ce,(ay,ar)))	((ay,ar),(ce,or))	((ce,ay),(ar,or))
$\Theta \sim G(2, 100), \tau \sim G(2, 200)$	46.9 ; 47.6	35.9 ; 37.8	-	-	-
$\Theta \sim G(2, 1000), \tau \sim G(2, 2000)$	35.1 ; 30.2	20.1 ; 24.6	11.7 ; 10.3	11.3 ; 10.1	8.7 ; 9.4
$\Theta \sim G(2, 1000), \tau \sim G(2, 200)$	32.4 ; 30.5	26.8 ; 27.8	9.6 ; 10.5	9.2 ; 9.7	9.7 ; 8.9
$\Theta \sim G(2, 100), \tau \sim G(2, 2000)$	38.3 ; 41.4	36.9 ; 34.5	-	-	-

Table 3: AUC values from *Phyllomedusa "araguari"*, *P. ayeaye* and *P. centralis* related to distinct ecological niche modeling algorithms.

Species	Euclidean distances	MAXENT	GARP	SVM
Phyllomedusa "araguari"	0.86	0.84	0.99	0.99
Phyllomedusa ayeaye	0.86	0.99	0.99	0.99
Phyllomedusa oreades	0.92	0.93	0.99	0.99

Supporting information

Fig. S1: Values of LnPr(X/K) (open squares) for K = 1-8 after analysis with STRUCTURE, and values of ΔK (black circles).

Fig. S2: Posterior probabilities of conspecificity in Bayesian implementation of GMYC. Cells are colored by corresponding sequences are conspecific, showing uncertainty in species limits. For relevant numerical values, see Fig. 4. Green = *Phyllomedusa ayeaye*; yellow = *P. centralis*; blue + red = *P. oreades* + *P. "araguari"*

Fig. S3: Reliable ecological niche models. (a) Euclidean distances, (b) MAXENT, (c) GARP and (d) SVM.

Fig. S4: Principal component analysis showing prior (black), posterior (red), and observed data (yellow) for (a) alloparapatry model in multinomial logistic regression selection. (b) allopatry and (c) alloparapatry models in non-linear local regression selection.

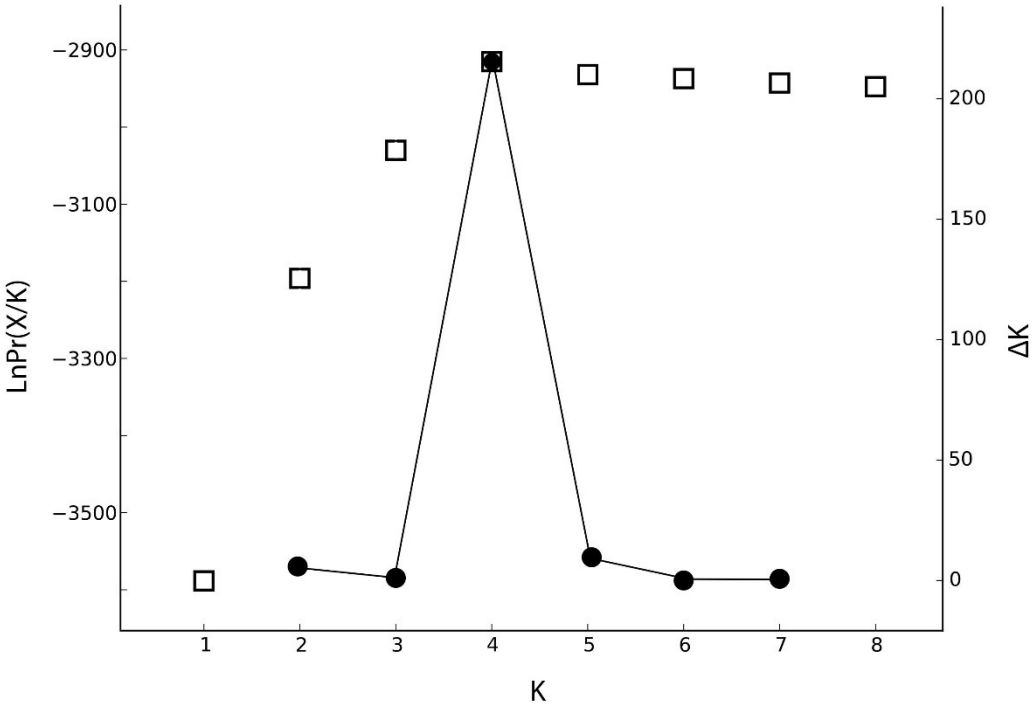
−2900
−3100
−3300
−3500
LnPr(X/K)
200
150
100
50
0
ΔK
1
2
3
4
5
6
7
8
K

