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Suelen da Silva Alves

FILOGEOGRAFIA DE ANFÍBIOS ANUROS DOS CAMPOS SUBTROPICAIS DO SUDESTE DA AMÉRICA DO SUL

Santa Maria, RS, Brasil

2019

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Tese apresentada ao Programa de Pós-Graduação em Biodiversidade Animal, da Universidade Federal de Santa Maria (UFSM, RS), como requisito parcial para a obtenção do título de **Doutora em Biodiversidade Animal**

Orientador: Prof. Dr. Tiago Gomes dos Santos
Coorientador: Prof. Dr. Celio Fernando Baptista Haddad
Colaboradores: Drª. Mariana Lyra e Dr. Arley Camargo

Santa Maria, RS, Brasil
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Aprovado em 30 de agosto de 2019:

Tiago Gomes

Tiago Gomes dos Santos, Dr. (UNIPAMPA-RS)
(Presidente/Orientador)

Célio Fernando Baptista Haddad, Dr. (UNESP-SP)
(Coorientador)

Brasileiro

Cinthia Aguirre Brasileiro, Drª. (UNIFESP-SP)

Igor Luis Kaefer

Igor Luis Kaefer, Dr. (UFAM-AM)

Maurício Beux dos Santos, Dr. (UFSM-RS)

Ana Lúcia Anversa Segatto

Ana Lúcia Anversa Segatto, Drª. (UFSM-RS)

Santa Maria, RS
2019

DEDICATÓRIA

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Na vida, tudo é uma questão de
***tempo**, seja ele em milhões de anos*
ou em questão de segundos!

Suelen. S. Alves

RESUMO

FILOGEOGRAFIA DE ANFÍBIOS ANUROS DOS CAMPOS SUBTROPICAIS DO SUDESTE DA AMÉRICA DO SUL

AUTORA: Suelen da Silva Alves
ORIENTADOR: Prof. Dr. Tiago Gomes dos Santos
COORIENTADOR: Prof. Dr. Célio Fernando Baptista Haddad

Os eventos climáticos e geológicos do período Neogeno, e as flutuações climáticas do Quaternário têm sido apontados como os principais responsáveis pela diversificação da biota em áreas abertas da América do Sul. Entretanto, pouco se sabe sobre os padrões filogeográficos de anuros dos campos dessa região e como esse ecossistema campestre moldou a diversidade da anurofauna. Nesse sentido, o objetivo geral da Tese foi investigar o padrão filogeográfico de múltiplas espécies de anuros com diferentes histórias de vida e co-distribuídas nos campos subtropicais do sudeste de América do Sul. A Tese está dividia em dois capítulos, conforme os objetivos específicos a seguir: (1) acessar a diversidade genética, padrões de estruturação populacional e histórica demográfica e, testar a congruência temporal no padrão demográfico; (2) reconstruir as relações filogenéticas, testar a congruência temporal no padrão de divergência, reconstruir o padrão de difusão filogeográfica no espaço e no tempo e, por fim, realizar a delimitação de espécies. As espécies selecionadas estão distribuídas em três padrões geográficos básicos: amplamente distribuídas ao longo da vegetação florestal e aberta da América do Sul, espécies distribuídas ao longo dos campos do bioma Pampa e campos de Altitude do bioma Mata Atlântica, e espécies restritas aos campos do Pampa. Sequenciamos um fragmento do gene 16S de 371 amostras de seis espécies nominais de anuros (*Leptodactylus latrans, Physalaemus cuvieri, Scinax uruguayus, Physalaemus gracilis, Phyllomedusa iheringii* e *Pseudis minuta*), coletadas ao longo de suas distribuições geográficas. Descobrimos que processos de diversificação nessa região são complexos e que as espécies associadas a áreas campestres apresentam respostas variáveis. Recuperamos diversidade genética variando de alta a baixa, espécies com e sem estruturação filogeográfica e histórias demográficas com algumas peculiaridades, mas congruentes em sua maioria. A maioria das linhagens de anuros dos campos mostrou sinais de expansão populacional durante os ciclos glaciais do Quaternário e quatro dessas linhagens em expansão populacional tiveram um pulso de expansão no mesmo tempo evolutivo (sincrônicos), enquanto uma espécie teve um pulso de expansão em um tempo aleatório. Esses pulsos de coexpansão e expansão coincidem com a dinâmica climática do Plioceno-Pleistoceno, i.e., Neógeno-Quaternário. Além disso, nossos modelos de paleodistribuição foram congruentes em demonstrar uma relação positiva da distribuição dessas espécies com o Último Máximo Glacial (UMG). Reportamos forte estruturação geográfica, alta divergência genética e grande número de passos mutacionais entre clados de duas espécies tipicamente campestres (*S. uruguayus* e *P. gracilis*). Recuperamos uma divergência profunda que remonta o Oligoceno e quebras filogeográficas dos principais clados datados do Mioceno, porém não congruentes temporalmente. Além disso, reportamos uma contínua diversificação durante o Plioceno-Quaternário, com o aumento da dispersão dessas linhagens durante o Quaternário. Por fim, delimitamos cinco linhagens coalescentes sob o nome de duas espécies tipicamente campestres. É provável que as transgressões marinhas do Mioceno sejam responsáveis pela diversificação inicial dessas linhagens e que a dinâmica climática e campestre juntamente com a topografia da região tenha contribuído para moldar a atual diversidade e distribuição dessas espécies, evidenciando uma forte interação entre as forças paleogeográficas e paleoclimáticas. Este é o primeiro estudo com filogeografia comparada de anuros nos campos subtropicais do sudeste da América do Sul.

Palavras-chave: Filogeografia Comparada. Anura. Campos Sulinos. Neogeno-Quaternário. Dinâmica Climática. Espécies Crípticas. Delimitação de Espécies.

ABSTRACT

PHYLOGEOGRAPHY OF ANURAN AMPHIBIANS FROM THE SUBTROPICAL GRASSLANDS OF SOUTHEASTERN SOUTH AMERICA

AUTHOR: Suelen da Silva Alves
ADVISOR: Prof. Dr. Tiago Gomes dos Santos
CO-ADVISOR: Prof. Dr. Célio Fernando Baptista Haddad

The climatic and geological events of the Neogene period and the climatic fluctuations of the Quaternary have been pointed out as the main responsible for the diversification of the biota in open areas of South America. However, little is known about phylogeographic patterns of anurans in the grasslands of this region, and how these grassland ecosystems shaped the diversity of the anurofauna. In this sense, the general objective of this Thesis was to investigate the phylogeographic pattern of multiple anuran species with different life histories and codistributed in the subtropical grasslands of southeastern South America. The Thesis was divided into two chapters, according to the specific objectives: (1) to access genetic diversity, patterns of population and historical demographic structuring, and to test temporal congruence on the demographic pattern; (2) to reconstruct the phylogenetic relationships, to test the temporal congruence in the divergence pattern, to reconstruct the phylogeographic diffusion pattern in space and time and, finally, to perform the species delimitation. The species selected are distributed in three basic geographic patterns: widely distributed throughout the open and forest vegetation of South America, species distributed throughout the Pampa grasslands and Highland grasslands of the Atlantic Forest biome, and species restricted to the Pampa grasslands. We sequenced a fragment of the 16S gene from 371 samples of six nominal species of anurans (*Leptodactylus latrans, Physalaemus cuvieri, Scinax uruguayus, Physalaemus gracilis, Phyllomedusa iheringii*, and *Pseudis minuta*), along their geographical distributions. We found that diversification processes in this region are complex and that the species associated with grasslands areas present variable responses. We recovered from high to low genetic diversity, species with and without phylogeographic structuring and demographic histories with some peculiarities, but congruent in their majority. Most grassland anuran lineages showed signs of population expansion during the Quaternary glacial cycles, and four of these lineages in population expansion had an expansion pulse at the same evolutionary time (synchronic), while one species had a pulse expansion at a random time. These coexpansion and expansion pulses coincided with the climatic dynamics of Pliocene-Pleistocene, i.e., Neogene-Quaternary. In addition, our paleodistribution models were congruent in demonstrating a positive relation of the species distribution with the Last Glacial Maximum (LGM). We report a strong geographic structure, high genetic divergence and a high number of mutational stages between clades of two typically grassland anuran species. We recover a deep divergence dating back to the Oligocene and phylogeographic breaks of the major clades dating from the Miocene, but not temporally congruent. In addition, we report a continuous diversification during the Pliocene-Quaternary, with increase of dispersion of these lines during the Quaternary. Finally, we delineate five coalescing lineages under the name of two typically grassland anuran species (*S. uruguayus* and *P. gracilis*). It is probable that the Miocene marine transgressions are responsible for the initial diversification of these lineages and that the climatic and grassland dynamics together with the topography of the region have contributed to shape the present diversity and distribution of these species, evidencing a strong interaction between paleogeographic and paleoclimatic forces. This is the first study with comparative phylogeography of anurans in the subtropical grasslands of southeastern South America.

