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INTEGRANDO MODELOS DE DISTRIBUIÇÃO POTENCIAL COM USO DE HÁBITAT PELAS ESPÉCIES DAS PERERECAS MACACO *PITHECOPUS AYEAYE* E *PITHECOPUS OREADES* (ANURA: PHYLLOMEDUSIDAE) NO ENCONTRO DE NOVAS POPULAÇÕES

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LABORATÓRIO DE FAUNA
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Orientador: Prof. Dr. Reuber Albuquerque Brandão

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“Minha alucinação é suportar o dia a dia
e meu delírio é a experiência com coisas reais”

Ilustração de Yuri Só

Antônio Carlos Gomes Belchior Fontenelle Fernandes - Álbum Alucinação (1976)

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Resumo

Modelagem preditiva é uma técnica amplamente utilizada para prever a distribuição das espécies através de modelos probabilísticos. Esses modelos têm aplicações em estudos de padrões biogeográficos baseados na diversidade, dispersão e extinção das espécies e, para trabalhar com modelo de distribuição, usamos espécies como unidade básica evolutiva. É possível fazer projeções mais realistas das distribuições geográficas associando fatores bióticos e abióticos e pelas interações que as espécies estabelecem entre si e com o meio. Partindo do conhecimento de nicho ecológico e do modelo de B.A.M., devemos considerar que essas interações são dinâmicas e, por isso, nem sempre a espécie necessariamente ocorrerá em todas as localidades indicadas pelo modelo. Através de técnicas moleculares de sequenciamento de DNA, é possível identificar as amostras a nível de espécie, principalmente para formas crípticas e, assim, poder aumentar a precisão dos seus limites de distribuição espacial. As pererecas-macaco-de-flancos-reticulados *Pithecopus ayeaye* e *P. oreades* são espécies irmãs que ocorrem em ambientes abertos de solos rupestres em áreas de altitude no Cerrado e enclaves de campos na Mata Atlântica. Utilizam vegetação ripária e arbustiva ao longo de riachos temporários, e, por isso, sua conservação está ligada fortemente à preservação desses ambientes sensíveis e raros na paisagem do Cerrado. Neste trabalho, desenvolvemos um protocolo combinando modelos preditivos de distribuição, imagens da ferramenta Google Earth™ (GE) e informações de história natural para ampliar o número de registros de *Pithecopus ayeaye* e *P. oreades* (Anura, Phyllomedusidae). Primeiramente, elaboramos mapas de ocorrência potencial para as espécies com base nas ocorrências registradas na literatura e coleções para, a partir deles, prever novas localidades de alta adequabilidade. Posteriormente, usamos imagens de alta resolução espacial GE para selecionar os riachos de primeira ordem e drenagens com potencial de ocorrência das espécies. Finalmente, validamos o modelo através de visitas às novas localidades selecionadas. Os indivíduos encontrados foram coletados para identificação molecular a partir da comparação de fragmentos mitocondriais Cyt-b. Seguindo esse protocolo, obtivemos 38 novos registros para as espécies (24 para *P. ayeaye* e 14 para *P. oreades*, sendo 3 localidades de hibridização). A partir da incorporação de

sequências de 204 indivíduos, geramos a filogenia com os novos indivíduos coletados. A acurácia do modelo preditivo foi alta para *P. ayeaye* e *P. oreades* (AUC = 0,935 e *AUC* = 0,923, respectivamente). Entender a distribuição e suas relações de parentescos entre elas é a base para estratégias visando a conservação, mesmo com espécies que possuem taxonomia complexa, sem padrões claros de distribuição espacial e sobreposições de limites geográficos em algumas regiões, como o caso dessas duas *Pithecopus*. Concluímos, então, que o protocolo para seleção de novas localidades foi excepcionalmente satisfatório para a predição da ocorrência dessas espécies.

Abstract

Predictive models are a modern technique that has been widely used to predict species distribution through probabilistic models. It is possible to make more realistic projections of geographic distributions associating biotic and abiotic factors and the interactions that species establish with each other and with the environment. Based on the ecological niche knowledge, we must consider these interactions are dynamic and, therefore, not all occurrence locations indicated by the model the species will occur. Through molecular DNA sequencing techniques, it is possible to identify species, especially for cryptic ones, and correctly identify its spatial distribution limits. The reticulated flank monkey tree frogs *Pithecopus ayeaye* and *P. oreades* are two closely related species that occur in open environments of rupestrian soils in high altitude areas in Cerrado and field enclaves in Atlantic Forest. They use riparian and shrub vegetation along temporary streams, and, therefore, their conservation is strongly linked to the preservation of these sensitive and rare environments in the Cerrado landscape. Herein, we aim to explore the development of a practical protocol combining predictive distribution models, images from Google Earth™ (GE) tool, and natural history information to increase the records of *P. ayeaye* and *P. oreades* (Anura, Phyllomedusidae). First, we prepared potential occurrence maps based on historical records, aiming to predict new locations of high suitability. Subsequently, we used high-resolution GE spatial images to select first-order streams and creeks with potential species occurrence. Finally, we validated the model in the field. The individuals found were collected for molecular identification from the comparison of mitochondrial Cyt-b fragments. Following this protocol, we obtained 39 new records for the species (24 for *P. ayeaye* and 15 for *P. oreades*, being three sympatric localities). From the incorporation of sequences from 204 individuals, we generated the phylogeny with the new individuals collected. The accuracy of the predictive model was high for *P. ayeaye* and *P. oreades* (AUC=0.935 and AUC=0.923, respectively). Understanding the distribution and its relatedness between them is the basis for strategies aimed conservation, even with species that have complex taxonomy, without clear patterns of spatial distribution and overlapping geographic boundaries in some regions, as in the case of these two Pithecopus. We conclude the protocol for

selecting new locations was exceptionally satisfactory for predicting the occurrence of these species in new localities.

Introdução Geral

Modelos preditivos de distribuição

Os modelos preditivos de distribuição de espécies (*species distribution modeling – SDM*), também chamados de modelo de nicho ecológico *(ecologial niche modeling – ENM*) ou modelos de envelope bioclimático (*bioclimatic envelope modeling – BEM*), são amplamente utilizados para predizer a distribuição das espécies através de modelos probabilísticos (Peterson et al., 2011; Qiao et al., 2016; Svenning et al., 2011). A ampla utilização desses modelos nos dias de hoje foi possível pela formação de bancos de dados informatizados de coleções biológicas, o acesso de tecnologias do Sistema de Informações Geográficas (GIS) e o desenvolvimento de modelos bioclimáticos (Booth et al., 2014; Colwell & Rangel, 2009). Tais modelos baseiam-se na premissa de que a distribuição de uma espécie é resultado do efeito de fatores abióticos (e.g. temperatura, umidade, altitude), bióticos (interações) e capacidade de dispersão atuando em conjunto sobre os indivíduos (Pearson et al., 2007; Smith & Boyer, 2012), premissa também conhecida como “B.A.M.” (veja Soberón, 2007).

Esses modelos possuem várias aplicações, como estimar a distribuição das espécies no passado, presente e futuro (Giannini et al., 2012; Guisan & Thuiller, 2005; Svenning et al., 2011) e entender padrões gerais de distribuição das espécies (Lemes et al., 2011; Pearson et al., 2007; Raxworthy et al., 2003). Conhecer a distribuição geográfica das espécies é de grande importância, pois permite compreender, quantificar e conservar a biodiversidade do planeta, detectando riqueza de espécies, hotspots, focos de endemismos e outros padrões biogeográficos (Raxworthy et al., 2003). As técnicas de modelagem possuem como base modelos estatísticos de distribuição que, através de programas computacionais, utilizam variáveis bioclimáticas características dos registros já existentes para prever a possível ocorrência de espécies em determinadas áreas. Desde então, modelos de nicho ecológico vêm sendo utilizados para estimar a distribuição geográfica de espécies, seja potencial ou real e, para isso, é necessário conhecer os fatores que delimitam seu nicho ecológico (Peterson et al., 2011; Qiao et al., 2016).

O nicho ecológico é um conceito muito discutido ao longo dos anos e, de acordo com Elton (conceito Eltoniano), o nicho está intimamente ligado às necessidades ecológicas e aos recursos que uma espécie consome (Tilman, 1982; Whittaker et al., 1973). Grinnell por sua vez (conceito Grinnelliano) infere que o nicho é dado pela distribuição das espécies considerando barreiras físicas e climáticas, desconsiderando relações bióticas (Whittaker et al., 1973). G. Evelyn Hutchinson, em 1957, consolidou a teoria de nicho ecológico e propôs a ideia do nicho *n*-dimensional, no qual a espécie é capaz de sobreviver, crescer e reproduzir, tendo uma relação direta com o espaço geográfico (Colwell & Rangel, 2009; Hutchinson, 1957; Hutchinson, 1959; Hutchinson, 1978; Whittaker et al., 1973). A escolha do conceito depende da pergunta/hipótese do seu trabalho. No entanto, há um grande desafio em transformar um conceito abstrato (nicho ecológico) em representação palpável (distribuição geográfica representada em mapas através da amostragem, presença/ausência de registros e resolução) (Soberón, 2007).

A premissa central dos modelos preditivos é que a distribuição inferida de uma espécie fornece informações sobre os requisitos ambientais aos quais a espécie está associada (Pearson & Dawson, 2003). No entanto, a capacidade dos modelos em descrever todas as condições que permitam a persistência da espécie (chamado de nicho fundamental) está intimamente relacionada ao quanto as dimensões ambientais examinadas realmente definem os limites dessa distribuição através das interações bióticas, abióticas e capacidade de dispersão (chamado de nicho realizado) (Hutchinson, 1957; Hutchinson, 1978; Pearson et al., 2007). Logo, nem todos os locais indicados pelos modelos preditivos realmente apresentam as condições reais adequadas para a presença da espécie (Anderson et al., 2002; Svenning & Skov, 2004).

Para trabalhar com biogeografia, é preciso ponderar que modelos preditivos de distribuição não incorporam o efeito das interações ecológicas ou características ambientais em fina escala. No entanto, apesar de cada ponto no biótipo corresponder a um ponto no espaço de nicho, um único espaço de nicho pode corresponder a muitos biótipos (Colwell & Rangel, 2009; Hutchinson, 1978). Portanto, é possível extrapolar as características ambientais para além dos locais amostrados e conhecidos de ocorrência e fazer previsões mais acuradas (Colwell & Rangel, 2009; Hutchinson, 1978).

A partir do conhecimento dos registros e história natural da espécie, técnicas de modelagem de nicho têm várias aplicações diante da necessidade de respostas fundamentadas para a perda de habitat, as mudanças climáticas e o impacto gerado pelo uso de recursos naturais pelo ser humano, entre outras (Giannini et al., 2012; Peterson et al., 2011). Através dessas respostas, podemos obter áreas de alta adequabilidade de distribuição (Lemes et al., 2011; Giannini et al., 2012; Peterson et al., 2011; De Siqueira & Durigan, 2007), que apoiam diversas atividades de manejo e conservação de espécies, principalmente as raras, ameaçadas ou de distribuição restrita (Engler et al., 2004), reintrodução de espécies ou para o manejo de espécies exóticas (Hirzel et al., 2002; Peterson et al., 2003; Peterson & Robins, 2003).

Além dessas aplicações na Biologia da Conservação, modelos preditivos têm sido utilizados também na filogeografia, comparando padrões de distribuição com relações de parentescos. A biogeografia usa evidências históricas para entender o atual *status* das espécies e sua distribuição, relacionando eventos relevantes para compreender as relações de parentescos entre as espécies no planeta. Desta forma, é possível formular hipóteses integrativas como distribuição geográfica, relações filogenéticas e estimativas da época e idade de divergência entre linhagens existentes e extintas (Costa et a., 2008; Fonseca, 2018; Giannini et al., 2012; Peterson et al., 2000).

Genética como ferramenta na identificação das espécies

Ordenar, classificar e entender padrões da natureza parece ser uma das maiores preocupações da mente humana, seja no presente, no passado e, certamente o será no futuro. Sempre foi confortável ao pensamento humano categorizar as coisas. Aristóteles, que propôs diversos sistemas de classificação, afirmou que “a ordem do pensar reflete a ordem do mundo” (Aristóteles, trad.

2006). A Taxonomia e a Sistemática são produtos desse pensar humano sobre padrões naturais, e mais recentemente, a compreensão dos padrões filogeográficos e as tentativas de desvendar a diversidade do planeta (Dubois, 2003; Larsen et al., 2017; Rivera-Correa et al., 2021; Vogel et al., 2017).

Comparações de caracteres morfológicos (fenótipo), como resultado da manifestação dos genes (genótipo), ainda têm sido utilizadas como metodologia primária para delimitação de espécies. Porém, estudos atuais vêm usando sequências de DNA como importante ferramenta para taxonomia, distribuição geográfica e conservação, o que contribui para o entendimento da história evolutiva das populações de maneira integrativa (Avise, 2009; Fujita et al., 2012).

Análises moleculares auxiliam a identificação de espécies distribuídas em amplas escalas espaciais, bem como suas relações evolutivas. Para isso, é preciso entender padrões de ancestralidade comum e classificar os seres vivos para acessar as relações de parentesco entre os organismos e agrupá-los ou ordená-los em categorias com características previamente definidas (Avise, 2009; Fujita et al., 2012; Vogel et al., 2017). Diante de tais necessidades, Hebert et al. (2003) propôs o sistema de identificação molecular por *Barcode* genético (DNA *barcoding*), tratando sequências específicas do DNA mitocondrial como “bioindicador”, semelhante aos códigos de barras universais, e o uso de *Barcode* vem sendo amplamente utilizada para identificação das espécies (Hebert et al., 2003; Valentini et al., 2009). Nesse contexto, análise do DNA mitocondrial tem se tornado uma forte ferramenta para estudos evolutivos, ajudando a compreensão sobre estruturas populacionais, fluxo gênico, hibridização, biogeográfica e relações filogenéticas (Pearson et al., 2000; Moritz et al., 1987).