Fig. S1

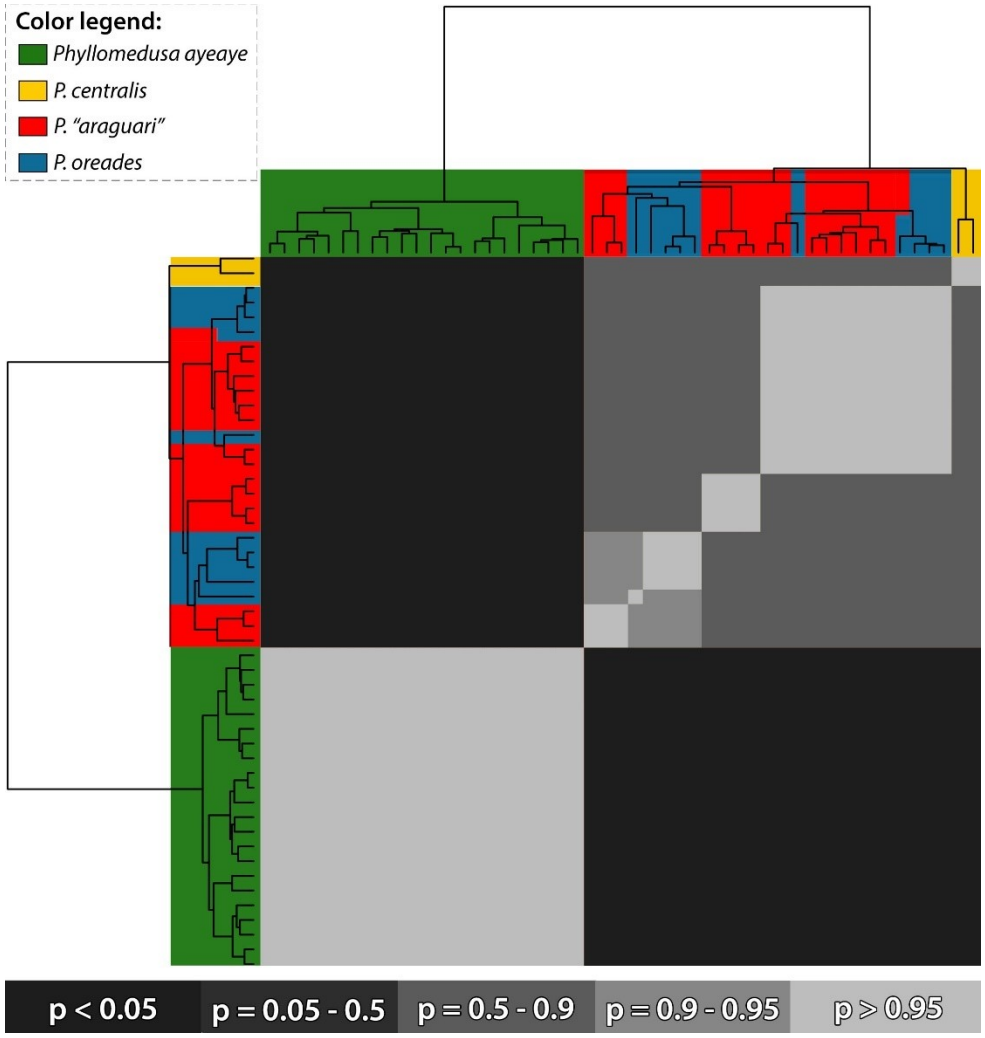
Color legend:
Phyllomedusa ayeaye
P. centralis
P. "araguari"
P. oreades
p < 0.05
p = 0.05 - 0.5
p = 0.5 - 0.9
p = 0.9 - 0.95
p ⩾ 0.95