Keywords: Comparative Phylogeography. Anura. Campos Sulinos. Neogene-Quaternary. Climate Dynamic. Cryptic Species. Delimitation of Species.

SUMÁRIO

CAPÍTULO 1: PARTIALLY SYNCHRONOUS RESPONSE TO NEOGENE-QUATERNARY CLIMATIC DYNAMICS IN CODISTRIBUTED FROGS FROM THE SOUTH AMERICAN SUBTROPICAL GRASSLANDS

CAPÍTULO 2: STRONG MITOCHONDRIAL DIVERGENCE REVEALS CRYPTIC DIVERSITY IN FROGS FROM THE SUBTROPICAL GRASSLANDS OF SOUTH AMERICA.

INTRODUÇÃO GERAL

Avanços recentes na área da biologia molecular, principalmente em métodos filogenéticos, revolucionaram o estudo da diversificação biológica (RULL, 2018) contribuindo positivamente para a conservação da biodiversidade. Apesar desse avanço, questões cruciais continuam sem resposta. Sete principais deficiências no conhecimento sobre a biodiversidade são apresentadas em HORTAL et al. (2015): deficiências na sistemática (Lacuna Linneana), biogeografia (Lacuna Wallaceana), biologia populacional (Lacuna Prestoniana), evolução (Lacuna Darwiniana), ecologia funcional “baseada em características” (Lacuna Raunkiaeran), tolerâncias abióticas (Lacuna Hutchinsonian) e interações ecológicas (Lacuna Eltonian). Aqui destacamos algumas dessas lacunas de conhecimento: de acordo com a Lacuna Linneana, há espécies que ainda não foram amostradas e espécies que já foram amostradas, mas ainda não foram descritas. A Lacuna Wallaceana diz que o conhecimento sobre a distribuição geográfica da maioria das espécies é incompleto. A Lacuna Prestoniana diz que a dinâmica populacional no espaço e no tempo são muitas vezes escassas e a Lacuna Darwiniana remete sobre falta de conhecimento sobre a evolução das espécies e sua característica. De fato, a diversidade de espécies é consequência de uma longa e complexa história de processos evolutivos, processos ecológicos e forças ambientais externas (RULL, 2011) e identificar e compreender esses processos são os principais objetivos da filogeografia (BEHEREGARAY, 2008). A filogeografia é um campo de estudo preocupado com os princípios e processos que regem as distribuições geográficas de linhagens genealógicas, especialmente aquelas dentro e entre espécies intimamente relacionadas (AVISE et al., 1987; AVISE, 2000). Estudar as causas da diversificação biológica e os principais fatores ambientais envolvidos é útil não apenas para o progresso fundamental da ciência, mas também para informar práticas conservacionistas (RULL, 2018). Assim, podemos dizer que a filogeografia, considerada uma subdisciplina da biogeografia (AVISE, 2000), tem um papel crucial no preenchimento dessas lacunas de conhecimento da biodiversidade.

O continente sul-americano é composto por várias regiões biogeográficas, apresenta a maior biodiversidade do globo, mas, apesar do crescimento nos estudos filogeográficos nessa região, foi pouco investigado quando aos padrões e processos que moldaram essa espantosa biodiversidade (TURCHETTO-ZOLET et al., 2013). Os eventos geológicos do Mioceno e do Plioceno, em conjunto com as mudanças climáticas do Pleistoceno, têm sido mecionados como os principais responsáveis por moldar um padrão complexo de eventos filogeográficos que impactaram a diversidade geral da América do Sul (RULL, 2008, TURCHETTO-ZOLET et al., 2013). O avanço e recuo do mar, o soerguimento de montanhas e as flutuações climáticas influenciaram na dinâmica dos ambientes florestais e abertos, causando vários eventos de retração e expansão desses ambientes ao longo da história evolutiva (BEHLING, 2002; ORTIZ-JAUREGUIZAR; CLADERA, 2006; OVERBECK et al., 2007), gerando complexos cenários para a diversificação de espécies. Informações paleoclimáticas da temperatura média terrestre e do nível médio do mar revelam que a história do clima foi caracterizada pela alternância de períodos quentes e frios (de OLIVEIRA et al., 2017; Figura 1). A liberação de gases vulcânicos nos últimos 100 milhões de anos, e provavelmente as mudanças de órbita da terra, foram as principais causas das flutuações climáticas, incluindo o período quente do Cretáceo e as eras glaciais do Plioceno-Pleistoceno (BERNER, 1983; VIAGGI, 2018). Os ciclos glaciais e interglaciais do Pleistoceno, por exemplo, teriam promovido migrações em grande escala e mudanças de distribuição que foram decisivas para a modelagem de padrões biogeográficos atuais e a composição de comunidades existentes (WILLIS; BHAGWAT, 2009). A ocorrência intercalada de eras glaciais (frias) e interglaciais (quentes) gera fenômenos periódicos denominados *ciclos de Milankovitch* (BROWN; LOMOLINO, 2006; de OLIVEIRA et al., 2017). Durante os estágios iniciais de glaciação, o desenvolvimento de gelo e neve aumentou sobre grandes áreas do planeta, reduzindo o aquecimento solar efetivo sobre a Terra e, posteriormente, aumentando as taxas de resfriamento, provocando uma glaciação mais rápida. Durante a deglaciação (i.e., interglacial), as geleiras derreteram mais rápido do que a sua formação, o que parece ter sido dirigido em parte por mudanças globais na concentração dos gases estufa, especialmente o dióxido de carbono e o metano (BROWN; LOMOLINO, 2006). Análises de perfurações no gelo produziram um registro de condições ambientais do passado que remontam até 420 mil anos e que abrangem quatro períodos glaciais anteriores (de OLIVEIRA et al., 2017). Os períodos glaciais-interglaciais mostram que existe um padrão de correlação entre a temperatura, o dióxido de carbono (CO_2), o metano (CH_4), a insolação e o oxigênio atmosférico (Figura 2).