Para identificar regiões específicas do DNA são utilizados marcadores moleculares. Esses marcadores são sequências específicas no DNA que caracteriza diferentes indivíduos (Nadeem et al., 2018). Os marcadores moleculares são identificados pela variação nos nucleotídeos (polimorfia), são herdados geneticamente e não são influenciados pelo ambiente, logo, sua descoberta gerou grandes avanços nos estudos moleculares (Nadeem et al., 2018; Valentini et al., 2009).

Existem diferentes tipos de marcadores moleculares, porém o que irá determinar a escolha do marcador será o tipo de estudos, aplicações e recursos. O gene Citocromo b (Cyt-b) é amplamente escolhido para estudos de vertebrados por ser um marcador mitocondrial conservado e é comumente utilizado para estudos populacionais e intraespecíficos (Meyer, 1994; Pearson et al., 2000). Os níveis de divergência genética associada às espécies estão em uma faixa na qual o gene Cyt-b é filogeneticamente informativo, sendo possível fazer comparações de sequências homólogas de DNA (Johns & Avise, 1998; Pearson et al., 2000).

A filogeografia como disciplina formal começou com a introdução de análises de DNA mitocondrial à genética de populações, o que permitiu a construção de árvores genealógicas em combinação com informações sobre a distribuição geográfica das populações (Avise, 2009; Avise et al., 1998). Dada esta ligação entre a geografia e a genética populacional, a filogeografia pode investigar a relação entre os processos micro (genético) e macro evolutivos (Avise, 2009), como distribuição espacial das espécies.

A dispersão dinâmica de organismos vivos pelo espaço e tempo, através de processos como especiações, migrações e extinções, é amplamente estudada pela biogeografia histórica (Wiens & Donoghue, 2004), a qual visa entender como processos ecológicos do passado atuam no padrão atual da distribuição das espécies. Em combinação com análises filogenéticas, podem ser realizadas investigações sobre a evolução da paisagem e sua influência na evolução dos organismos (Bermingham & Moritz, 1998), permitindo avaliar processos relacionados às taxas de especiação, ao fluxo gênico, à radiação adaptativa e a eventos de extinção de linhagens.

A compreensão de tais questões se inicia na identificação e delimitação das espécies, sendo espécie tratada como unidade básica evolutiva da classificação biológica (Aleixo, 2009; Coyne, 1994; De Queiroz, 1998; De Queiroz, 2005; Mayr, 1969; Orr, 1996). Contudo, ainda há um debate atual sobre o conceito de espécie que, por muitas vezes, fomenta uma confusão entre conceituar e delimitar espécies (De Queiroz, 2007). Para isso, De Queiroz (2007) propôs um conceito unificado de espécie que visa tratar o fenômeno de sua existência como unidades evolutivas significantes (UES), cuja proposta leva em consideração o acúmulo de evidências de características únicas. Para definir uma espécie considerando essa abordagem, devemos incluir o conhecimento de isolamento reprodutivo intrínseco, monofilia, uso de diferentes hábitats, morfologia distinta, diferentes frequências alélicas, entre outros conceitos (De Queiroz, 2007). Esse acúmulo de características leva metapopulações (De Queiroz, 2005; Hanski & Gaggiotti, 2004) portadoras da matéria-prima própria (genes ou caracteres), a evoluir de forma independente no tempo presente (Magalhães et al., 2017; De Queiroz, 1998; De Queiroz, 2007).

Espécies não são definidas por conceitos excludentes e são entidades que evoluem constantemente no tempo presente, porém de diferentes formas (De Queiroz, 1998; De Queiroz, 2007). Assim, é possível compreender e identificar processos evolutivos e ecológicos responsáveis pela origem, distribuição e manutenção da biodiversidade, tópico de grande interesse na biologia evolutiva (Beheregaray & Caccone, 2007).

Nesse contexto, considerando o conceito filogenético de espécies como o menor grupo monofilético com um ancestral comum (Cracraft, 1983; De Queiroz & Donoghue, 1990; De Queiroz & Gauthier, 1990), as espécies são examinadas não apenas enquanto grupo monofilético, mas como unidades evolutivas significantes (UES) (Magalhães et al., 2017; De Queiroz, 2007). Portanto, o conhecimento acerca dos limites populacionais dessas UES é crucial para o entendimento dos processos ecológicos e evolutivos relevantes para a conservação da biodiversidade.

Uma das aplicações do sequenciamento de DNA é a delimitação de espécies crípticas, que são aquelas com duas ou mais espécies distintas comumente classificadas como uma única espécie, dada a morfologia semelhante (Bickford et al., 2007; Bragg et al., 2016). A história evolutiva das espécies não é medida diretamente, mas estimada usando genealogias inferidas para *loci* particulares (Bragg et al., 2016), considerando espécies como hipóteses que podem ser testadas. Porém, existem questões a serem resolvidas sobre as espécies crípticas, como se elas são mais comuns em determinados habitats, latitudes ou grupos taxonômicos (Bickford et al., 2007).

Todas essas técnicas aliadas ajudam a revelar limites geográficos e genealógicos de populações, principalmente de espécies crípticas (Ramos et al., 2018) e para espécies com distribuição restrita. Esses avanços também dão embasamento teórico e prático para trabalharmos com questões importantes em teorias evolutivas, biogeográficas ou para a conservação da biodiversidade (Bickford et al., 2007; Magalhães et al., 2017). As pererecas *Pithecopus ayeaye* e *Pithecopus oreades* são exemplos de espécies com distribuição ampla no Cerrado, porém restritas a ambientes abertos e rupestres de altitude (Borges, 2018; Brandão, 2002; Brandão et al., 2009; Frost, 2021; Oliveira, 2007), sendo boas espécies-alvo para testar hipóteses integrativas.

Pithecopus ayeaye Lutz, 1966 e Pithecopus oreades (Brandão, 2002)

O gênero *Pithecopus* Cope, 1866 é um grupo Neotropical de pererecas caracterizado por ter polegares opositores, com especializações na morfologia, na reprodução e no uso de hábitat e que divergiu recentemente (há aproximadamente 17.1 milhões de anos (14.8-19.3 m.a.) do gênero irmão *Callimedusa* (Caramaschi, 2006; Duellman et al., 2016). Tais pererecas, como diversas outras da família Phyllomedusidae, se movem por marcha em ritmo lento e, ao escalarem, se seguram com os dedos oponíveis e não por fricção ou aderência, como a maioria dos anuros costumam fazer (Brandão, 2002; Lutz, 1966; Oliveira, 2007).

Essa modificação no caminhar e os padrões de desenhos reticulados escuros nos flancos e coxas sobre a coloração de fundo variando entre laranja e vermelho (Caramaschi, 2006; Duellman et al., 2016; Faivovich et al., 2010), conferem a elas o nome popular de pererecas-macaco-de-flancos-reticulados (Lutz, 1966). O nome *Pithecopus* deriva do grego *pithekodes*, e significa “semelhante a um macaco” (Duellman et al., 2016). *Pithecopus ayeaye* e *P. oreades* são pererecas arborícolas com ocorrência restrita a formações campestres em áreas de maior altitude (geralmente acima de 800m), sendo *P. ayeaye* conhecida para a região sudeste do Cerrado e enclaves de campos da Mata Atlântica (Frost, 2021; Oliveira, 2007) e *P. oreades* nas encostas do

Planalto Central brasileiro (Borges, 2018; Brandão, 2002; Brandão et al., 2009; Frost, 2021).

Essas espécies utilizam vegetação ripária e arbustiva associado a riachos temporários de leito rochoso (drenagens) (Borges, 2018; Brandão & Álvares, 2009; Oliveira, 2007), essenciais para sua reprodução. Os machos vocalizam na vegetação marginal das drenagens e, durante o amplexo, os ninhos são feitos em folhas de arbustos que pendulam acima da lâmina d’água (Brandão & Álvares, 2009; Oliveira, 2007). Possuem reprodução prolongada, na qual os adultos estão ativos nos três primeiros meses de chuva (Brandão & Álvares, 2009; Magalhães et al., 2017; Oliveira, 2007). O pico de reprodução é observado especialmente com a formação de poços dentro das drenagens, nos quais os girinos, ao eclodirem, podem escorrer pela folha e cair na coluna d’água (Brandão & Álvares, 2009; Oliveira, 2007).

As principais características diagnósticas dessas duas espécies são o porte pequeno, a cabeça curta e estreita, porém com paratóides distintas (Brandão, 2002; Lutz, 1966). Possuem dorso liso geralmente verde, que varia do verde-limão ao verde-escuro, mas pode apresentar tons arroxeados, e partes ocultas dos flancos e coxas com padrão reticulado preto sobre fundo laranja a vermelho (Brandão, 2002; Lutz, 1966) (Figura 1).

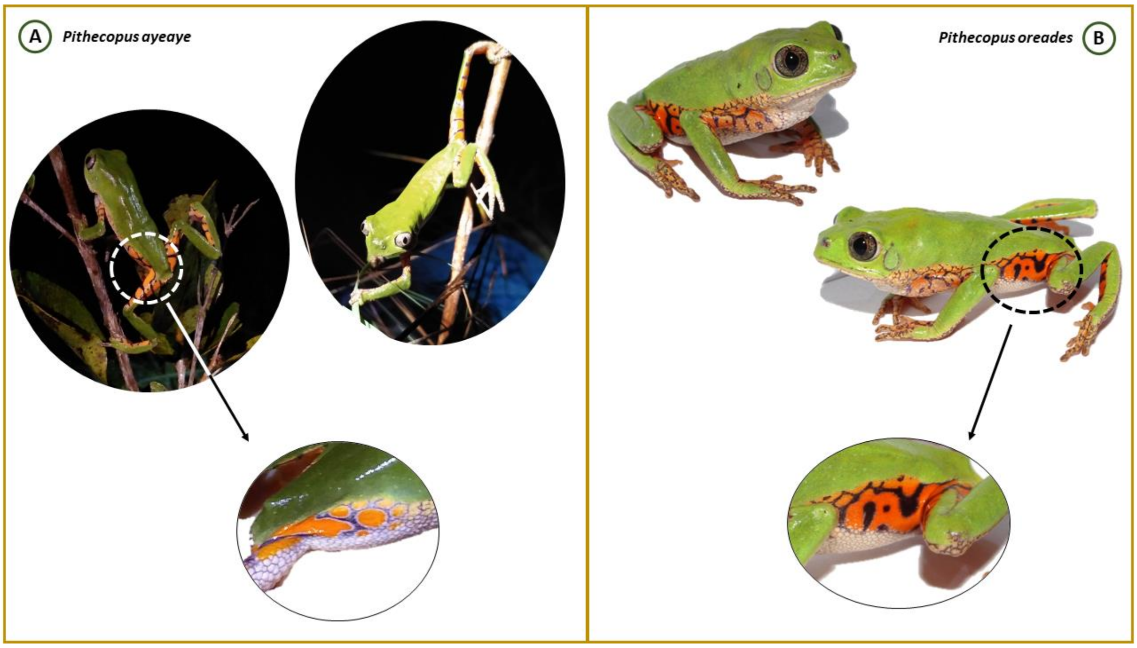

Figura 1. A: Indivíduo adulto de *Pithecopus ayeaye* da Serra de Andrequicé - MG; B: Indivíduo adulto de *Pithecopus oreades* de Patrocínio - MG. Fotos: Ana Cecilia Holler.

Essas espécies de *Pithecopus* são restritas a áreas abertas e fitofisionomias campestres em altitudes acima de 800 metros e, por isso, sua conservação está ligada fortemente à preservação desses ambientes sensíveis e raros na paisagem do Cerrado (Brandão & Álvares, 2009; Magalhães et al., 2017). Além disso, *Pithecopus ayeaye* está classificada como CR (“Critically Endangered”) de acordo com a *Red List* da IUCN (Caramaschi, 2016) e como LC (“Least Concern”) no Livro Vermelho da Fauna Ameaçada de Extinção, (ICMBIO/MMA, 2018). *Pithecopus oreades* está classificada como DD (“Data Deficient”) de acordo com a *Red List* da IUCN (Silvano, 2016) e como LC (“Least Concern”) no Livro Vermelho da Fauna Ameaçada de Extinção, (ICMBIO/MMA, 2018). Desta forma, há incongruências das categorias de amaças entre as listas mundial (IUCN) e nacional (ICMBio).

Nesse contexto, é importante reavaliar o status de conservação dessas duas espécies, especialmente através do encontro de novas populações, na delimitação da distribuição geográfica das mesmas e na identificação de ameaças locais (Magalhães et al., 2018; Magalhães et al., 2020).

Objetivo Geral

Desenvolver um protocolo de localização de novas populações de espécies de anfíbios hábitat especialistas através da combinação de modelos preditivos de distribuição, ferramenta espacial (Google Earth^{TM} - GE), ferramentas moleculares e conhecimento de história natural. Para isso, iremos usar as espécies *Pithecopus ayeaye* e *P. oreades* (Anura, Phyllomedusidae) como espécies-modelo para testar nosso protocolo.