Fig. S2

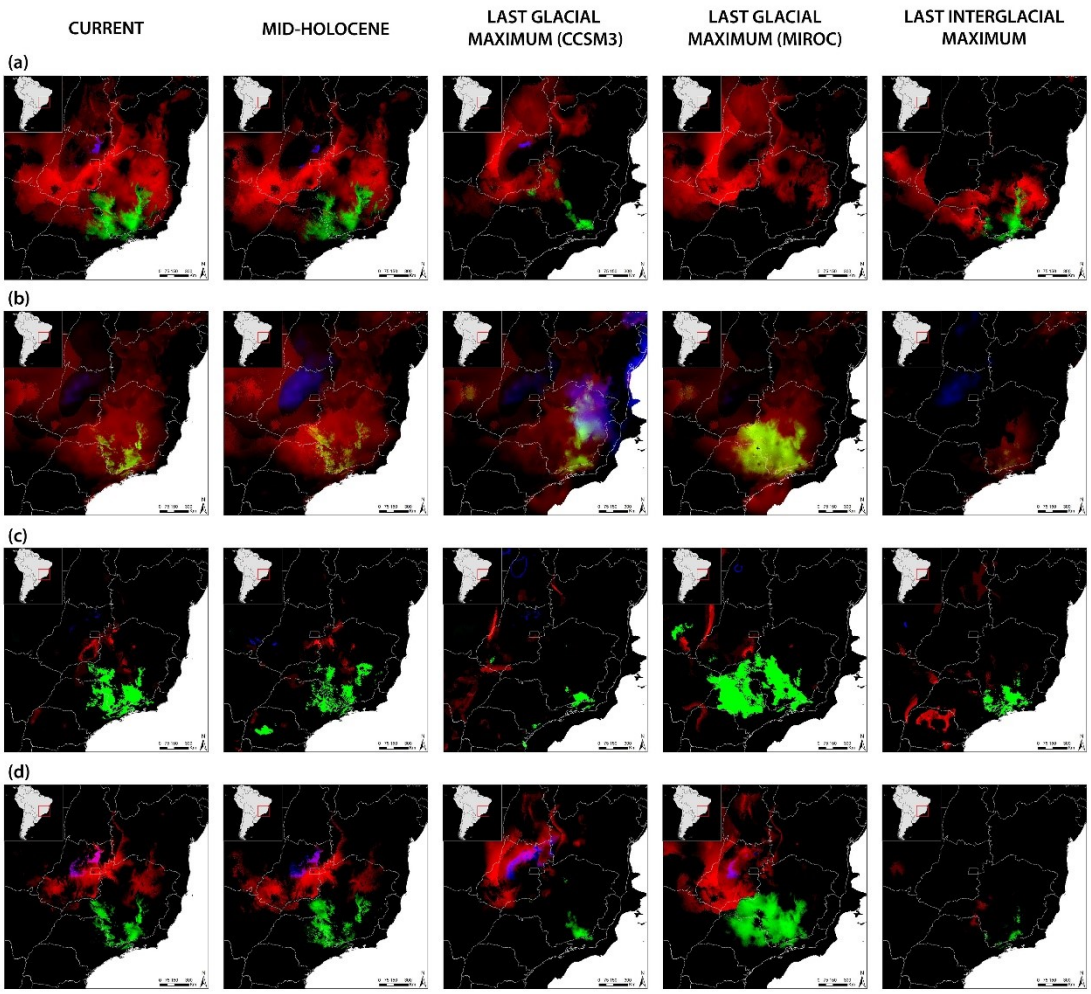
CURRENT
MID-HOLOCENE
LAST GLACIAL MAXIMUM (CCSM3)
LAST GLACIAL MAXIMUM (MIROC)
LAST INTERGLACIAL MAXIMUM
(a)
(b)
(c)
(d)