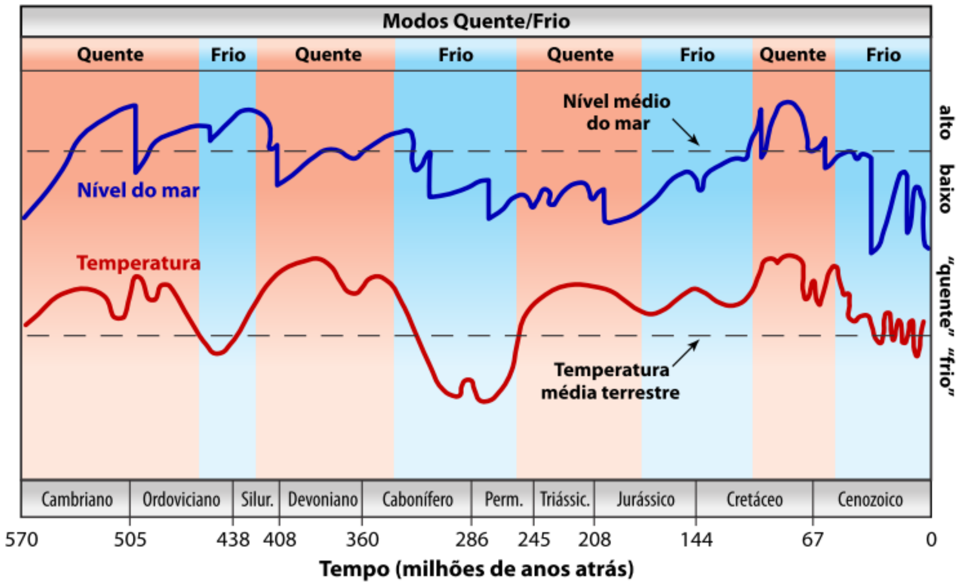

Figura 1. Variações estimadas do nível eustático do mar e da temperatura global durante o Fanerozoico, ilustrando os Modos Quentes e Frios. **Fonte: de Oliveira et al. (2017).**

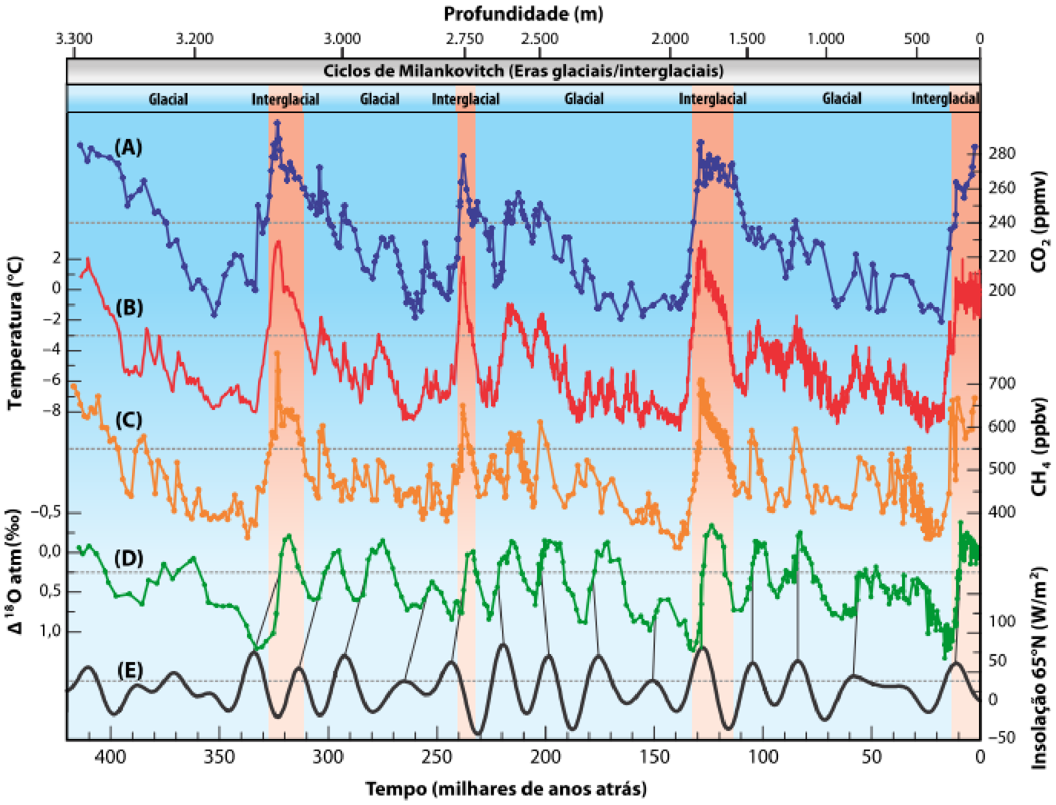

Figura 2. Ciclos de Milankovitch registrados nos testemunhos de gelo. Séries de Vostok (curvas coloridas), insolação (curva preta) e períodos glaciais e interglaciais (preenchimentos verticais azuis e vermelhos, respectivamente). Séries com relação ao tempo (escala com indicação de profundidades correspondentes no eixo superior): (A) Dióxido de carbono (CO_2); (B) Temperatura isotópica da atmosfera; (C) Metano (CH_4); (D) Composição isotópica de oxigênio ($\Delta^{18}O$ atm); (E) Insolação, em meados de junho, na latitude 65ºN. **Fonte: de Oliveira et al. (2017).**

Uma revisão dos estudos filogeográficos na América do Sul foi compilada por TURCHETTO-ZOLET et al. (2013) os quais destacaram que dentre 214 estudos reportados, 120 (56%) estão concentrados nas regiões tropicais, 57 (27%) nas regiões temperadas e apenas 14 (6%) na região subtropical, sendo Amazônia e Andes as mais estudadas. Destes estudos, 125 revelaram a presença de estrutura filogeográfica intraespecífica e apenas 13 discutiram sobre possíveis casos de espécies crípticas. Além disso, alterações demográficas foram identificadas em 58% dos estudos e sugeriram uma interação importante entre os cenários demográficos e organismos de áreas florestais e abertas. As faixas geográficas de espécies associadas a ambientes florestais foram reduzidas devido às condições mais secas durante os períodos glaciais (período que favoreceu a expansão das áreas campestres) e 13% dos estudos indicaram expansão populacional durante períodos interglaciais e/ou fragmentação durante os períodos glaciais. As espécies associadas a ambientes abertos apresentaram um padrão mais variável, mas, a maioria delas teve expansão populacional durante os períodos glaciais e fragmentação durante os períodos interglaciais. No entanto, apenas 28% dos estudos filogeográficos focaram em espécies associados a habitats naturalmente abertos. Muitas plantas e animais que não conseguiram rastrear suas oscilações ambientais eram capazes de permanecer *in situ* pela adaptação às condições alteradas, já as espécies remanescentes incapazes de mudar ou sofrer adaptação, foram extintas (BROWN; LOMOLINO, 2006). Os biomas naturalmente abertos da América do Sul ocorrem através de uma variedade de condições ambientais e são sazonalmente estressados pela seca, mas apesar de apresentarem semelhanças, respondem de maneira diferente às mudanças climáticas e ambientais (WERNECK, 2011).

Os campos são áreas abertas amplamente definidas como ecossistemas em que gramíneas e plantas semelhantes a gramíneas formam uma “camada herbácea” relativamente contínua de vegetação consideradas antiga, composta por alta riqueza de espécies, e altas taxas de endemismo (VELDMAN et al., 2015). No entanto, estes ecossistemas são pouco conhecidos e preservados, bem como estão sob intensa modificação (por exemplo, uso da terra), particularmente nas regiões tropicais e subtropicais (BOND; PARR, 2010). Os campos subtropicais da porção sudeste da América do Sul (faixa latitudinal entre 25°S e 35°S) são comumente divididos em Campos do Pampa (presentes no Uruguai, parte da Argentina e metade sul do Rio

Grande do Sul, no Brasil), e Campos de Altitude (formando um mosaico natural associado à floresta araucária no sul do Brasil (ANDRADE et al., 2015; OVERBECK et al., 2015; ANDRADE et al., 2019; Figura 3). Apesar da imensa biodiversidade encontrada em vários grupos de plantas e animais, e da diversidade ambiental proporcionada pelos campos, ainda são pouco esclarecidos os processos que influenciaram a diversificação das espécies em regiões campestres da América do Sul (TURCHETTO-ZOLET et al., 2013).