Objetivos Específicos

1. Compilar a distribuição conhecida das espécies e reproduzir modelos preditivos de distribuição associadas às variáveis ambientais locais para identificar áreas de adequabilidade;
2. sobrepor os modelos preditivos a imagens de satélite de alta resolução espacial (GE) para identificar ambientes com características físicas específicas;
3. validar o protocolo através de expedições a campo com a localização de novas populações e comparar a proporção de acertos (presença vs. ausência de registro) com a aplicação do método;
4. Refinar a identificação das amostras das populações através de ferramentas moleculares que identificam fragmentos mitocondriais;
5. inferir o limite de distribuição das duas espécies e refinar os critérios de ameaça da IUCN para eventual revisão do *status* dessas espécies.

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Capítulo 1 – Combining distribution modeling, landscape imaging, barcoding, and natural history for improving records of the habitat specialist frogs *Pithecopus* ayeaye and *Pithecopus oreades*

Capítulo a ser submetido à revista Journal for Nature Conservation (https://www.journals.elsevier.com/journal-for-nature-conservation), que possui fator de impacto 2.482 e Qualis A2 (Qualis nova avaliação).

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Combining distribution modeling, landscape imaging, barcoding, and natural history for improving records of the habitat specialist frogs *Pithecopus* ayeaye and *Pithecopus oreades*

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Abstract

Unveiling the limits of species geographic distribution is essential for a better understanding of potential threats and extinction risks associated with populations. It is possible to use q predictive model of distribution to make more accurate projection of species geographic mapping by associating biotic and abiotic factors and the interactions that species establish with each other and with the environment. Herein, we propose a practical protocol for selecting locations of potential occurrence for habitat specialist species, combining predictive models of distribution, Google Earth™ (GE) tools, and natural history data to improve the new populations records of *Pithecopus ayeaye* and *P. oreades* (Anura, Phyllomedusidae). We firstly associated spatial distribution records with bioclimatic variables aiming to predict new locations of high suitability. Posteriorly, we used high-resolution GE spatial images to select potential reproductive sites of the species (i.e, first-order rocky streams) in open fields, placed within predicted areas with higher climatic suitability and, finally, we validated the model in the field. The individuals found were collected for molecular identification from the comparison of mitochondrial Cyt-b fragments. Following this protocol, we obtained 37 new records for the species. From the incorporation of sequences from 204 individuals, we generated the phylogeny with the new individuals collected. The accuracy of the predictive model was high for *P. ayeaye* and *P. oreades* (AUC=0.935 and AUC=0.923, respectively). Understanding the distribution and its relatedness is the basis for strategies aiming conservation, even with species that have complex taxonomy, without clear patterns of spatial distribution and overlapping geographic. However, for species conservation, it is important to unveil their boundaries and geographic distribution. These data are basilar for threatened species category classification since there are inconsistencies in the national and IUCN Red Lists for these species. Thereby, we conclude the protocol for selecting new locations was exceptionally satisfactory for predicting the occurrence of these species.

Keywords: Anura, conservation, *campo rupestre*, modeling, Phyllomedusidae, rocky streams

1. Introduction

The understanding of biogeographic patterns based on phylogeography in association of species diversity, dispersion, and extinction through time has been a great challenge. Concurrently, cartographic modeling using statistical techniques became an important tool in determine the species distribution, with the advantage of permitting the inference of past, present, and future distribution (Giannini et al., 2012; Guisan & Thuiller, 2005; Lemes et al., 2011; Pearson et al., 2007; Peterson et al., 2011; Raxworthy et al., 2003). It has also been used to infer unsampled areas with potential for occurrence of species (Lemes et al., 2011; Pearson et al., 2007; Qiao et al., 2016). The wide use of these models is possible by the computerized databases of biological collections, the access of Geographic Information System (GIS) technologies and the development of bioclimatic models (Booth et al., 2014; Colwell & Rangel, 2009).

By using ecological niche modeling, it is possible to map their preferred habitats and accessed the species distribution (Lemes et al., 2011; Pearson et al., 2007; Sales et al., 2021; Smith & Boyer, 2012), including ecological complexity involved in species distribution and habitat use (Smith & Boyer, 2012). To understand the species geographic distribution, species records are associated with environment variables to predict where they can potentially occur in addition to the known localities (Giannini et al., 2012; Guisan & Thuiller, 2005; Pearson et al., 2007; Smith & Boyer, 2012; Svenning et al., 2011). However, the practical applications use of these models is a challenge especially for unknown tropical landscapes with scarce distribution data, cryptic species and species that are difficult to detect and/or with distribution often limited to small samples from observed locations (Pearson et al., 2007; Qiao et al., 2016; Raxworthy et al., 2003).

Understanding the dynamic processes of populations or communities under extinctions risks of extinctions demands knowledge of several factors such as habitat loss, climatic changes, and other impacts caused by human actions (Giannini et al., 2012). By identifying areas of high suitability of distribution of some species using predictive models (Giannini et al., 2012; Peterson et al., 2011; Siqueira & Durigan, 2007) we can improve conservation plans (Raxworthy et al., 2003; Smith & Boyer, 2012) specially for rare, threatened, or distribution restricted species (Engler et al., 2004). However, to improve the efficiency of predictive models, it is necessary to add data on the natural history of the target species and how they interact between themselves and with the environment in which they live (Silva & Alves-Silva, 2013).

These predictive models, also known as Ecological Niche Modeling (ENM), use probabilistic models that project species distributions (Peterson et al., 2011; Qiao et al., 2016; Svenning et al., 2011). Such models are based on the premise that the distribution of a species is the result of the effect of abiotic factors (e.g. temperature, humidity, altitude), biotic (interactions) and dispersal capacity acting together on individual organisms (Pearson et al., 2007; Sales et al., 2021; Smith & Boyer, 2012). This premise is also known as "B.A.M." (see Soberón, 2007). So, to improve our understanding of a species distribution and its ecological/environment demands we need to know the attributes associated with its ecological niche (Peterson et al., 2011). Applications of Hutchinson's *n*-dimensional niche concept are often focused on the role of interspecific competition in the species distribution patterns, in which the species is capable to survive, grow, and reproduce, often having a strict relation to geographic space and available resources (Colwell & Rangel, 2009; Hutchinson, 1978; Whittaker et al., 1973).

The central premise of predictive models is that the observed distribution of a species can provide useful information regarding the environmental requirements to which that species is associated (Pearson & Dawson, 2003). However, the fundamental niche described in those models is closely related to the environmental dimensions used to define the limits of that distribution (called realized niche) (Hutchinson, 1957; Hutchinson, 1978). So, it is recommended to be careful with the high suitability locations indicated by the models, because predictive models do not consider ecological relationships (Anderson et al., 2002; Svenning & Skov, 2004).

To complement such predictive models, the use of the Google Earth™ (GE) tool has shown itself promising (Bird Life International, 2009; Butler, 2005; Folco et al., 2019; Garcia-Milagros & Funk, 2010). Also, it creates the possibility of investigating the species geographic distribution based on a specific type of habitat (Silva & Alves-Silva, 2013) and testing phylogeographic hypothesis. In GE, we can look for places and directions with useful geographical tools and images with an exceptional spatial resolution (Silva & Alves-Silva, 2013). It also enabled us to save personal and financial resources for a future field trip in the previously identified localities. Furthermore, by being free, it can be largely used in low-income municipallity, often those that harbor most of the world's biodiversity.

Finally, we can bring together predictive models and GE tools with the natural history of selected species to improve the finding of new populations. *Pithecopus ayeaye* Lutz, 1966 and *P. oreades* (Brandão, 2002) are two Phyllomedusidae monkey frogs with specialized morphology, reproduction, and habitat use (Duellman et al., 2016). These species are endemics to the Cerrado biome, occurring only in mountain ranges (above 800m a.s.l.), using only small streams and its marginal vegetation for reproduction (Brandão et al., 2009; Borges, 2018). In addition, these *Pithecopus* are active only in the first three months of the rainy season, spawning in leaves that hang above the water (Brandão & Álvares, 2009; Magalhães et al., 2017; Oliveira, 2007).

The relation between these *Pithecopus* and the open and rupestrian phytophysiognomies in highlands makes its conservation strongly linked to the preservation of these sensitive and rare environments in the Cerrado dominion (Brandão & Álvares, 2009; Magalhães et al., 2017). Furthermore, *Pithecopus ayeaye* is classified as CR ("Critically Endangered") according to the IUCN Red List (Caramaschi, 2016) and as LC ("Least Concern") in the Red Book of Endangered Fauna, (ICMBIO/MMA, 2018). *Pithecopus oreades* is classified as DD ("Data Deficient") according to the IUCN Red List (Silvano, 2016) and as LC ("Least Concern") in the Red Book of Fauna Threatened with Extinction, (ICMBIO/MMA, 2018). Thus, there are inconsistencies in the categorization between the world (IUCN) and national (ICMBio) lists.

Knowledge about species distribution may influence the categorization of a given species conservation status. Faced with the uncertainty about where species occur, an inference about such distribution is usually used (Lemes et al., 2011), especially when used in red lists, since most species are categorized by geographic criteria. In general, the distribution application on conservation has some bottleneck for most species, based on gaps in knowledge about their distribution, knowing as Wallacean deficit (Lomolino, 2004; Whittaker et al., 2005). In this context, it is important to evaluate the conservation status of these two *Pithecopus*, especially through new records from field improving its distribution limits and identifying local threats.

Herein, we propose a practical protocol for selecting locations of potential occurrence for habitat specialist species, combining predictive models of distribution, Google Earth^{TM} (GE) tools, and natural history data to improve the record of new occurrence sites. We tested the protocol by searching for new sites where samples of two reticulated monkey tree-frogs *Pithecopus ayeaye* and *P. oreades* (Anura, Phyllomedusidae) could be found.

2. Material and methods

2.1. Sampling site selection

First, we surveyed all geographical distribution records for *Pithecopus ayeaye* and *P. oreades* available in scientific collections and in the literature. Then, we modeled their geographical distribution using predictive models of distribution to predict areas with high suitability to the species presence (Figure 1).

Posteriorly, we used Google Earth (GE) to select the potential habitats of these species searching for high altitude regions with geomorphological traits characteristics for the species occurrence. First, we made a cutout above 800 m a.s.l. and, inside this cut, we made a screening to determine some characteristics that described the rocky field environment. To do that, we prioritized the search for moderate slope, grassy soil coverage, and the presence of first-order streams with shrub vegetation as the main creeks in the landscape (Figure 1). This step was made adapting the methodology described by Silva & Alves-Silva (2013).

Finally, to validate the model, we made eight field expeditions to the altitude rocky fields indicated by the combination of our predictive models of distribution plus GE tools in three Brazilian states, Minas Gerais, Goiás, and Distrito Federal (Figure 1). These locations were sampled from October of 2019 to February of 2020, during the Cerrado rainy season, when these tree-frogs show their activity peak, especially during the first three months of the rainy season (Brandão & Álvares, 2009). Some individuals of these populations were collected for later identification based on Cyt-b sequencing.

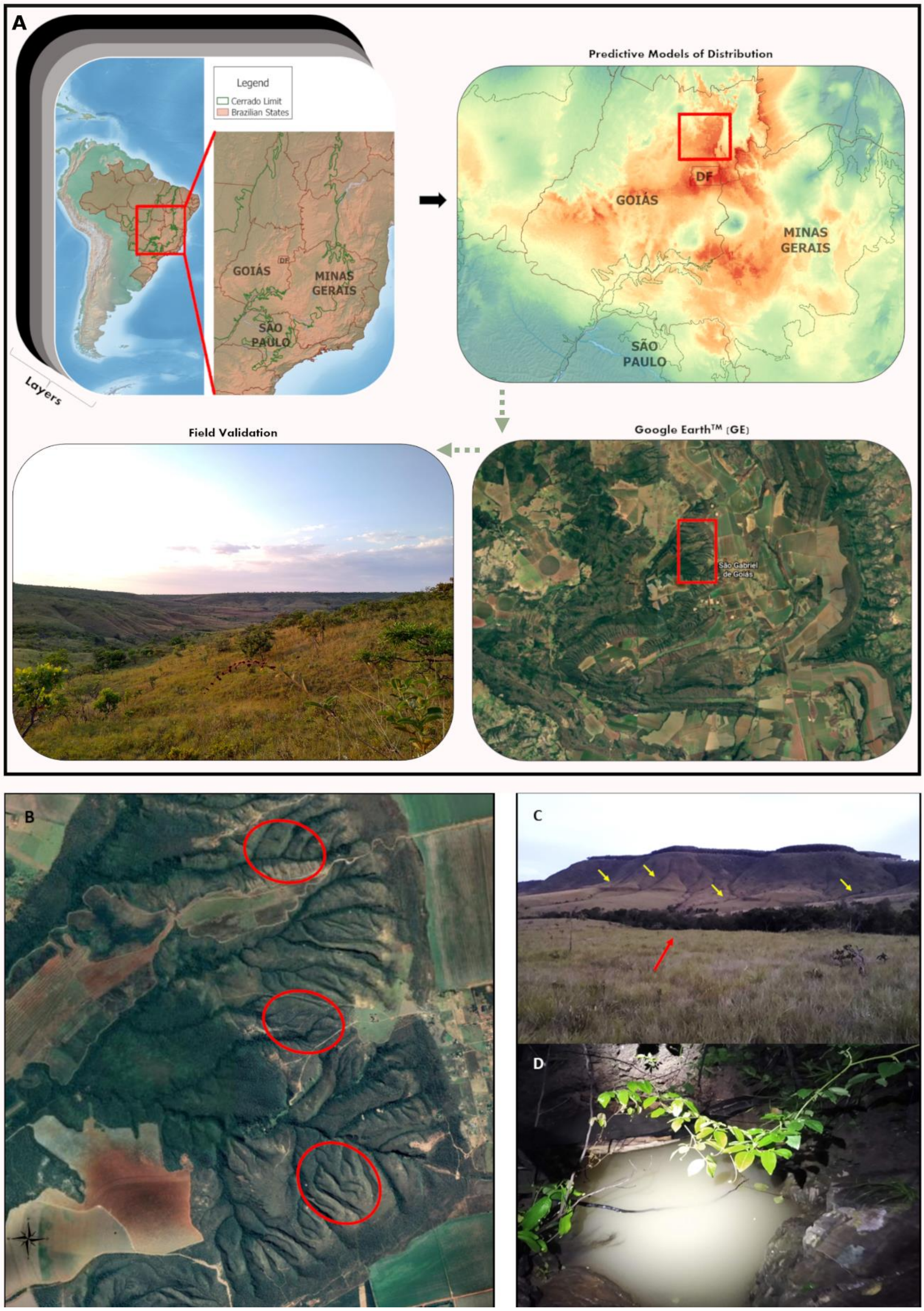

Figure 1. A: Steps of the methodology for selection of potential occurrence areas of *Pithecopus ayeaye* and *Pithecopus oreades*. “Layers” represents the selected variables bioclimatic overlapping; B: First-degree rocky stream identified on GE tool; C: Creeks in altitude rocky stream

habitat. The red circles correspond to regions magnified in the subsequential scale of analysis. The red arrows correspond to the mains creeks (formation of gallery forests along a perennial stream) and the yellow arrows correspond to the slope creeks, breeding habitat used by *Pithecopus ayeaye* and *Pithecopus oreades*; D: Characteristic vegetation of the reproduction sites of *Pithecopus ayeaye* and *Pithecopus oreades*.