Fig. S3

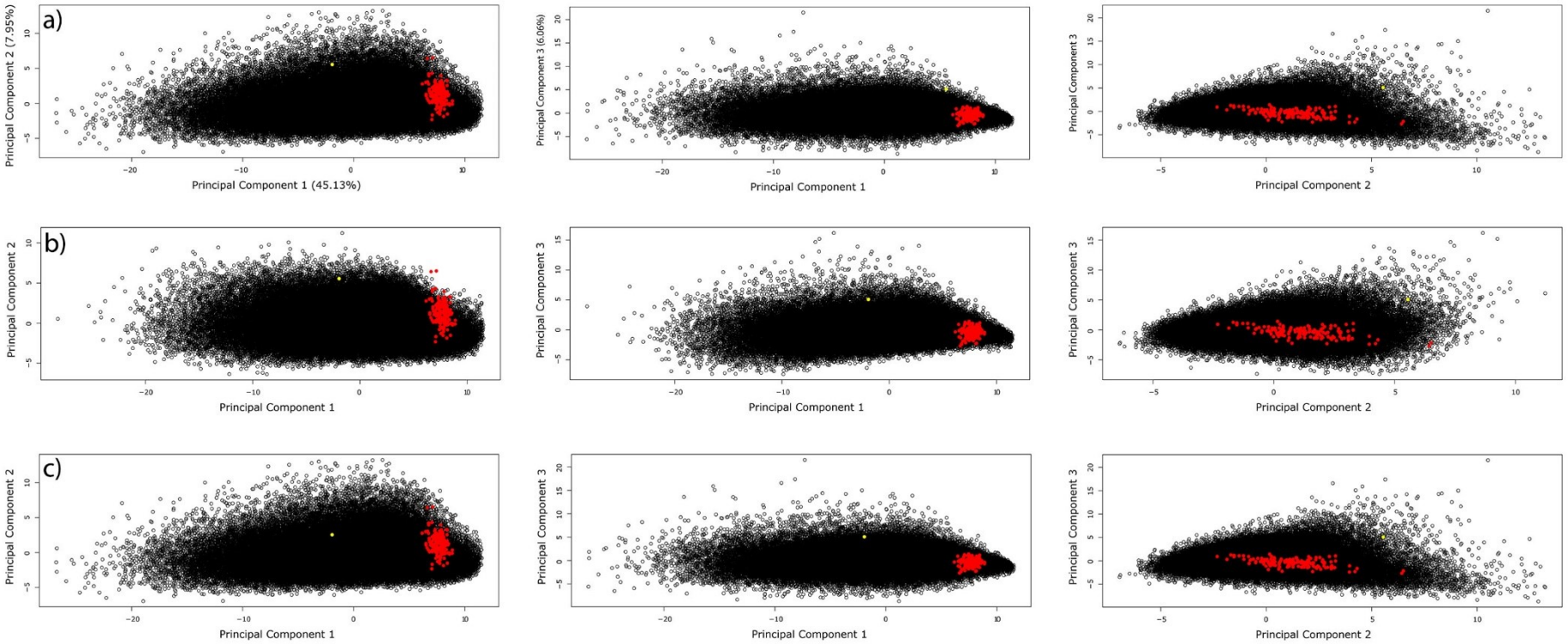
a)
b)
c)
Principal Component 2 (7.95%)
Principal Component 1 (45.13%)
Principal Component 3 (6.06%)
Principal Component 1
Principal Component 3
Principal Component 2
Principal Component 2
Principal Component 1
Principal Component 3
Principal Component 1
Principal Component 3
Principal Component 2
Principal Component 2
Principal Component 1
Principal Component 3
Principal Component 1
Principal Component 3
Principal Component 2

Fig. S4

Table S1 (part 1): Occurrence points used in ecological niche modelling.