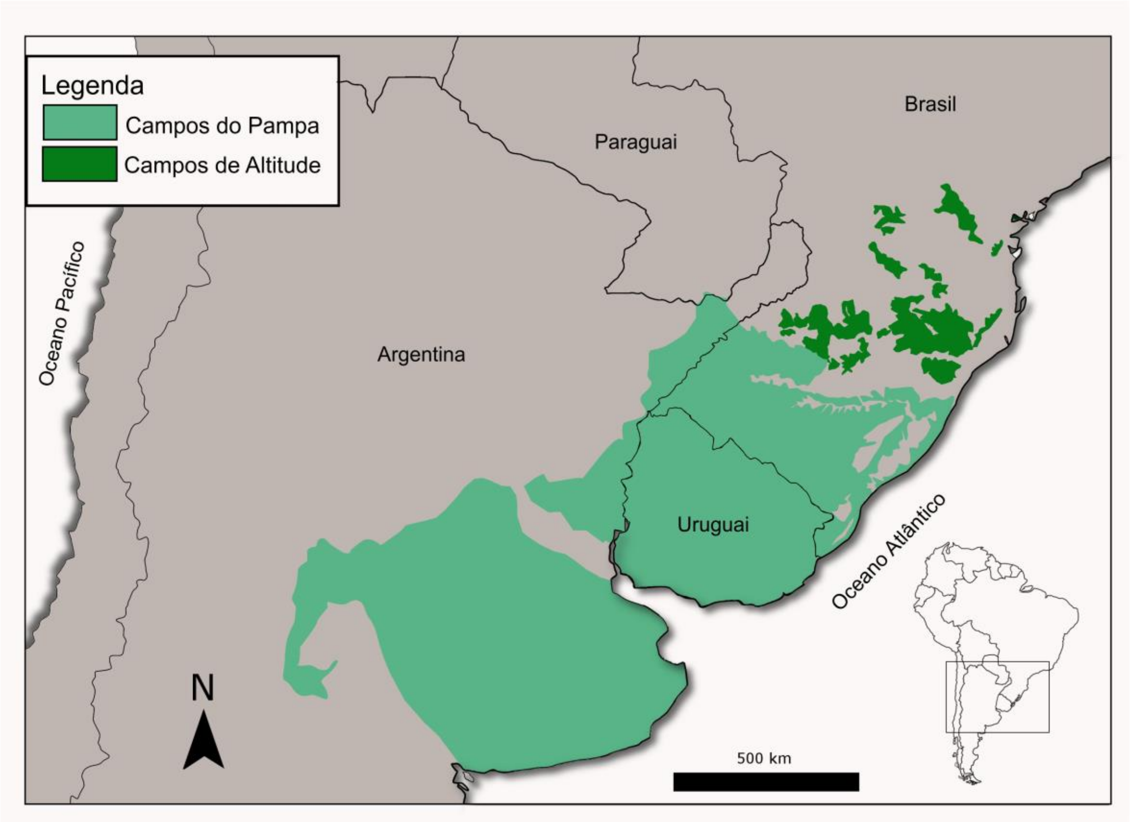

Figura 3. Distribuição original dos campos naturais subtropicais do sudeste da América do Sul. Adaptado de Andrade et al. (2015).

Ao longo de sua história evolutiva, a distribuição dos campos foi moldada por ciclos de expansão e retração, provavelmente pressionando que as espécies desse ambiente desenvolvessem adaptações e/ou acompanhassem a dinâmica dos campos (ABREU-JARDIM, 2018). Alguns trabalhos que examinaram os padrões filogeográficos de organismos que habitam os ecossistemas campestres subtropicais da América do Sul incluem plantas (FREGONEZI et al., 2013; MÄDER et al., 2013; LONGO et al., 2014; TURCHETTO et al., 2014; MAIA et al., 2017; SILVA et al., 2018; JOHN et al., 2019), artrópodes (PERES et al., 2015; CRISTIANO et al., 2016) mamíferos (WLASIUK et al., 2003; MORA et al., 2006; MONTES et al., 2008; KITTLEIN; GAGGIOTTI, 2008; LOPES et al., 2013; RORATTO et al., 2014; QUINTELA et al., 2015), peixes (GARCÍA, 2006), lagartos (FELAPPI et al., 2015; VILLAMIL et al., 2019) e, mais recentemente, anfíbios (BARRASO, 2014; LANGONE et al., 2016; ABREU-JARDIM, 2018; de OLIVEIRA MIRANDA et al., 2019; BRUSQUETTI et al., 2019). Eventos climáticos e geológicos do Neógeno e flutuações climáticas do Quaternário têm sido apontados como os principais responsáveis pela diversificação da biota nessa região (ORTIZ-JAUREGUIZAR; CLADERA, 2006). No entanto, como apontado por TURCHETTO-ZOLET et al. (2013), espécies associadas a áreas aberta apresentaram respostas variáveis às oscilações climáticas, por exemplo, sugerindo padrões filogeográficos mais complexos na biota da América do Sul. Mesmo com o aumento no número de publicações, os estudos relacionados à filogeografia e à demografia histórica ainda são muito escassos na América do Sul, quando comparados com outras regiões do mundo (BEHEREGARAY, 2008; TURCHETTO-ZOLET et al., 2013). Além disso, entre os estudos existentes, nenhum deles testou comparativamente se espécies co-distribuídas responderam de forma sincrônica aos eventos do Neógeno-Quaternário nesta região. De fato, conhecer a história evolutiva das espécies e como elas responderam a eventos passados, também pode nos fornecer informações sobre como elas poderiam responder às mudanças ambientais atuais e futuras (TURCHETTO-ZOLET et al., 2013).

A classe Amphibia, que inclui as ordens Gymnophiona – cecílias, Caudata – salamandras, e a ordem Anura – sapos, rãs e pererecas, é considerada amplamente diversificada, totalizando mais de 8.000 espécies atualmente conhecidas (FROST, 2019), e uma média de 150 novas espécies descritas por ano ao redor do mundo (AMPHIBIAWEB, 2019). Esses animais são considerados excelentes modelos em estudos de hipóteses filogeográficas (ZEISSET; BEEEBEE, 2008; NUÑEZ et al., 2011). Eles têm um ciclo de vida geralmente dependente de ecossistemas aquáticos e terrestres, pele altamente permeável, fisiologia ectotérmica, baixa capacidade de dispersão (WELLS, 2007; VITT; CALDWELL, 2013), são considerados extremamente suscetíveis a diversificar em espécies crípticas (BICKFORD et al., 2007; FOUQUET et al., 2007), assim, têm um alto potencial para a descoberta e descrição de novas espécies (KÖHLER et al., 2005). Por causa de seu baixo poder de dispersão, esses animais tendem a apresentar uma estrutura genética forte (JOHNS; AVISE, 1998), com áreas geográficas restritas (i. e., altas taxas de endemismo), sendo, portanto, mais propensos à extinção do que aqueles com ampla distribuição (COOPER et al., 2008; OCHOA-OCHOA et al., 2012; BRUM et al., 2013). No entanto, ao mesmo tempo em que são descobertas novas espécies, muitas outras também estão desaparecendo. Os anfíbios são um dos táxons mais ameaçados (FICETOLA et al., 2015) e o grupo mais ameaçado dentre os vertebrados (WAKE, 1991; STUART et al., 2004). Atualmente, a perda/fragmentação do habitat e as mudanças climáticas foram identificadas como as principais causas do declínio global dos anfíbios (STUART et al., 2004; FICETOLA et al., 2015; COHEN et al., 2018). O uso da terra para atividades humanas foi diretamente apontado como mais importante do que o clima na determinação da distribuição de anfíbios ameaçados, não apenas individualmente, mas estruturada filogeneticamente, ou seja, ameaçando linhagens inteiras (BRUM et al., 2013). Contudo, as mudanças climáticas estão sendo intensificadas por mudanças no uso e cobertura da terra (PIELKE, 2005) e alguns estudos recentes evidenciaram que as mudanças climáticas podem causar alterações nos padrões espaciais de diversidade de anfíbios (DUAN et al., 2016; VASCONCELOS et al., 2018), destacando ainda que os anuros são o grupo que mais pode sofrer com as mudanças climáticas no futuro (LOYOLA et al., 2014; VILELA et al., 2018; VASCONCELOS et al., 2018). Entre os anuros, a superfamília Hyloidea (i.e., que congrega a maioria das espécies) também mostra, filogeneticamente, um maior risco de extinção e está geograficamente concentrada na América Central e do Sul, e na Austrália, regiões que sofrem as maiores ameaças ambientais (COREY; WAITE, 2008). Levar em conta essa concordância filogenética e geográfica é essencial para a conservação desse grupo (COREY; WAITE, 2008) e considerando que a maioria das espécies permanece desconhecida, esforços para catalogar e explicar a origem e manutenção da biodiversidade merecem prioridade (BICKFORD et al., 2007).