2.2. C*ampo rupestre* (Open altitude highlands)

In some mountain tops in the Cerrado biome, there is a unique formation of mosaic vegetation with extremely interaction between biotic and abiotic factors, causing high diversity and endemism (Antonelli et al., 2018; Magalhães et al., 2021; Miola et al, 2021; Perrigo et al., 2020). This environment is of extreme importance for the local biodiversity, its distribution, and its maintenance. The ground is covered by grasses and plants growing on rocks, associated with shallow and nutrient-poor soils (Körner et al., 2017; Morellato e Silveira, 2018; Perrigo et al., 2020; Silveira *et al*., 2016). In general, the *campo rupestre* (or rupicolous) phytophisiognomie, is an ecoregion defined by the WWF (World Wide Fund for Nature) that occurs mainly above 900m a.s.l., often with outcrops rocky of quartzite, sandstone, and/or iron rich terrains (Vasconcelos, 2011).

Considered by some researchers as one of the oldst geologic and biologic environments on the planet (Miola et al., 2021; Silveira *et al*., 2019), more than 300 Ma in the past, these mountains used to reach thousands of meters high that, over the years, have been eroded and lowered till the current conformation (Antonelli et al., 2018; Ross, 1995). The great biodiversity of *campos rupestres* were adapting to the conditions and the isolation in mountain top, its characteristic relief and weather (Miola et al., 2021; Silveira *et al*., 2019), as they face drastic variations in temperature, water restriction and intense solar radiation (Körner, 2004; Pepin *et al*., 2015). Even in the rainy season, the water rapidly drains over the stony and shallow soil, with no storage of groundwater (Miola et al., 2021; Silveira *et al*., 2019). Water only lasts longer in some sandy and flat places, or along creeks located on hill slopes. Along these creeks, which often correspond to temporary streams, there is the formation of deeper pools that constitute the reproductive habitat for the life cycle of *Pithecopus ayeaye* and *P. ayeaye*.

All these environmental characteristics also make the rocky streams easy to identify even using images produced by remote sensing devices (Figure 1B), making the GE an effective tool to search for these habitats. Based on these features, we visit several localities and search for the species along the creeks (Figure 1C, Figure 2). When individuals were found, we recorded the local coordinates for the new information to GE tools and use for the species distribution modeling.

Figure 2. Typical rocky field landscape of the spots visited.

2.3. Data collection

In the field we conducted the active searches as described in Heyer et al. (2014), seeking out vocalizing males through the creeks and searching for individuals in every development stages. We searched both in the vegetation bordering the creeks, as well as from inside them, prioritizing places where the formation of pools occurred, forming the habitat used for laying eggs and where tadpoles thrive. We registered the points coordinate using a mobile applicative

Alpine-Quest (http://alpinequest.net/) and classifying the locations as “record” and “no record”.

The sampling effort was based on the number of individuals sampled per point. The tadpole has diurnal activity being sought between afternoon and twilight (using hand net) and the adults has nocturnal activity being sought at night. The searches were conduct till we collected five individuals per locality or until the adults stop vocalizing. For tadpoles, we were careful to select the individuals at the most distant stages of development (see Gosner, 1960) and in different drainages to avoid close kinship. These species tadpoles have an elongated and trapezoidal shape, tail narrow with an elongated and acute tip, large eyes and dorsolaterally placed, small and anterior mouth (Brandão, 2002; Lutz, 1966).

The adults found were weighed with a 10g Pesolla® Dynanometer and several morphometric measurements were taken with a digital caliper with 0.01mm precision (Figure 3). Subsequently, the individuals were transported to the laboratory and the tadpoles and froglets were stored separated by lots, indicating the creeks and locality of origin (Figure 4). The individuals collected will be deposited in the Herpetological Collection of Universidade de Brasília (CHUNB) and the samples were analyzed in the Biodiversity and Molecular Evolution Laboratory (LBEM) from Universidade Federal de Minas Gerais (UFMG).

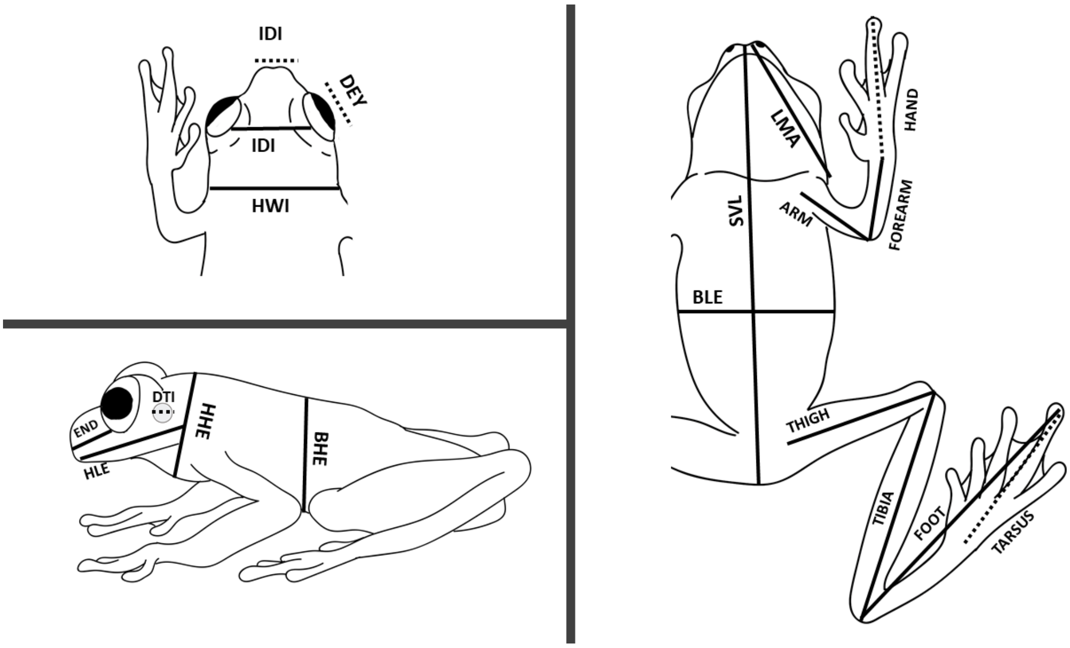

Figure 3. Morphometric measures: HWI - head width; HLE - head length (square bone to mandible); HHE – head height; IDI - internal diameter; DEY - diameter of the eye; IDI – interorbital distance; IDE - internal distance from the eardrum; END - eye-nostril distance; SVL – snout-vent length; BHE - body height; BLE - body length; LMA - length of the mandible (end of the mouth opening to the nostril). Scheme: Yuri Só.

Figure 4. Sample collected of tadpoles or froglets for molecular analysis. The red highlight indicates the muscle for different parts of the body, depending on the stage of development.

2.4. Molecular assignment

To check the specimen's taxonomy identification and assignment to a species name from new localities, sample tissues were collected from 184 individuals for 37 localities visited (Table A.1). The specimens were stored in ethanol 70% and the tissues in alcohol absolute.

The extraction of DNA from liver and muscle tissue, was made based on the protocol phenol-chloroform (adapt from de Sambrook & Russel, 2001). We generated an 896-pb Cytocrhome b (Cyt-b) fragment using the primers forward MVZ15 (GAA CTA ATG GCC CAC ACW WTA CGN AA) (Moritz et al., 1992) and reverse ARH (TAW AAG GGT CTT CTA CTG GTT G) (Goebel et al., 1999).

The DNA amplification was made by polymerase chain reactions (PCR) using the protocol described in Magalhães et al. (2017), and we use electrophoresis in 1% of agarose gel to verify the amplification quality. The amplicons were purified in 20% polyethylene glycol (PEG) (modified from Sambrook & Russel, 2001 by Santos Júnior et al., 2015). For the sequencing, we used the BigDye Terminator v3.1 kit (Life Technologies) in a sequencer ABI 3130xl genetic analyzer (Applied Biosystems).

2.5 Ecological niche modelling

Firstly, we spatially thinned 84 occurrence localities by 20 km to minimize a clustering of records due to biased sampling by using "*thin*()" available in the R package spThin (Aiello-Lammens et al., 2015). We used 19 bioclimatic variables as current candidate predictors with a spatial resolution of 0.08333° (~10km) from the global dataset of Worldclim (Fick & Hijmans, 2017). To minimize collinearity among predictors and thus avoid biased ENMs predictions, we computed a factor analysis with a varimax rotation [R package 'psych' (Revelle, 2015)] (Lima-Ribeiro et al., 2017; Terribile et al., 2012). Considering such analysis, we selected five variables out of 19 most represented in each axis: mean diurnal range, temperature seasonality, mean temperature of warmest quarter, precipitation seasonality, and precipitation of wettest quarter (see Table B.1).

The spatial distribution records were associated with environmental data to characterize environmental conditions experienced by species to predict their geographic distribution. We used the ensemble forecasting (Araújo & New, 2007) of ecological niche modelling (Peterson & Soberón, 2012), and the output models were combined to generate a single prediction. We selected four modeling methods: Bioclim, Gower distance, Maximum Entropy (MaxEnt), and Support Vector Machine (SVM). These were all implemented in the 'dismo' package of R (Hijmans et al., 2017), except SVM was from the 'kernlab' package (Karatzoglou et al., 2004). While the presence-only methods (Bioclim and Gower distance) estimate species niche-based on climatic envelope and environmental distance, the machine learning methods (MaxEnt and SVM) use presence and background data to estimate species niche with more complex relationships between response and predictor variables (Peterson et al., 2011). Because these modelling methods are based on different conceptual approaches and should capture different relationships between the occurrence data and predictors, they are essential for evaluating model uncertainty (Yates et al., 2018).

We used the area under the curve (AUC) of the receiver operating characteristic (ROC) to assess the accuracy of ecological niche models (ENMs; Fielding & Bell, 1997). A ROC curve estimates the proportion of omission and commission errors across all possible thresholds between 0 and 1 (Jiménez-Valverde, 2012). AUC values of 0.5–0.7 correspond to low accuracy, 0.7–0.9 indicate good accuracy and values above 0.9 indicate high accuracy (Sweets, 1988). The true skill statistic (TSS) is calculated as sensitivity + specificity -1 (Allouche *et al*., 2006) and ranges from -1 to +1. For each species, we generate ensemble forecasting using the output models for each species. We classified continuous predictions into presence/absence maps based on maximizing the true skill statistics (TSS, Allouche *et al*., 2006). Further, we assembled the binary maps by simple mean values for each species from single methods under current climate conditions.

2.6. Data analysis

We generated suitability results for each locality visited in RStudio (RStudio Teams, 2021) and made distribution maps using the software QGis 3.16. We used the Geospatial Conservation Assessment Tool (GeoCat - http://geocat.kew.org/) from IUCN to calculate the species extent of occurrence (EOO) and area of occupancy (AOO) for both species.

We use *Pithecopus megacephalus* (GenBank: MF171789) as and outgroup to reconstruct the phylogeny. We edited and revised the chromatograms in the program SeqScape v. 2.6.0 (Applied Biosystems), and we aligned the sequences in the MUSCLE module in AliView 1.1 (Edgar, 2004). We estimated the Cyt-b gene tree using the BEAST v.2.6.5 software (Bouckaert et al., 2019) to co-estimating the site models in *bModelTest* package through *transitionTransvertionSplit* option (Bouckaert & Drummond, 2017), and assuming a relaxed log-Normal clock model (Drummond *et al*., 2006), and a Yule tree model Birth-Death. This analysis was made with three replicates (runs), with a burn-in of 5% and 10^6 interactions.