Species	Locality	Latitude	Longitude	Source
Phyllomedusa "araguari"	Perdizes - MG	-19.2888	-47.2974	R.A.B
	Brasília - DF	-15.7760	-47.7978	CHUNB
	Pirenópolis - GO	-15.8260	-49.0110	CHUNB
	Caldas Novas - GO	-17.7708	-48.6892	CHUNB
	Cristalina - GO	-16.8991	-47.5435	UFMG
	Brasilândia de Minas - MG	-17.0097	-46.0094	UFMG
	Chapada Gaúcha - MG	-15.3290	-45.9753	CHRWT
Phyllomedusa ayeaye	Alpinópolis - MG	-20.8597	-46.4203	AAG
	Lavras - MG	-21.2013	-44.9413	CHRWT
	Minduri - MG	-21.5934	-44.5719	UFMG
	Nova Lima - MG	-20.0552	-44.0135	UFMG
	Ouro Preto - MG	-20.4309	-43.4904	UFMG
	Pedregulho - MG	-20.2256	-47.4539	CFBH
	Poços de Caldas - MG	-21.9172	-46.5686	UFMG
		-21.8612	-46.5086	UFMG
		-21.7772	-46.6192	UFMG
	Sacramento - MG	-19.9316	-47.4183	CFBH
	São Roque de Minas - MG	-20.2669	-46.5553	UFMG
		-20.1939	-46.6306	UFMG
		-20.2792	-46.5208	UFMG
	Arantina - MG	-21.8609	-44.2165	R.A.B.
	Brumadinho - MG	-20.1014	-43.9888	UFMG
		-20.0940	-44.0210	UFMG
		-20.0837	-43.9951	UFMG
		-20.1016	-43.9884	R.A.B.
	Carrancas - MG	-21.5939	-44.6272	R.A.B.
	Congonhas do Campo - MG	-20.4536	-43.8742	UFMG
		-20.1278	-43.9835	UFMG
		-20.4381	-43.9398	UFMG
		-20.4867	-43.9431	UFMG
		-20.4334	-43.8740	R.A.B.
		-20.4827	-43.9368	R.A.B.
	Itabirito - MG	-20.2309	-43.8524	UFMG
		-20.2934	-43.9306	R.A.B.
	Lavras - MG	-21.3277	-44.9797	UFMG
	Luminárias - MG	-21.5535	-44.8187	R.A.B.
	Minduri - MG	-21.5941	-44.5739	UFMG
	Nova Lima - MG	-20.1015	-43.9886	UFMG
		-20.0051	-43.9281	UFMG
		-20.0083	-43.9309	R.A.B.

Table S1 (part 2): Occurrence points used in ecological niche modelling.

Species	Locality	Latitude	Longitude	Source
Phyllomedusa ayeaye	Ouro Branco - MG	-20.5143	-43.6197	UFMG
		-20.5089	-43.6155	UFMG
		-20.4797	-43.5931	MZUFV
		-20.5086	-43.6131	MZUFV
		-20.5149	-43.6262	R.A.B.
	Ouro Preto - MG	-20.4323	-43.4917	UFMG
		-20.4315	-43.4870	UFMG
		-20.4321	-43.4877	UFMG
		-20.2777	-43.5257	MZUFV
		-20.3333	-43.4833	R.A.B.
		-20.4777	-43.6874	R.A.B.
	Poços de Caldas - MG	-21.7766	-46.6178	CFBH
		-21.9088	-46.5467	R.A.B.
		-21.8977	-46.5470	R.A.B.
	Pedregulho - SP	-20.2148	-47.4264	R.A.B.
	Sacramento - MG	-20.2197	-47.1062	CFBH
	São Roque de Minas - MG	-20.2682	-46.5549	UFMG
		-20.2283	-46.4564	R.A.B.
		-20.2778	-46.5221	R.A.B.
		-20.2977	-46.5244	R.A.B.
		-20.2682	-46.5549	R.A.B.
Phyllomedusa centralis	Chapada do Guimarães - MT	-15.4341	-55.8564	UFMG
		-15.3857	-55.8389	UFMG
		-15.4606	-55.7497	CFBH
		-15.1071	-55.5396	UFMT
	Barra do Garças - MT	-15.3876	-52.5486	AAG
Phyllomedusa oreades	Minaçu - GO	-13.4958	-48.3974	CHUNB
	Cavalcante - GO	-13.7740	-47.8743	UFMG
		-13.6424	-47.7217	CHUNB
	Alto Paraíso - GO	-14.4787	-47.5781	CZUFG
		-14.1622	-47.5233	CHUNB
		-14.2056	-47.6294	UFMG
		-14.0788	-47.5096	UFMG
		-14.1325	-47.5100	UFMG