Evitar a perda de habitat talvez seja o maior desafio para a conservação da biodiversidade global, e as medidas de conservação de habitat dependem frequentemente da estimativa da riqueza e do endemismo de espécies (BICKFORD et al., 2007). Inventários de biodiversidade de anuros baseados apenas na morfologia podem ser enganosos já que a evolução morfológica conservadora obscurece linhagens evolutivas divergentes que merecem reconhecimento e proteção (STUART et al., 2006). A especiação nem sempre é acompanhada por alterações morfológicas (BICKFORD et al., 2007) e a relação entre diferenciação genotípica e fenotípica nos estágios iniciais da especiação ainda é pouco compreendida (KAEFER et al., 2013). Assim, espécies crípticas, ou táxons que são morfologicamente semelhantes, mas geneticamente divergentes, são os principais desafios da delimitação de espécies (SINGHAL et al., 2018). No entanto, a delimitação de espécies é um tema complexo, em parte porque não há uma definição universalmente aceita de espécies (MALLET, 2013; RANNALA, 2015). Para o grupo dos anfíbios, o gene 16S provou ser suficientemente variável para identificar inequivocamente a maioria das espécies (VENCES et al., 2005), sendo ainda sensível à história evolutiva recente (KAEFER et al., 2013). Entre os métodos complementares para delimitação de linhagens candidatas a espécies, está seleção de modelos bayesianos, considerado apropriado para a identificação de espécies morfologicamente crípticas (LEACHÉ et al., 2018). Porém, vale ressaltar que o mais adequado nesse caso é considerar “espécies” que foram delimitadas nesses modelos como linhagens coalescentes e apenas apoiar espécies biológicas quando houver outros dados que suportem tal delimitação (i.e., morfologia, vocalização, fase larval, comportamento, etc.). Mas a partir do momento que a diferenciação genética é estabelecida, essas linhagens coalescentes podem também ser chamadas de Unidades de Manejo - MUs ou Unidades Evolutivamente Significativas - ESUs (MORITZ, 1994; DANTAS, 2013), que devem ser usadas como uma categoria para fins de conservação (MORITZ, 2002). Geralmente, as ESUs coincidem com espécies ou categoria sub-específicas que são comumente usadas para definir padrões atuais de bioviversidade (RULL, 2018). Podemos dizer que as MUs e as ESUs são populações dentro de espécies que estão se diferenciando ao longo do tempo evolutivo, a ponto de ser considerado um possível caminho (passo) para a especiação (DANTAS, 2013).

VIEITIES et al. (2009) e PADIAL et al. (2010), em estudos de anuros utilizando o gene 16S propuseram uma classificação para putativas espécies novas, assumindo três categorias: (i) espécies candidatas confirmadas (CCS - *confirmed candidate species*), consideradas aquelas que diferem claramente por caracteres morfológicos e bioacústicos e geralmente apresentam alta diferenciação genética, sendo supostamente distintas, mas formalmente não descritas e não nomeadas; (ii) espécies candidatas não confirmadas (UCS - *unconfirmed candidate species*) como linhagens genealógicas profundas - bioacusticamente e morfologicamente não estudadas e geralmente derivadas de populações geograficamente distantes - para as quais existem indicações gerais de que são distintas e não descritas; e (iii) linhagens genéticas profundas (DCL - *deep conspecific lineages*) aquelas linhagens genealógicas profundamente divergentes, estudadas, mas não tendo claras diferenças morfológicas ou bioacústicas com espécies descritas. Claro que, além do desafio de delimitar espécies ou linhagens evolutivamente distintas, também é crucial entender os fatores geográficos, climáticos e/ou ecológicos que podem apoiar a origem e a diversificação das linhagens dentro das espécies. Nesse sentido, a reconstrução de padrões filogeográficos de espécies crípticas poderia revelar locais de endemismo e diversidade ainda desconhecidos que poderiam justificar uma reconsideração de habitats ou locais de conservação específicos (BICKFORD et al., 2007).

Na presente Tese, nós utilizamos a filogeografia comparada para explorar a diversidade, os padrões de estrutura genética e a história demográfica de seis espécies de anuros das famílias Leptodactylidae e Hylidae (pertencentes à superfamília Hyloidea), que são codistribuídas nos campos subtropicais do sudeste da América do Sul (i.e., campos do Pampa e campos de Altitude). Levando em conta que as diferenças de história de vida entre as espécies podem afetar diferencialmente seus níveis e padrões de diversidade (FRANÇOSO et al., 2016), selecionamos duas espécies com baixa especificidade de habitat e ampla distribuição geográfica ao longo de biomas gramíneos e florestais do Sul, e quatro anuros com alta e moderada especificidade de habitat e distribuição restrita a habitats campestres. Se as espécies compartilham a mesma distribuição geográfica com padrões filogeográficos congruentes, isso indica que elas foram historicamente co-distribuídas e diferenciadas em resposta aos mesmos eventos geológicos ou ambientais (ZINK, 1996). Portanto, testamos se os processos históricos responsáveis pela demografia e diversificação atuaram de forma sincronizada nessas espécies co-distribuídas. Seguindo uma nova perspectiva no campo da filogeografia preditiva baseada em características (PAPADOPOULOU; KNOWLES, 2016; SULLIVAN et al., 2019), hipotetizamos que espécies com históricos de vida e distribuições geográficas semelhantes tenderão a mostrar padrões filogeográficos congruentes e a responder de forma semelhante às mudanças ambientais. Além disso, diferentes mecanismos de diversificação produzem diferentes padrões de diversidade entre regiões e grupos taxonômicos, de modo que estudos de casos específicos sobre grupos taxonômicos e/ou regiões geográficas específicas sugerem relações de diversificação causais razoavelmente detalhadas, embora muito heterogêneas (RULL, 2018). Portanto, um estudo utilizando anfíbios em uma abordagem comparativa de importantes regiões da América do Sul, como os Campos, ajudará a elucidar os padrões filogeográficos de maneira mais precisa e a compreender melhor a origem e a manutenção da biota sul-americana.

A Tese segue as normas da MDT (Manual de Dissertação e Teses) da UFSM, porém os capítulos estão organizados de acordo com as normas dos periódicos científicos aos quais pretendemos submeter. Assim, o primeiro capítulo segue as normas do periódico *Journal of Biogeography*, enquanto o segundo capítulo segue as normas do periódico *Journal of Evolutionary Biology.*

OBJETIVOS

Objetivo Geral

Investigar o padrão filogeográfico de múltiplas espécies de anuros com diferentes histórias de vida e co-distribuídas nos campos subtropicais do sudeste de América do Sul, levando em conta que as diferenças ecológicas entre as espécies podem impactar diferencialmente os níveis e padrões de diversidade registrados (Françoso et al., 2016). A Tese está dividia em dois capítulos, conforme os objetivos específicos a seguir: (1) acessar a diversidade genética e possíveis padrões de estruturação populacional, identificando a existência de congruência temporal e de história demográfica e como essas espécies responderam ás mudanças climáticas passadas e; (2) identificar a existência de congruência temporal em padrões de divergência, reconstruir o padrão de difusão filogeográfica no espaço e no tempo, bem como utilizar análises de delimitação de espécies para grupos genéticos profundamente divergentes.