Stationarity and convergence were made on TRACER v.1.7.1 software (Bouckaert et al., 2019). The trees of independent runs were merged using LogCombiner v.2.6.3 (Bouckaert et al., 2019) and the resultant maximum clade credibility (MCC) tree was annotated in TreeAnnotator v.2.6.3 (Bouckaert et al., 2019). Finally, we edited the MCC tree on FigTree v1.4.4 (Rambaut, 2012).

3. Results

Based on the association between the previous records of *Pithecopus ayeaye* and *P. oreades* and the bioclimatic data, we generated suitability maps for each species. The selected sites were elected from the suitability maps generated by the predictive models for field validation. These sites are associated with altitude rocky streams in open fields within the Cerrado, the environment used by *Pithecopus ayeaye* and *P. oreades*. We visited 77 localities in 26 municipallities and three states (Goiás, Distrito Federal and Minas Gerais) (Figure

5). We visited 37 localities (48% of visited localities) with *Pithecopus* records, totalizing 95 individuals collected (Table A.2).

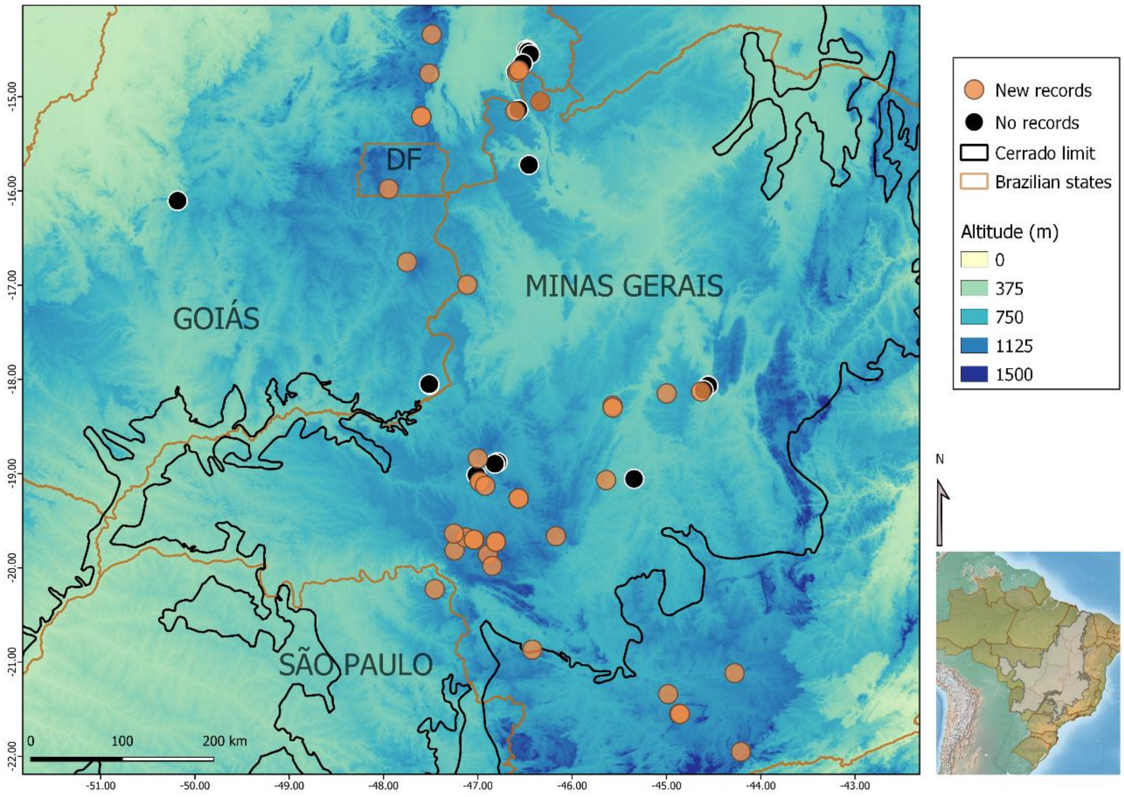

Figure 5. Visited localities (78 sites) divides between “records” (blue circles) and “no records” (black circles) of *Pithecopus*.

Based on 184 samples analyzed and through molecular analysis (Table A.1), it was possible to determine the species of the new collected individuals. The final Cyt-b gene tree included the new individuals collected of *Pithecopus ayeaye* and *P. oreades*, also recovering the monophyly of the two species (Figure 7; Figure 7; Figure C. ***1***). In the nodes, we put only the support values that presented statistical support above 95% (posterior probability).

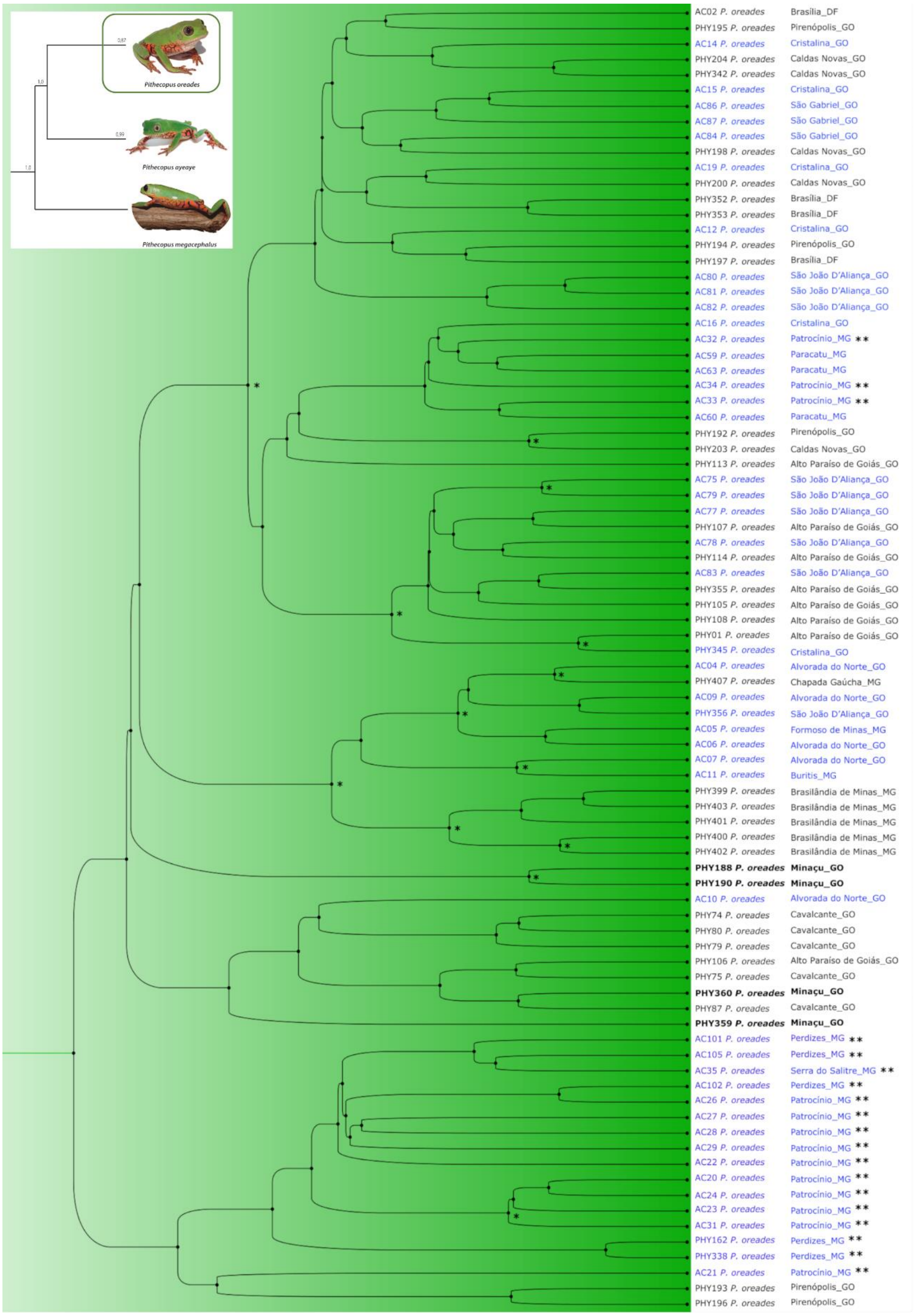

Figure 6. Best Cyt-B gene phylogenetic tree composed by 23 populations of *Pithecopus oreades*. The nodes asterisks (*) represents the statistical support equal or above 95% (posterior probability). The bold names represent the individuals collected in the type locality. The blue names represent the new sampled localities. The double asterisks (**) in the names represent a

sympatric zone (localities where were collected *Pithecopus oreades* and *Pithecopus ayeaye* in the same locality).

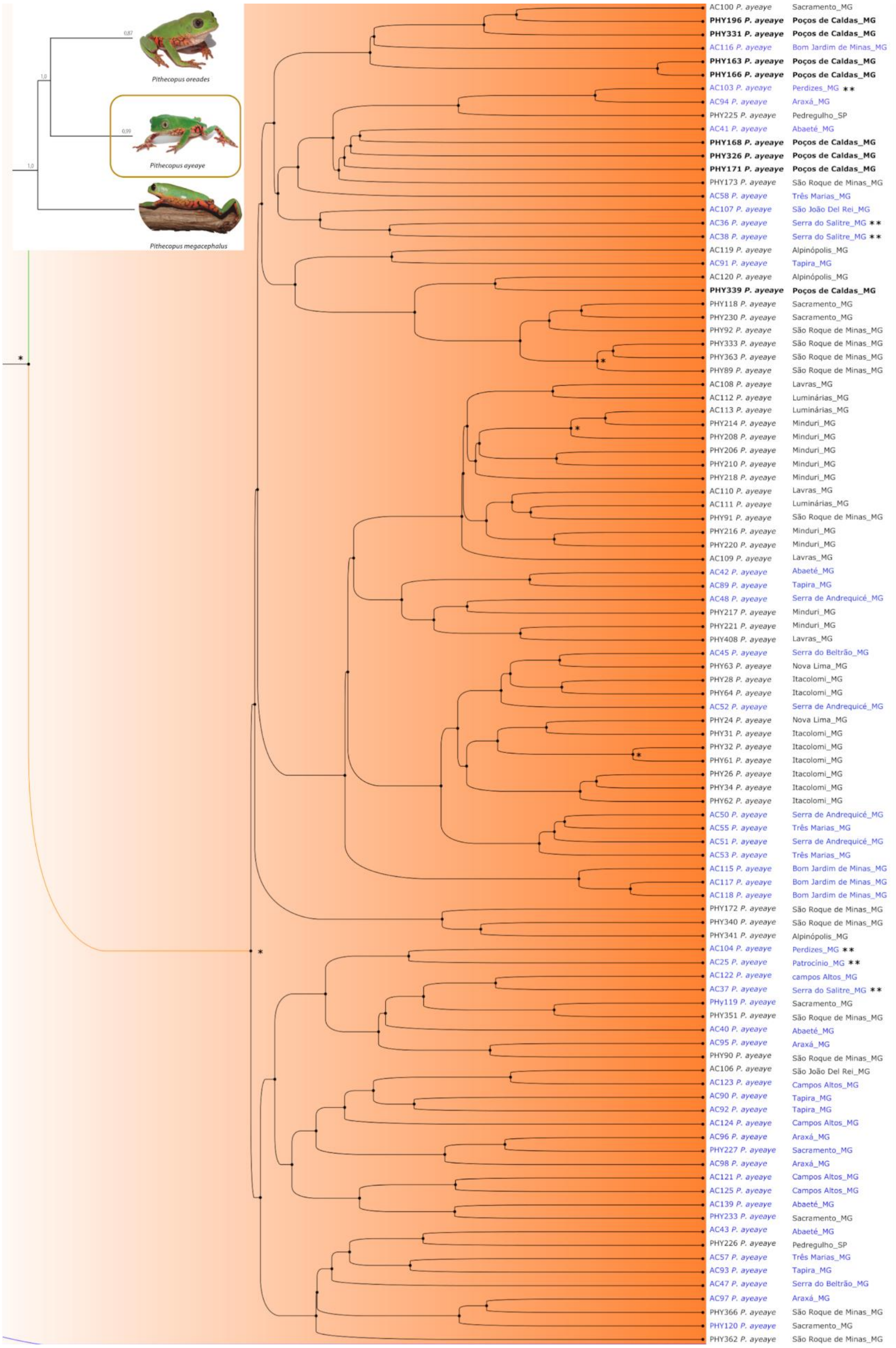

Figure 7. Best Cyt-B gene phylogenetic tree composed by 25 populations of *Pithecopus ayeaye*. The nodes asterisks (*) represents the statistical support equal or above 95% (posterior probability). The bold names represent the individuals collected in the type locality. The blue

names represent the new sampled localities. The double asterisks (**) in the names represent a sympatric zone (localities where were collected *Pithecopus oreades* and *Pithecopus ayeaye* in the same locality).

Based on the molecular assignments, we update the distribution ranges of *Pithecopus ayeaye* and *Pithecopus oreades*, containing both previously known and new records (Figure 8). We also included the localities visited but with no records.