Coleção Herpetológica "Alfred Russel Wallace", tissues – UFLA (CHARW-T), Coleção Herpetológica "Ariovaldo Antônio Giaretta" (AAG), Coleção Herpetológica "Célio Fernando Baptista Haddad" (CFBH), Coleção Herpetológica da Universidade de Brasília (CHUNB) and Coleção Herpetológica of the Centro de Coleções Taxonômicas of the Universidade Federal de Minas Gerais (UFMG), R.A. Brandão data (R.A.B.), and Museu de Zoologia da Universidade Federal de Viçosa (MZUFV).

P.S.: Voucher numbers will be included in final version.

Table S2: Summary statistics for sampled loci. Parameters shown are nucleotide diversity per locus (π) and number of segregating sites (ss). Or: *Phyllomedusa oreades*, Ar: *P. "araguari"* and Ay: *P. ayeaye.*

Fragment	Length (bp)	Model	π	ss	Tajima's D	Species	π_{within}	$\pi_{between}$ (Dxy)		
								Or-Ar	Or-Ay	Ar-Ay
cyt-*b*	896	HKY + I	18.34982	83	0.33588	Or	5.23529	6.64832	30.86720	30.54464
						Ar	6.20553			
						Ay	3.69728			
A-fib	508	HKY + I	3.27975	31	-1.08365	Or	2.65775	3.22580	3.72872	3.21056
						Ar	1.83671			
						Ay	2.79342			
β-fib	568	K80 + I	2.23507	39	-1.95169	Or	2.55080	3.03880	2.20952	2.36288
						Ar	2.81282			
						Ay	1.35667			
c-myc	430	K80 + I	2.14311	20	-0.99602	Or	1.16934	1.49210	2.02530	2.14140
						Ar	1.38841			
						Ay	0.99179			
POMC	601	HKY + I	3.17624	24	-0.62112	Or	3.19964	3.69615	3.59398	3.81635
						Ar	3.26004			
						Ay	2.14685			
Anonymous	633	HKY + Γ	14.74709	58	1.26207	Or	6.79310	27.75705	24.13629	17.38218
						Ar	5.04231			
						Ay	11.50586			
RPL3	518	K80 + Γ	11.18074	53	0.44517	Or	2.60250	5.98808	14.54026	12.48898
						Ar	2.83932			
						Ay	14.18977			
TNS3	495	F81	1.50392	11	-0.6459	Or	1.41889	2.13345	1.12860	1.86615
						Ar	1.22415			
						Ay	0.76478			

CONSIDERAÇÕES FINAIS

Nossos resultados fornecem dados sobre a importância do isolamento geográfico em ilhas de altitude nos padrões de diversificação observados nos campos rupestres. O tempo de diversificação para as *Phyllomedusa* indica eventos de divergência inter- e intraespecífica todos ocorridos durante o Pleistoceno, indicando a importância das mudanças paleoclimáticas na formação da diversidade e endemismos em ilhas de altitude brasileiras. Apesar dos poucos estudos relacionados à evolução da biota dessas áreas, a hipótese de diversificação impulsionada pelo clima é reforçada pelos resultados deles. A fixação de características fenotípicas diferenciais entre distintas espécies está diretamente relacionada ao tempo, uma vez que o processo evolutivo é gradual. Eventos de especiação recente geralmente estão relacionados à diversificação críptica. Se isto for um padrão geral, os campos rupestres podem ser um 'hotspot' para estudos de especiação críptica no Brasil.

Quanto ao potencial evolutivo das espécies, nossos resultados também apontam para a ocorrência de múltiplas linhagens intraespecíficas, o que pode ser um padrão geral em espécies endêmicas dos campos rupestres. Estas linhagens são a "matéria-prima" para futuros eventos de diversificação. Por este motivo, nós encorajamos a expansão do conhecimento sobre especiação e diversificação críptica nos campos rupestres. No futuro, estes dados poderão ser utilizados para a criação de políticas de conservação específicas que considerem o padrão de isolamento geográfico das espécies endêmicas dessa região.