Objetivos Específicos

Capítulo 1

Explorar a diversidade, os padrões de estrutura genética e a história demográfica de seis espécies nominais de anuros das famílias Leptodactylidae e Hylidae pertencentes a superfamília Hyloidea, que são co-distribuídos nos campos subtropicais do sudeste da América do Sul: I) espécies amplamente distribuídas em regiões florestais e abertas da América do Sul [*Leptodactylus latrans* (STEFFEN, 1815) e *Physalaemus cuvieri* Fitzinger (1826)]; II) espécies distribuídas ao longo dos campos do Pampa e campos de Altitude [*Scinax uruguayus* (SCHMIDT, 1944) e *Physalaemus gracilis* (BOULENGER, 1883)]; e III) espécies restritas aos campos do Pampa [*Phyllomedusa iheringii* (BOULENGER, 1885) e *Pseudis minuta* Günther (1858)]. Se as espécies compartilham a mesma distribuição geográfica com padrões filogeográficos congruentes, isso indica que elas foram historicamente codistribuídas e diferenciadas em resposta aos mesmos eventos geológicos ou ambientais (ZINK, 1996). Usamos a filogeografia comparada para testar se os processos históricos responsáveis pela demografia agiram de forma sincronizada nessas espécies cod-istribuídas e a modelagem de paleodistribuição para entender como era a provável distribuição dessas espécies mediante as mudanças climáticas passadas (i.e., a períodos glaciais e interglaciais).

Capítulo 2

Investigar o padrão filogeográfico recuperado para os dois anuros nominais co-distribuídos nos campos do Pampa e campos de Altitude: *Physalaemus gracilis* (BOULENGER, 1883) e *Scinax uruguayus* (SCHMIDT, 1944). Utilizamos a filogeografia comparada para testar a congruência temporal em eventos de divergência, isto é, se as espécies compartilham a mesma distribuição geográfica com padrões filogeográficos congruentes, isso indica que elas foram historicamente codistribuídas e diferenciadas em resposta a os mesmos eventos geológicos ou ambientais (ZINK, 1996). Nós reconstruímos o padrão de difusão filogeográfica no espaço e no tempo, e usamos análise de delimitação de espécie para verificar a existência de mais de uma linhagem evolutiva sob o mesmo nome de *Physalaemus gracilis* e *Scinax uruguayus*.

CAPÍTULO 1: PARTIALLY SYNCHRONOUS RESPONSE TO NEOGENE-QUATERNARY CLIMATIC DYNAMICS IN CODISTRIBUTED FROGS FROM THE SOUTHEASTERN AMERICAN SUBTROPICAL GRASSLANDS.

Ambiente de reprodução utilizado por anfíbios anuros, Campos Mistos de Andropogônias e Compostas, município de São Gabriel, RS, Brasil.
Foto: Suelen da Silva Alves Saccol.

Artigo em preparação para submissão no periódico ***Journal of Biogeography***

To be submmited to Journal of Biogeography

RESEARCH PAPER

Partially Synchronous Response to Neogene-Quaternary Climatic Dynamics in Codistributed Frogs from the Southeastern American Subtropical Grasslands.

Comparative phylogeography of frogs

Suelen S. A. Saccol[1,*] | Mariana L. Lyra[2] | Arley Camargo[3] | João G. R. Giovanelli[2]| Raul Maneyro[4] | Sonia T. Z. Cechin[1] | Célio F. B. Haddad[2]| Tiago G. Santos[5]

[1]*Departmento de Ecologia e Evolução, UFSM – Universidade Federal de Santa Maria, Centro de Ciências Naturais e Exatas, Prédio 17, sala 1140 - D, Cidade Universitária Camobi, Km 29 CEP 97105-900, Santa Maria, RS, Brazil*

[2]*Departamento de Zoologia e Centro de Aquicultura (CAUNESP), UNESP – Universidade Estadual Paulista, Campus Rio Claro, Av. 24 A, No. 1515, Bela Vista, CEP 13506-900 Rio Claro, SP, Brazil*

[3]*Centro Universitario de Rivera, Universidad de la República, Ituzaingó 667, Código Postal 40.000, Rivera, Uruguay*

[4]*Universidad de la República de Uruguay, Faculty of Sciences, Montevideo, Uruguay*

[5]*Universidade Federal do Pampa, Campus São Gabriel, Av. Antônio Trilha, 1847 CEP 97300-350, São Gabriel, RS, Brazil*

Correspondence: Saccol, S.S.A., Email: suelenbio.ufsm@gmail.com

ABSTRACT

Aim To reconstruct the historical phylogeographic pattern of codistributed frog species with constrating life histories in the subtropical grasslands of Southeastern South America (*Campos*), using comparative phylogeography.

Location Grasslands of Southeastern South America.

Taxa Anura, Amphibia

Methods We used mitochondrial DNA markers to access the genetic diversity and to characterize the geographical distribution patterns of six nominal frogs codistributed in the *Campos*. We used haplotypes networks, neutrality tests, Bayesian Skyline Plots, hierarchical bayesian computation to explore demographic history and temporal congruence of coexpansion. In addition, we modelled the distribution of these species to predict their contemporary and historical habitat.

Results We recovered a genetic diversity ranging from high to low values, species with and without genetic structuring and demographic histories with some peculiarities, but usually congruent, with demographic expansions on the Pliocene-Pleistocene period. Our models of paleodistribution have detected the effects of LGM and LIG on the distribution dynamics of target species that seems to have been favored by colder periods.

Main conclusions Our discovery highlights the presence of deep divergent lineages in frogs codistributed in the *Campos* and that climate dynamics of the Neogene-Quaternary were important in the demographic history of these species. In addition, it was possible to verify that the phylogeographic patterns recovered so far for typical species of open areas are complex and reinforce the idea that there is no single pattern of diversification acting. We point out that this is the first phylogeographic comparative study of frogs in the subtropical grasslands of Southeastern South America.

Keywords

Anurans, South America’ Grasslands, Conservation, Comparative Phylogeography, Climate Change, Demography History.

1 | INTRODUCTION

Despite the high biodiversity found in several groups of plants and animals, and the environmental diversity provided by the grasslands, little is known about the processes that have influenced the species diversification associated with grassland of South America (Turchetto-Zolet, Pinheiro, Salgueiro & Palma-Silva, 2013). Grassland ecosystems are open areas in which grassy or grassy-like plants, forbs, and shrubs form a relatively continuous “herbaceous layer” considered as old-growth vegetation harboring, high species richness, high endemism, and unique species compositions (Veldman et al., 2015). However, these ecosystems are poorly known and preserved, and they are under intense modification (e.g., land use), particularlly in the tropical and subtropical regions (Bond & Parr, 2010). The subtropical grasslands in Southeastern portion of South American (latitudinal range between 25°S and 35°S), referred hereafter as *Campos*, are commonly divided into the Pampa grasslands (present at Uruguay, part of Argentina and in the Brazilian Southern portion) and the Highland grasslands (forming a natural mosaic associated to the Araucarian forest in part of Southern Brazil (Andrade et al., 2015; Overbeck et al., 2015; Andrade et al., 2019). Phylogeographic studies of South American taxa can provide valuable information about the historical processes of diversification in this region (Turchetto-Zolet et al., 2013).