We recorded 24 new populations of *Pithecopus ayeaye* in 11 new municipallities, all in Minas Gerais states (Abaeté, Araxá, Bom Jardim de Minas, Campos Altos, Corinto, São João del Rei, Serra do Salitre, Patrocínio, Perdizes, Tapira, and Três Marias). We recorded 13 new populations of *Pithecopus oreades* in eight municipallities (Alvorada do Norte, São Gabriel, and São João D'Aliança in state of Goiás; Buritis, Formoso de Minas, Paracatu, Patrocínio, and Serra do Salitre in state of Minas Gerais). We also observed three locations where *P. ayeaye* and *P. oreades* co-occur, evidencing a sympatric zone between these two species.

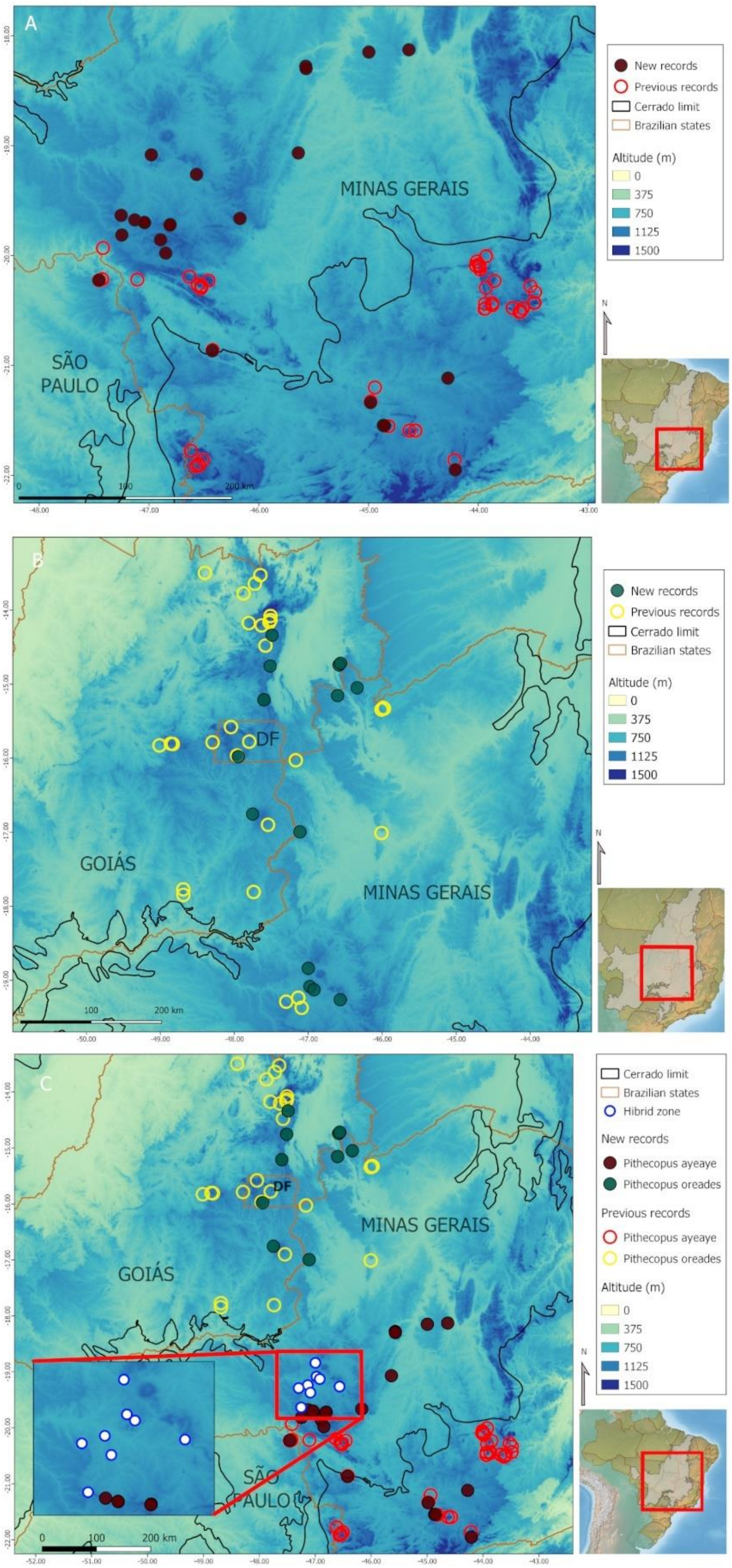

Figure 8. A: Previous and new distribution of *Pithecopus ayeaye*; B: Previous and new distribution of *Pithecopus oreades*; C: Previous and new distribution record for *Pithecopus ayeaye* and *Pithecopus oreades* highlighting in white spots the sympatric zone (localities where both species were found together).

After generating maps with the new distributions and based on the suitability data, we plotted the new records over the modeling map (Figure 9**Figure *9***) for checking the overlap between the model and the successful record of new populations. The predictive accuracies of the models were relatively high for both species with an AUC of 0.935 (st. dev = 0.090) for *P. ayeaye* and 0.923 (st. dev = 0.097) for *P. oreades*. By using the binary maps for *P. ayeaye* (TSS = 0.672 and sd = 0.025), the high habitat suitability areas were Serra da Canastra (MG) region and south of state of Minas Gerais. While for *P. oreades* (TSS = 0.50 and sd = 0.024), the high habitat suitability areas were Chapada dos Veadeiros (GO) region and north of Serra da Canastra in Minas Gerais.

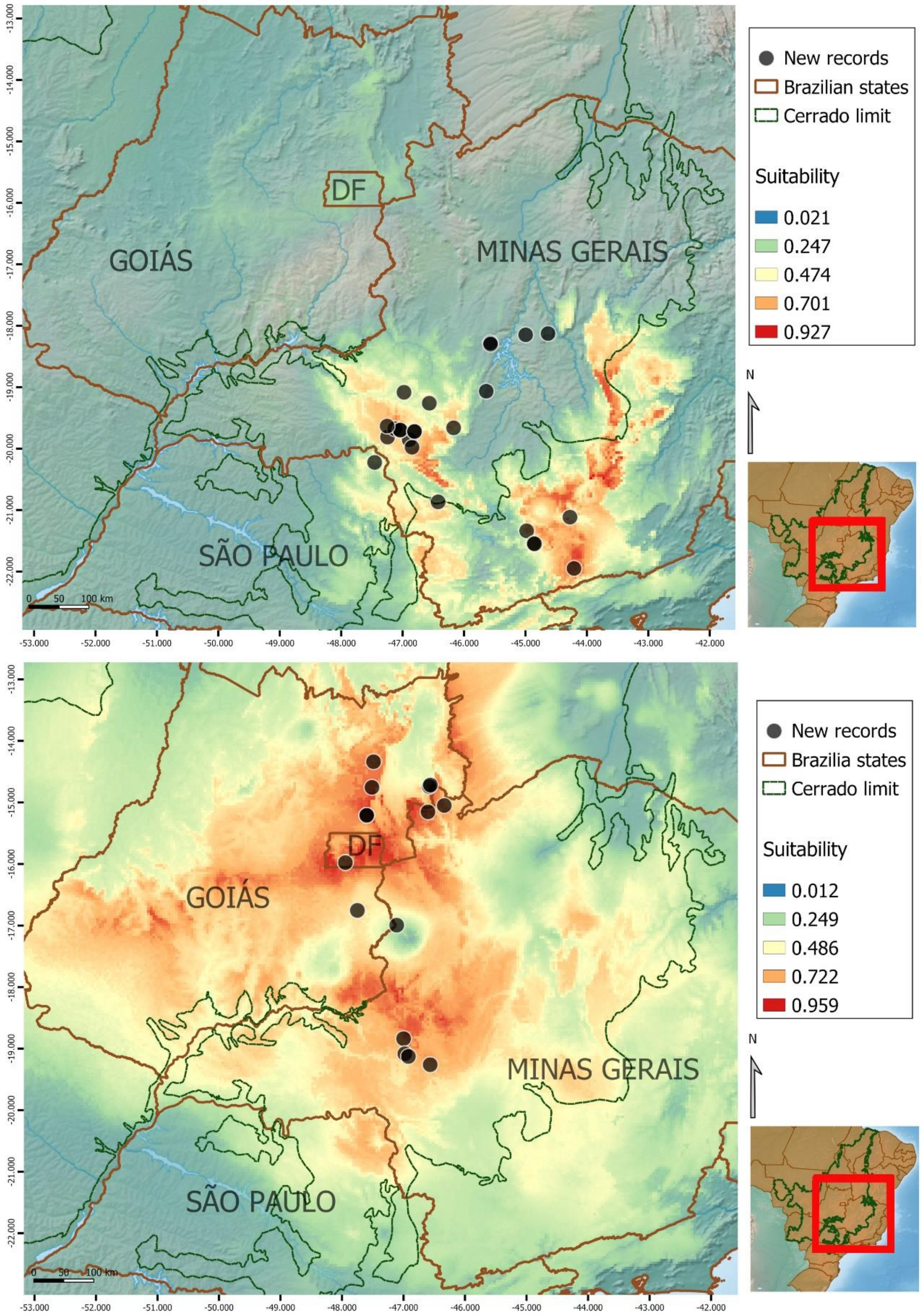

Figure 9. A: New populations records in the suitability areas of *Pithecopus ayeaye*; B: New populations records in the suitability areas of *Pithecopus oreades*.

To evaluate the efficacy of our predictive model to identify new localities of occurrence, we drew a histogram (Figure 10) showing the density of records or no records relating to modeled suitability of the places visited for each species.

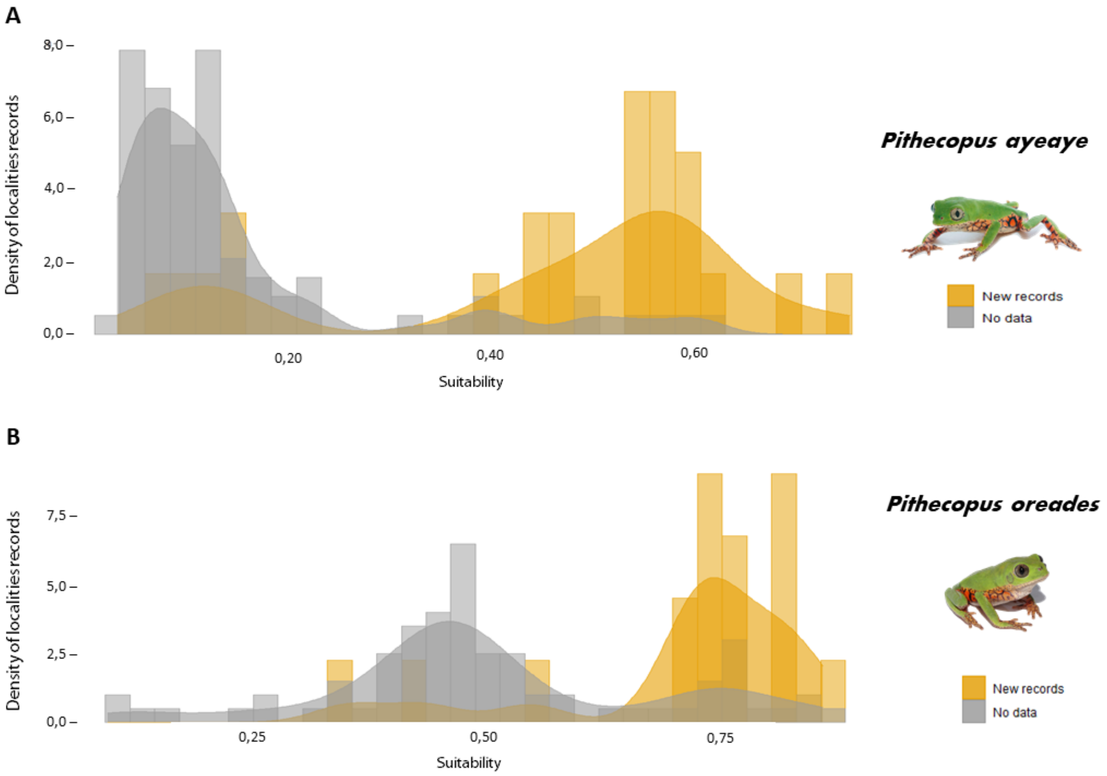

Figure 10. Density of localities visited with new records (orange) and no records (gray) represented in lines and bars for *Pithecopus ayeaye* (A) and *Pithecopus oreades* (B).

3.1. Extension of Occurrence (EOO) and Area of Occupation (AOO)

With the correct species determined by molecular analysis and with the new distribution, we could calculate new EOO and AOO for both species (Table 1). Based on these data and the threats suffered by the species, *Pithecopus ayeaye* and *P. oreades* should be categorized as LC (“Least concern”) based on EEO, and as EN (“Endangered”) based on AOO, according to IUCN.

Table 1. Current Extension of Occurrence (EOO) and Area of Occupation (AOO) for *Pithecopus ayeaye* and *Pithecopus oreades* with the new respective area increased (%) calculated based on the new populations recorded in the present study.

	Extension of Occurrence EOO (km^2)	**Increased EOO (this work - %)**	**Area of Occupation AOO (km^2)**	**Increased AOO (this work - %)**
Pithecopus ayeaye	128332.78	43.5	252000	34.9
Pithecopus oreades	151883.98	4.2	172000	32.5

4. Discussion

Through multiple approaches, we developed and tested a methodology to improve our ability to find habitat specialist species combining predictive models of distribution, GE tools, and natural history data. Additional molecular evidence was especially important in determining the species identity, refining distributions records, and, allowing us to carry out accurate estimations of the species extent of occurrence (EOO) and area of occupancy (AOO), with important implications in its categorization in Red Lists (IUCN, 2019).

Based on the molecular analysis, we recover a mitochondrial phylogenetic tree with the identifications and relationships of the populations collected, which is essential to understand the distributions patterns and the evolutionary relationship between these two *Pithecopus*. Molecular identification is also important for species conservation. By calculating the correct EEO and AOO we can update the species in Red Lists, such as their occurrence records, and natural history data (Ely et al., 2017; Magalhães et al., 2018).

By the correct taxa and the new geographical distribution, we can verify that *P. ayeaye* have a greater tolerance than predicted by the model and can occur in several localities of what was known before this project. For *P. oreades* the new records are in a more conservative area compared to their previous known distribution. We also found a sympatric area between these *Pithecopus* species. Both species are sympatric in Perdizes, Patrocínio, and Serra do Salitre municipallity and, by sharing the same reproductive habitat and having similar morphology, is possible to find gene flow between them at these locations (see Magalhães et al., 2021).

The developed protocol was effective. We recovered a strong AUC (high accuracy) for both predictive models in search for high suitability localities, with 37 new localities for *Pithecopus ayeaye* and *P. oreades* (24 and 13 new localities, respectively). Moreover, we recovered 19 new municipallity, being 11 new to *P. ayeaye* (Abaeté, Araxá, Bom Jardim de Minas, Campos Altos, Corinto, São João Del Rei, Serra do Salitre, Patrocínio, Perdizes, Tapira, and Três Marias in state of Minas Gerais) and eight to *Pithecopus oreades* (Goiás: Alvorada do Norte, São Gabriel, and São João D'Aliança in state of Goiás and Buritis, Formoso de Minas, Paracatu, Patrocínio, and Serra do Salitre in state of Minas Gerais).

Comparing both predictive models with new records overlapped, we can relate the suitability areas. For *P. ayeaye* there some records made in an area indicated as low suitability by the model. This same area, in predictive model for *P. oreades*, is indicating as high suitability (Figure 9) and it is reflected on the histogram (Figure 10) with displacement of the bars indicated for visited areas but without registration for an average value of suitability (approximately 0.45-0.5). This is an unexpected record due to niche conservation, reflecting the importance of molecular validation for cryptic species.

Therefore, for species conservation, it is important to unveil their correct taxa, geographic distribution and determine the boundaries between these *Pithecopus* (Lemes et al., 2001). As these species of *Pithecopus* are endemic from high plateaus in mountain tops in Cerrado (Brandão et al., 2009; Oliveira, 2007), there is a strong tendency for these populations to remain isolated in altitude areas. Associating the isolation with past and present climatic and other global changes, may contribute to the formation of independent evolutionary lineages (Silveira et al, 2019), and must be protected due the strong relation between these species and its environment. These *Pithecopus* live in rocky streams, an environment strongly subject to human action through different forms of land use incompatible with the preservation/conservation of species (Prette et al., 2020; Gomes et al., 2006). These ecosystems are rare in Cerrado biome and overly sensitive (Brandão, 2002; Brandão et al., 2009; Magalhães et al., 2020). In addition to being a heterogeneous environment with high diversity and rare and endemic species, it is home to springs of important rivers and hydrographic basins that are born in fields of rocky outcrops (Scariot et al., 2005).

The International Union for Conservation of Nature (IUCN) Red List of Threatened Species is an important instrument to evaluate the conservation status of living organisms. These data are basilar for threatened species category classification, since there are inconsistencies in the national and international Red Lists for these species. Another extremely important factor in unveiling these distributions, in addition to the discovery of new locations not previously registered, is that we can update the areas of occupation (AOO) and the extension of occurrence (EOO). This has direct implications for the conservation of species, including conservation policies, as there are inconsistencies of conservation status in the global and national threat lists.

The current EOO for both *Pithecopus* increased, but for *P. ayeaye* this new area was extremely higher proportionally (more than 55.000 km^2 that correspond to 43.3% of increase). This increase was possible due to the discovery of several localities placed beyond areas previously predicted by the model (low suitability). Taking several factors into account, such as the distribution increase and new limits between *Pitheocpus* species, the sympatric localities, incongruities between the Red Lists and the strong association and specialization between *Pithecopus* species and its environment, we strongly suggest that *P. ayeaye* and *P. oreades* should be re-categorized. Reinforcing the importance of a rigorous taxonomic approach and its boundaries geographic distribution to improving Red List assessments.

Collecting accurate data and determining population and species limits is extremely important for investigations on threatened status in Red Lists (Vogel et al., 2017). *Pithecopus ayeaye* and *P. oreades* present common characteristics of neotropical amphibian species that show decline and extinction (Collins, 2010), as wide distribution in central Brazil but restrict to high altitudes in mountain tops, out-of-water egg-laying, use of riparian environments and high habitat specificity (Brandão, 2002; Caramashi, 2006). In addition, there is a knowledge gap for this species and outdated knowledge that there still are great challenges to unveil the evolutionary story of these *Pithecopus* species (Magalhães et al., 2017).

The differential here is the combining of methodologies for detect target localities. Since predictive models used on conservation it is not an innovation, the combination with high spatial resolution satellite images (GE) proved to be essential for searching for appropriated environment by physical characteristics. By being free and easy to use, this software could help the researchers to save resources, time, and effort. The molecular validation was also essential here for dealing with cryptical species, helping to refine the model.

The complementation of these methodologies is reflected through different scales worked. In predictive models we work on a large scale (macro-geography), in GE on a landscape scale (searching for first order stream and marginal vegetation), in the field on a local level (searching the exactly spot of species occurrence). We also reinforce the importance of knowledge of species natural history, because, if needed, we can make decisions about the search location. Finally, the molecular validation confers the ultimate scale confirmation of the protocol developed. We conclude here that this protocol for new localities selection is exceptionally good to predict the occurrence for the species habitat specialists *Pithecopus ayeaye* and *P. oreades.*

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Appendix A

Table A.1 *Pithecopus species* from molecular analysis results.

Species	**Municipallity - State**	**Individuals (present work)**	**Individuals (adicional data - UFMG)**
Pithecopus oreades	Alto Paraíso de Goiás - GO	-	8
Pithecopus oreades	Alvorada do Norte – GO	5	-
Pithecopus oreades	Brasilândia de Minas - MG	-	5
Pithecopus oreades	Buritis – MG	1	-
Pithecopus oreades	Caldas Novas - GO	-	5
Pithecopus oreades	Cavalcante - GO	-	5
Pithecopus oreades	Chapada Gaúcha - MG	-	2
Pithecopus oreades	Cristalina – GO	5	1
Pithecopus oreades	FAL/UnB - DF	1	3
Pithecopus oreades	Formoso de Minas – MG	1	-
Pithecopus oreades	Minaçu - GO	-	4
Pithecopus oreades	Paracatu – MG	3	-
Pithecopus oreades	Patrocínio – MG	13	-
Pithecopus oreades	Perdizes – MG	3	2
Pithecopus oreades	Pirenópolis - GO	-	5
Pithecopus oreades	São Gabriel - GO	3	-

Pithecopus oreades	São João D'Aliança - GO	8	-
Pithecopus oreades	Serra do Salitre - MG	1	-
Pithecopus ayeaye	Abaeté – MG	5	-
Pithecopus ayeaye	Alponópolis – MG	2	1
Pithecopus ayeaye	Araxá – MG	5	-
Pithecopus ayeaye	Bom Jardim de Minas – MG	4	-
Pithecopus ayeaye	Campos Altos – MG	5	-
Pithecopus ayeaye	Lavras – MG	3	1
Pithecopus ayeaye	Luminárias – MG	3	-
Pithecopus ayeaye	Minduri - MG	-	9
Pithecopus ayeaye	Nova Lima - MG	-	2
Pithecopus ayeaye	Patrocínio – MG	1	-
Pithecopus ayeaye	Pedregulho - SP	-	2
Pithecopus ayeaye	Perdizes – MG	2	-
Pithecopus ayeaye	Pico de Itacolomi - MG	-	8
Pithecopus ayeaye	Poços de Caldas - MG	-	8
Pithecopus ayeaye	Sacramento - MG	1	6
Pithecopus ayeaye	São João Del Rei – MG	2	-
Pithecopus ayeaye	São Roque de Minas - MG	-	12
Pithecopus ayeaye	Serra de Andrequicé - MG	4	-
Pithecopus ayeaye	Serra do Beltrão – MG	2	-
Pithecopus ayeaye	Serra do Salitre – MG	3	-
Pithecopus ayeaye	Tapira – MG	5	-
Pithecopus ayeaye	Três Marias - MG	4	-
Total indivíduos	-	95	89
		184	

Table A.2 List of localities (37 spots) visited with records of *Pithecopus,* and its respective number of individuals collected.

Locality	Municipallities	State	Latitude	Longitude	Altitude (m)	Individuals
Água Limpa Farm – FAL/UnB	Brasília	DF	-15,977532	-47,945545	1161	3
-	Planaltina	GO	-15.209496	-47.598974	1147	1
Mountain range	Alvorada do Norte	GO	-14,739804	-46,58354	919	1
-	Formoso de Minas	MG	-15,049097	-46,33801	882	1
Mountain range	Alvorada do Norte	GO	-14,717897	-46,563691	942	5
Mountain range	Buritis	MG	-15,153369	-46,602581	918	1
-	Cristalina	GO	-16,754617	-47,750866	1011	5
Mountain range – North of Patrocínio	Patrocínio	MG	-18,83816	-46,999459	1078	5
Mountain range – North of Patrocínio	Patrocínio	MG	-19,084006	-46,978434	1080	5
Mountain range – North of Patrocínio	Patrocínio	MG	-19,127486	-46,921171	1168	5

Mountain range	Serra do Salitre	MG	-19,261847	-46,566624	977	4
-	Abaeté	MG	-19,067027	-45,639417	739	5
Beltrão mountain	Corinto	MG	-18,127894	-44,632761	754	4
Andrequicé mountain	Andrequicé	MG	-18,148793	-44,996989	734	5
-	Três Marias	MG	-18,278023	-45,571653	796	1
-	Três Marias	MG	-18,298646	-45,568833	781	5
-	Paracatu	MG	-16,994877	-47,111198	855	5
-	São João D'Aliança	GO	-14,338185	-47,487957	1056	5
-	São João D'Aliança	GO	-14,754688	-47,513633	1100	4
-	São Gabriel de Goiás	GO	-15,209496	-47,598974	1150	5
Rural region	Tapira	MG	-19.857417	-46.893861	1247	3
Rural region	Tapira	MG	-19.978389	-46.847028	1207	2
Rural region	Pedregulho	SP	-20.225361	-47.456583	997	1
-	Sacramento	MG	-19.813444	-47.247083	938	1
Lenheiro mountain	São João Del Rei	MG	-21.115833	-44.276306	1000	2
Highway Lavras-Luminárias	Lavras	MG	-21.338583	-44.981556	1121	3
Luz mountain	Luminárias	MG	-21.546028	-44.865222	1084	2

Luz mountain	Luminárias	MG	-21.549361	-44.854778	1149	2
Alpinópolis mountain	Alpinópolis	MG	-20.867694	-46.422417	1009	2
Rural region	Campos Altos	MG	-19.662639	-46.170611	1126	5
Rural region	Araxá	MG	-19.676083	-47.128861	1199	1
Rural region	Araxá	MG	-19.700611	-47.039722	1222	1
Rural region	Araxá	MG	-19.698639	-47.041222	1227	1
Rural region	Araxá	MG	-19.718667	-46.806611	1356	1
Rural region	Araxá	MG	-19.722806	-46.808278	1318	1
Rural region	Perdizes	MG	-19.634361	-47.253194	1127	5
Christ the Redeemer of Bom Jardim	Bom Jardim de Minas	MG	-21.950361	-44.209667	1319	4

Appendix B

Table B.1 Loadings of the bioclimatic variables based on the first five axes of Factor Analysis with a varimax rotation from current climate. Bioclimatic variables with the highest loading to each factor (in bold) were selected as predictors to build the ENMs.

Bioclimatic variables	I	II	III	IV	V
1	0.928	-0.268	0.129	0.000	0.209
2	0.110	-0.263	0.150	**0.896**	0.200
3	0.207	-0.656	0.121	0.000	0.578
4	-0.298	0.558	-0.431	0.000	**-0.638**
5	0.916	0.000	0.136	0.294	0.187
6	0.000	-0.271	0.110	-0.270	0.369
7	0.000	0.338	0.000	-0.331	-0.331
8	0.966	0.000	0.000	0.000	-0.130
9	0.796	-0.339	0.252	0.000	0.424
10	**0.996**	0.000	0.000	0.000	0.000
11	0.801	-0.368	0.253	0.000	0.397
12	0.000	0.279	0.949	0.000	0.000
13	0.000	-0.216	0.960	0.000	0.000
14	-0.285	0.888	-0.155	0.000	-0.230
15	0.000	**-0.922**	0.136	0.000	0.242
16	0.000	-0.198	**0.975**	0.000	0.000
17	-0.267	0.912	-0.125	0.000	-0.215
18	-0.369	0.199	0.254	0.000	-0.495
19	0.000	0.541	0.204	0.000	0.107

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.