The species diversity is consequence of a long and complex history of evolutionary processes, ecological processes, and external environmental forces (Rull, 2011), and to identify and to understand these processes are the main objectives of phylogeography (Beheregaray, 2008). Some studies that have examined the phylogeographic patterns of organisms inhabiting subtropical grassland ecosystems of South America include, for example: plants (e.g., Fregonezi, Turchetto, Bonatto & Freitas, 2013; Maia, Zwiener, Morokawa, Silva-Pereira & Goldenberg, 2017; Silva, Antonelli, Lendel, Moraes & Manfrin, 2018; John, Mäder, Fregonezi, & Freitas, 2019), arthropods (Peres et al., 2015; Cristiano, Cardoso, Fernandes-Salomão & Heinze, 2016), mammals (e.g., Montes, Oliveira, Bonatto, Callegari-Jacques & Mattevi, 2008; Lopes, Ximenes, Gava & De Freitas, 2013; Roratto, Fernandes & Freitas, 2014; Quintela, Gonçalves, Bertuol, Gonzalez & Freitas, 2015), fishes (García, 2006), lizards (Felappi, Vieira, Fagundes & Verrastro, 2015; Villamil et al., 2019), and more recently, amphibians (Barraso, 2014; Langone, Camargo & de Sá, 2016; Abreu-Jardim, 2018; de Oliveira Miranda et al., 2019; Brusquetti, Netto, Baldo & Haddad, 2019). Neogene climatic, geological events and Quaternary climate fluctuations have been pointed out as the main responsible for the diversification of the biota in this region (Ortiz-Jaureguizar & Cladera, 2006). However, as pointed out by Turchetto-Zolet et al., (2013), species associated with open vegetation areas have presented variable responses to climatic oscillations, for example, suggesting more complex phylogeographic patterns in the biota of South America. Even with an increase in publications, the studies related to phylogeography and historical demography are still very scarce in South America when compared to other regions of the world (Beheregaray, 2008; Turchetto-Zolet et al., 2013). In addition, among the existing studies, none of them tested comparatively if codistributed species or genetic groups responded in a synchronous way to the events of Neogene-Quaternary in this region. This information type is very important to know the evolutionary history of species and to understand how they have responded to past events, besides to provide us with hints on how they could respond to current environmental changes (Turchetto-Zolet et al., 2013).

Amphibians are considered excellent models in studies of phylogeographic hypotheses (Zeisset & Beeebee, 2008; Nuñez, Wood, Rabanal, Fontanella & Sites Jr, 2011). They have a life cycle generally dependent on aquatic and terrestrial ecosystems, a highly permeable skin, an ectothermic physiology, low dispersal abilities (Wells, 2007; Vitt & Caldwell, 2013), and are considered extremely susceptible to diversify into cryptic species (Bickford et al., 2007; Fouquet et al., 2007). Due to the low dispersal power, amphibians tend to present a strong genetic structure (Johns & Avise, 1998), with species restricted to narrow geographic ranges (i.e., high rates of endemism), being thus, more prone to extinction than those with wide distributions (Cooper, Bielby, Thomas & Purvis, 2008; Ochoa-Ochoa, Rodríguez, Mora, Flores-Villela & Whittaker, 2012; Brum et al., 2013). Amphibians are one of the most imperilled animal taxa (Ficetola, Rondinini, Bonardi, Baisero & Padoa-Schioppa, 2015) and the most threatened group of vertebrates (Wake, 1991; Stuart et al., 2004), being currently the habitat loss/fragmentation and climate changes identified as of the major causes of their global decline (Stuart et al., 2004; Ficetola et al., 2015; Cohen, Civitello, Venesky, McMahon & Rohr, 2018). According to Brum et al. (2013), land use was directly more important than climate in determining the distribution of threatened amphibians, not only individually, but phylogenetically structured, that is, threatening entire lineages. Some recent studies have also shown that climate change may cause shifts in spatial patterns of amphibian diversity (Duan, Kong, Huang, Varela & Ji, 2016; Vasconcelos, Nascimento & Prado, 2018) and, that the anurans are potentially, the most susceptible group to future climate changes (Loyola, Lemes, Brum, Provete & Duarte, 2014; Vilela, Nascimento & Vital, 2018; Vasconcelos et al., 2018). Among the amphibians, the superfamily Hyloidea (i.e., most frog species) presents the higher risk of extinction and are geographically concentrated in Central and South America and Australia, who also suffer from the greatest threats (Corey & Waite, 2008). Taking into account this phylogenetic and geographical concordance is essential for the conservation of this group (Corey & Waite, 2008).

Herein, we used comparative phylogeography to explore the diversity, patterns of genetic structure, and the demographic history of six nominal frogs from the Leptodactylidae and Hylidae families (hyloid frogs), that are codistributed in the *Campos*. Considering that distinct life histories between species may differentially affect the levels and patterns of diversity (Françoso, Zuntini, Carnaval & Arias, 2016), we selected two species with low habitat specificity and wide geographical distribution along grassy and forested biomes of South America, and four nominal frogs with high to moderate habitat specificity and distribution restricted to grassland habitats. If species share the same geographic distribution with congruent phylogeographic patterns, this indicates that they have been historically codistributed and differentiated in response to the same geological or environmental events (Zink, 1996). Therefore, we tested whether the historical processes responsible for demography have acted synchronously in these codistributed species. Following a new perspective in the field towards trait-based, predictive phylogeography (Papadopoulou & Knowles, 2016; Sullivan et al., 2019), we expect that species with similar life histories and geographical distributions will show congruent phylogeographic patterns, and to respond in a similar fashion to environmental changes. In addition, different mechanisms of diversification have produce different patterns of diversity between regions and taxonomic groups, so specific case studies on particular taxonomic groups and/or geographical regions suggest fairly detailed, although very heterogeneous, causal diversification relationships (Rull, 2018). Therefore, assessing a frog group from an important region of South America, such as the *Campos*, using a comparative approach will help us to elucidate phylogeographic patterns more accurately and to understand better the origin and maintenance of the South American biota.

2 | MATERIAL AND METHODS

2.1 | Selection of species and sampling

We selected nominal species with constrating life histories, including specialist and generalist ecologies, differential reproductive modes, and distribution area in natural grasslands of Southeastern South America. The selected species show three basic geographical patterns (Santos, Iop & Alves, 2014): I) widely distributed along forested and open vegetation of South America [*Leptodactylus latrans* (Steffen 1815) and *Physalaemus cuvieri* Fitzinger 1826]; II) species restricted to both Pampa and Highland grasslands [*Scinax* *uruguayus* (Schmidt 1944) and *Physalaemus gracilis* (Boulenger 1883)]; and III) species restricted to Pampa grasslands [*Phyllomedusa iheringii* (Boulenger 1885) and *Pseudis minuta* Günther 1858] (Fig. A2.1). Currently all these target species are included in the least concern category (LC) by the International Union for Conservation of Nature (IUCN 2019).

For all species we tried to sample one to 16 individuals per locality along the known distribution, but with emphasis in the subtropical grasslands of Southeastern South America - *Campos* (Fig. 1., modified from Andrade et al., 2015, using GNU Image Manipulation Program - GIMP 2.8 software), and considering information on ongoing taxonomic revisions. For that, we first generated distribution maps based on the IUCN report (Fig. 2 a. to f., see Table A1.1-6.) (Kwet, Garcia, Silvano &Langone, 2004; Kwet, Lavilla, Faivovich & Langone, 2004; Silvano, García, Kwet, Lavilla & Langone, 2004; Heyer et al., 2010; Lavilla, Kwet, Segalla, Langone & Baldo, 2010; Mijares, Rodrigues & Baldo, 2010), using ArcGIS® software, version 10.1 (ArcGIS 2011). We then updated the species distribution information to avoid sample populations that are other species confirmed. About nominal species, perhaps *Physalaemus gracilis*, from the Highland grasslands of Brazilian Atlantic Forest belongs to the recently described *Physalaemus carrizorum* Cardozo & Pereyra (2018) from part of Argentina. However, there are no publications testing this hypothesis and in this case, we decided to keep samples from that region in our analyzes without taxonomic distinction from the nominal *P. gracilis*. For *Physalaemus cuvieri* we regarded Lourenço et al. (2015) and data from de Oliveira Miranda et al. (2019), sampling localities just within the distribution of the monophyletic south clade (clade A; de Oliveira Miranda et al. 2019) which are believed to be the *P. cuvieri sensu stricto* (central-south Brazil). *Leptodactylus latrans* is currently considered as a species complex (De Sá et al., 2014). For this species we selected localities based on ongoing taxonomic revision that includes South populations and *L. latrans sensu* species (CFBH, MLL pers. Comm.). The species were sampled according to the maps, the availability of tissues in the biological collections and the nomenclature used following Jetz & Pyron (2018).