Apprendix C

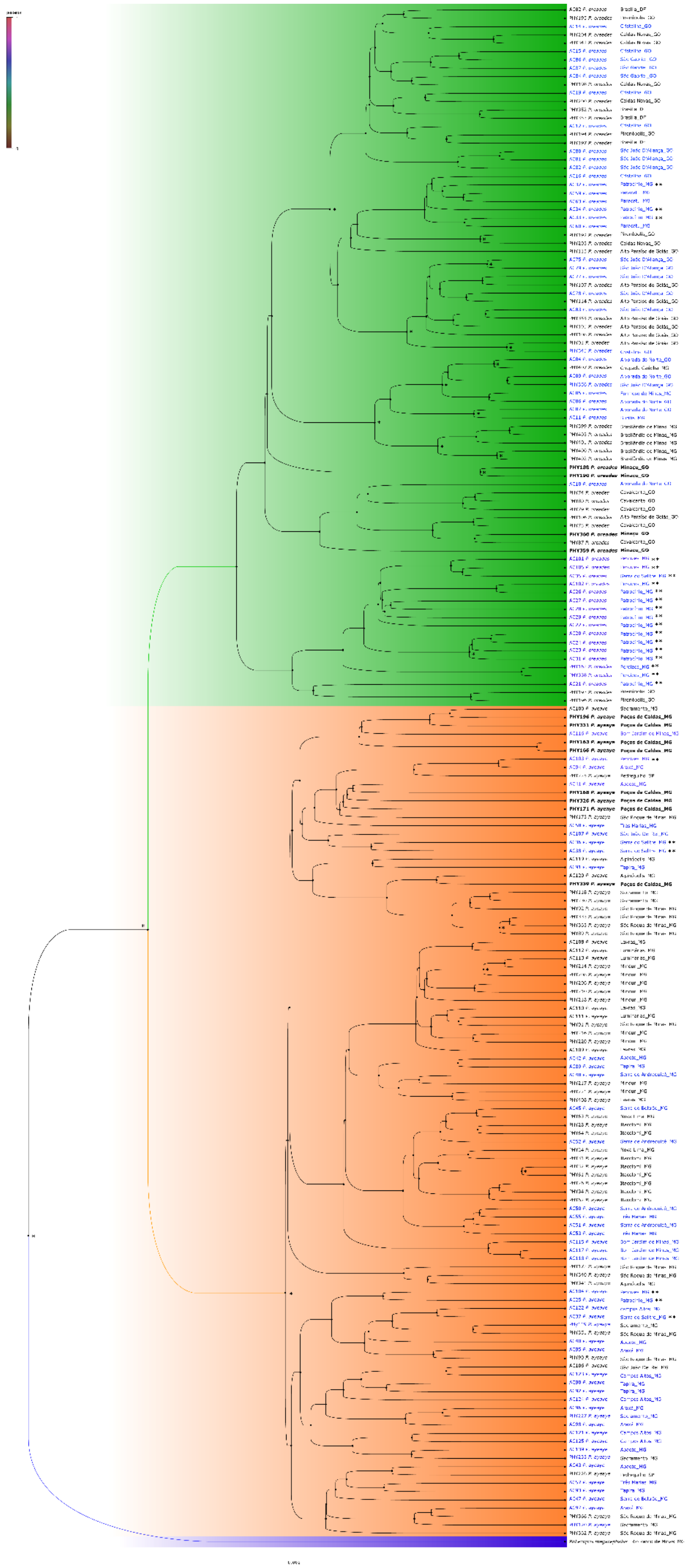
AC02 P. oreades Brasília_DF
PHY193 P. oreades Pirenópolis_GO
AC14 P. oreades Cristalina_GO
PHY204 P. oreades Caldas Novas_GO
PHY[illegible] P. oreades Caldas Novas_GO
AC15 P. oreades Cristalina_GO
AC86 P. oreades São Gabriel_GO
AC87 P. oreades São Gabriel_GO
AC84 P. oreades São Gabriel_GO
PHY198 P. oreades Caldas Novas_GO
AC19 P. oreades Cristalina_GO
PHY2[illegible] P. oreades Caldas Novas_GO
PHY352 P. oreades Brasília_DF
PHY35[illegible] P. oreades Brasília_DF
AC12 P. oreades Cristalina_GO
PHY194 P. oreades Pirenópolis_GO
PHY197 P. oreades Brasília_DF
AC80 P. oreades São João D'Aliança_GO
AC81 P. oreades São João D'Aliança_GO
AC82 P. oreades São João D'Aliança_GO
AC16 P. oreades Cristalina_GO
AC32 P. oreades Patrocínio_MG ◆*
AC59 P. oreades Paracatu_MG
AC63 P. oreades Paracatu_MG
AC34 P. oreades Patrocínio_MG ◆*
AC33 P. oreades Patrocínio_MG ◆*
AC60 P. oreades Paracatu_MG
PHY192 P. oreades Pirenópolis_GO
PHY203 P. oreades Caldas Novas_GO
PHY113 P. oreades Alto Paraíso de Goiás_GO
AC75 P. oreades São João D'Aliança_GO
AC79 P. oreades São João D'Aliança_GO
AC77 P. oreades São João D'Aliança_GO
PHY107 P. oreades Alto Paraíso de Goiás_GO
AC78 P. oreades São João D'Aliança_GO
PHY114 P. oreades Alto Paraíso de Goiás_GO
AC83 P. oreades São João D'Aliança_GO
PHY355 P. oreades Alto Paraíso de Goiás_GO
PHY101 P. oreades Alto Paraíso de Goiás_GO
PHY10[illegible] P. oreades Alto Paraíso de Goiás_GO
PHY91 P. oreades Alto Paraíso de Goiás_GO
PHY341 P. oreades Cristalina_GO
AC04 P. oreades Alvorada do Norte_GO
PHY407 P. oreades Chapada Gaúcha_MG
AC09 P. oreades Alvorada do Norte_GO
PHY350 P. oreades São João D'Aliança_GO
AC05 P. oreades Formoso de Minas_MG
AC06 P. oreades Alvorada do Norte_GO
AC07 P. oreades Alvorada do Norte_GO
AC11 P. oreades Buritis_MG
PHY399 P. oreades Brasilândia de Minas_MG
PHY403 P. oreades Brasilândia de Minas_MG
PHY401 P. oreades Brasilândia de Minas_MG
PHY400 P. oreades Brasilândia de Minas_MG
PHY402 P. oreades Brasilândia de Minas_MG
PHY188 P. oreades Minaçu_GO
PHY190 P. oreades Minaçu_GO
AC10 P. oreades Alvorada do Norte_GO
PHY74 P. oreades Cavalcante_GO
PHY80 P. oreades Cavalcante_GO
PHY79 P. oreades Cavalcante_GO
PHY106 P. oreades Alto Paraíso de Goiás_GO
PHY75 P. oreades Cavalcante_GO
PHY300 P. oreades Minaçu_GO
PHY87 P. oreades Cavalcante_GO
PHY359 P. oreades Minaçu_GO
AC101 P. oreades Perdizes_MG *◆
AC105 P. oreades Perdizes_MG *◆
AC35 P. oreades Serra do Salitre_MG *◆
AC102 P. oreades Perdizes_MG *◆
AC26 P. oreades Patrocínio_MG ◆*
AC27 P. oreades Patrocínio_MG ◆*
AC28 P. oreades Patrocínio_MG ◆*
AC29 P. oreades Patrocínio_MG ◆*
AC22 P. oreades Patrocínio_MG ◆*
AC20 P. oreades Patrocínio_MG ◆*
AC21 P. oreades Patrocínio_MG ◆*
AC23 P. oreades Patrocínio_MG ◆*
AC31 P. oreades Patrocínio_MG ◆*
PHY15[illegible] P. oreades Perdizes_MG ◆*
PHY338 P. oreades Perdizes_MG *◆
AC21 P. oreades Patrocínio_MG ◆*
PHY19[illegible] P. oreades Pirenópolis_GO
PHY19[illegible] P. oreades Pirenópolis_GO
AC100 P. ayeaye Sacramento_MG
PHY196 P. ayeaye Poços de Caldas_MG
PHY331 P. ayeaye Poços de Caldas_MG
AC116 P. ayeaye Bom Jardim de Minas_MG
PHY163 P. ayeaye Poços de Caldas_MG
PHY166 P. ayeaye Poços de Caldas_MG
AC103 P. ayeaye Perdizes_MG *◆
AC94 P. ayeaye Araxá_MG
PHY2[illegible] P. ayeaye Pedregulho_SP
AC41 P. ayeaye Araxá_MG
PHY168 P. ayeaye Poços de Caldas_MG
PHY326 P. ayeaye Poços de Caldas_MG
PHY171 P. ayeaye Poços de Caldas_MG
PHY173 P. ayeaye São Roque de Minas_MG
AC58 P. ayeaye Três Marias_MG
AC107 P. ayeaye São João Del Rei_MG
AC36 P. ayeaye Serra do Salitre_MG ◆*
AC38 P. ayeaye Serra do Salitre_MG ◆*
AC119 P. ayeaye Alpinópolis_MG
AC91 P. ayeaye Tapira_MG
AC120 P. ayeaye Alpinópolis_MG
PHY330 P. ayeaye Poços de Caldas_MG
PHY118 P. ayeaye Sacramento_MG
PHY[illegible] P. ayeaye Sacramento_MG
PHY92 P. ayeaye São Roque de Minas_MG
PHY3[illegible] P. ayeaye São Roque de Minas_MG
PHY303 P. ayeaye São Roque de Minas_MG
PHY89 P. ayeaye São Roque de Minas_MG
AC108 P. ayeaye Lavras_MG
AC112 P. ayeaye Luminárias_MG
AC113 P. ayeaye Luminárias_MG
PHY214 P. ayeaye Minduri_MG
PHY2[illegible] P. ayeaye Minduri_MG
PHY209 P. ayeaye Minduri_MG
PHY2[illegible] P. ayeaye Minduri_MG
PHY213 P. ayeaye Minduri_MG
AC110 P. ayeaye Lavras_MG
AC111 P. ayeaye Luminárias_MG
PHY91 P. ayeaye São Roque de Minas_MG
PHY216 P. ayeaye Minduri_MG
PHY220 P. ayeaye Minduri_MG
AC109 P. ayeaye Lavras_MG
AC42 P. ayeaye Araxá_MG
AC89 P. ayeaye Tapira_MG
AC48 P. ayeaye Serra do Andrequicé_MG
PHY217 P. ayeaye Minduri_MG
PHY2[illegible] P. ayeaye Minduri_MG
PHY108 P. ayeaye Lavras_MG
AC45 P. ayeaye Serra da Babilônia_MG
PHY53 P. ayeaye Nova Lima_MG
PHY28 P. ayeaye Itacolomi_MG
PHY64 P. ayeaye Itacolomi_MG
AC52 P. ayeaye Serra do Andrequicé_MG
PHY54 P. ayeaye Nova Lima_MG
PHY[illegible] P. ayeaye Itacolomi_MG
PHY[illegible] P. ayeaye Itacolomi_MG
PHY61 P. ayeaye Itacolomi_MG
PHY[illegible] P. ayeaye Itacolomi_MG
PHY34 P. ayeaye Itacolomi_MG
PHY5[illegible] P. ayeaye Itacolomi_MG
AC50 P. ayeaye Serra do Andrequicé_MG
AC55 P. ayeaye Três Marias_MG
AC51 P. ayeaye Serra do Andrequicé_MG
AC53 P. ayeaye Três Marias_MG
AC115 P. ayeaye Bom Jardim de Minas_MG
AC117 P. ayeaye Bom Jardim de Minas_MG
AC118 P. ayeaye Bom Jardim de Minas_MG
PHY17[illegible] P. ayeaye São Roque de Minas_MG
PHY340 P. ayeaye São Roque de Minas_MG
PHY341 P. ayeaye Alpinópolis_MG
AC104 P. ayeaye Perdizes_MG ◆*
AC25 P. ayeaye Patrocínio_MG ◆*
AC122 P. ayeaye Campos Altos_MG
AC37 P. ayeaye Serra do Salitre_MG *◆
PHY[illegible] P. ayeaye Sacramento_MG
PHY351 P. ayeaye São Roque de Minas_MG
AC40 P. ayeaye Araxá_MG
AC95 P. ayeaye Araxá_MG
PHY90 P. ayeaye São Roque de Minas_MG
AC106 P. ayeaye São João Del Rei_MG
AC123 P. ayeaye Campos Altos_MG
AC90 P. ayeaye Tapira_MG
AC92 P. ayeaye Tapira_MG
AC124 P. ayeaye Campos Altos_MG
AC96 P. ayeaye Araxá_MG
PHY227 P. ayeaye Sacramento_MG
AC98 P. ayeaye Araxá_MG
AC121 P. ayeaye Campos Altos_MG
AC125 P. ayeaye Campos Altos_MG
AC114 P. ayeaye Araxá_MG
PHY233 P. ayeaye Sacramento_MG
AC43 P. ayeaye Araxá_MG
PHY226 P. ayeaye Pedregulho_SP
AC57 P. ayeaye Três Marias_MG
AC93 P. ayeaye Tapira_MG
AC47 P. ayeaye Serra da Babilônia_MG
AC97 P. ayeaye Araxá_MG
PHY366 P. ayeaye São Roque de Minas_MG
PHY120 P. ayeaye Sacramento_MG
PHY362 P. ayeaye São Roque de Minas_MG
Pithecopus megacephalus Rio Pardo de Minas_MG

Figure C. 1. Best Cyt-b gene phylogenetic tree. Pithecopus oreades in green and Pithecopus ayeaye in orange. The nodes asterisks (*) represents the statistical support equal or above 95% (posterior probability). The bold names represent the individuals collected in the type locality. The blue names represent the new sampled localities. The double asterisks (**) in the names represent a sympatric zone (presence of *Pithecopus oreades* and *Pithecopus ayeaye* in the same locality).