We obtained tissue samples from 371 individuals, considering the material available in herpetological collections and in field expedictions. The number of individuals, localities sampled per species and list of abbreviations used in the code of the individuals are available at Table A1.1 to A1.6 and Table A1.13, in Appendices. Voucher specimens of most samples are deposited in the following collections: 'Célio F. B. Haddad' amphibian collection (CFBH) at the Departamento de Zoologia, Instituto de Biociências, Universidade Estadual Paulista – UNESP, Rio Claro, SP, Brazil; Herpetological collection of the Universidade Federal de Santa

Maria (UFSM) and Herpetological collection of the Universidad de la República (Udelar, Uruguay).

2.2 | Laboratory protocols

We isolated the Total genomic DNA using ammonium acetate precipitation method (Maniatis, Fritsch & Sambrook, 1982) or glass fiber extraction protocol (CCDB - Canadian Center for DNA Barcoding, Ivanova, Dewaard & Hebert, 2006). We PCR amplified a fragment of the 16S ribosomal RNA gene (16S, ~543 bp) using primers 16SAR 'CGCCTGTTTATCAAAAACAT' and 16SBR 'CCGGTCTGAACTCAGATCACGT' described in Kessing et al. (1989). We used Taq DNA Mastex mix (Ampliqon II) to perform PCR amplifications with a combination of standard and touchdown thermal-cycling profiles used in frogs (Lyra, Haddad & De Azeredo–Espin, 2017). We purified the PCR products with enzymatic reactions and sent them to Macrogen Inc. (Korea) for sequencing. We visualized the resulting chromatograms, edited and cleaned using Geneious® 9.1.4 program (www.geneious.com, Biomatters; Kearse et al., 2012), and subsequently aligned the sequences of each species using MUSCLE software (Edgar, 2004) implemented in MEGA 7.0 (Kumar, Stecher & Tamura, 2016). We searched each sequence against the NCBI database using BLAST (Basic Local Alignment Search Tool, blast.ncbi.nlm.nih.gov/Blast.cgi; Altschul, Gish, Miller, Myers & Lipman., 1990) to confirm identity. We submitted the sequences to GenBank (accession numbers will be provided upon manuscript acceptance).

2.3 | Genetic diversity and phylogeographic patterns

For each species, we calculated the index of nucleotide diversity (π), number of haplotypes (h), haplotype diversity (Hd), and number of polymorphic sites (s), using DNAsp 5 (Librado & Rozas, 2009). To graphically represent the genetic variation of the 16S, we collapsed the sequences into haplotypes using DnaSP 5 (Librado & Rozas, 2009) and constructed haplotype networks in PopART (Leigh & Bryant, 2015) with Median-Joining network method (Bandelt, Forster & Rohl, 1999). We edited manually the networks using contrasting colors to represent genetically and geographycally structured group. To estimate the average genetic distances within species and between/within clade we used the uncorrected *p*-distance and pairwise deletion in the software MEGA 7.0 (Kumar et al., 2016), based in 1000 boostrap replications.

2.4 | Demographic histories

We used several methods to explore the demographic history of species. We performed the neutrality Fu's test Fs* (Fu, 1997), based on the diversity of haplotypes. We also estimated D* and F* of Fu and Li (Fu & Li, 1993), that considers the total number of derived nucleotide variants and the mean pairwise difference between sequences, respectively (Ramírez-Soriano, Ramos-Onsins, Rozas, Calafell & Navarro, 2008).

To estimate the change in population size over time, we used a Bayesian Skyline plot (BSP) analyses (Drummond, Rambaut, Shapiro & Pybus, 2005), implemented in BEAST software v1.8.4 (Drummond, Suchard, Xie & Rambaut, 2012). Analyses were conducted for each species and for clades that co-occurs in the *Campos* region when we found geographycally structured population within species (i.e, for *Leptodactylus latrans*, *Physalaemus gracilis* and *Scinax uruguayus*; see tree topology results), since population size changes might be affected by population structure (Heller, Chikhi & Siegismund, 2013). The best-fit substituition model chosen by jModelTest v.2.0 (Darriba, Taboada, Doallo & Posada, 2012), we used a random starting tree, and the tree prior we assumed the coalescent Bayesian skyline demographic process. Markov chain Monte Carlo (MCMC) analyses were performed assuming the model "Strict Clock" calibrated with the mutation rate of the 0.26% (0.0026 substitutions/million years, SD = 0.0001) following Evans, Kelley, Tinsley, Melnick & Cannatella (2004), and Lemmon, Lemmon & Cannatella (2007). All BSP analyses consisted in one independent runs of 50 million generations and sampled every 5.000 steps with the first 10% discarded as burn-in. Plots were reconstructed in Tracer v1.6 (Rambaut, Suchard, Xie & Drummond, 2014), which summarizes the population size (Ne) parameters sampled across branches of coalescent trees as a function of time. Results were considered when Effective Sample Size (ESS) ≥ 200.

Additionally, we used an approximate and hierarchical approximate bayesian computation (hABC) for detecting the temporal congruence in expansion events. According to Chan, Schanzenbach & Hickerson (2014), this method combines population genetic data sets from multiple taxa into a single analysis to estimate the proportion of a community sample that demographically expanded in a temporally clustered pulse and when the pulse occurred. Thus, based on the results of the previous demographic analyzis, i.e., for species that showed signs of demographic expansion (five species, see results below), we used the hBayeSSC software to test for shared expansion times (github.com/UH-Bioinformatics/hBayeSSC) (Chan et al., 2014). We estimated the hyperparameter Z (ζ) that represents the proportion of species that coexpanded, ranging from Z = 0 (all asynchronous expansions), $0 < Z < 1$

(partially synchronous), to Z = 1 (all synchronous coexpansions) (Chan et al., 2014). We sampled from prior, species-specific parameter distributions using 500,000 coalescent simulations for each possible Z value (Z = 0.0, 0.2, 0.4, 0.6, 0.8, and 1; 3 million simulations total). Prior distributions of parameters are described in the Appendice A3.1. We approximated the posterior distribution of Z with algorithm ‘neural net’ using the R package 'abc' (Csillery, Francois & Blum, 2012). In addition, we performed a principal component analysis (PCA) of the species-specific summary statistics to identify which species did not coexpand with the other four (Chan et al., 2014). The mutation rate considered in this analysis was 0.26% and a generation time of two years. We used as reference the sexual maturation recently reported for some species of this region, which varied from one to three years (Caldart, Loebens, Brum, Bataioli & Cechin, 2019 and A.J.C. Brum, 2019; T.F. Theis, 2019 unpublished data).

2.5 | Ecological niche modeling

We use ecological niche modeling to produce maps of potential distribution, past and present, in order to identify areas within the distribution of our nominal species that most likely to have remained stable during the Pleistocene. We used the points of presence of the species, listed in Appendice (Table A1.1-6.). We used the maximum entropy algorithm implemented in MAXENT 3.4.1 (Phillips, Anderson & Schapire, 2006). As model parameters in Maxent, we used 500 interations, 5,000 background points, auto features, random seed, analysis of variable importance, and response curves. We used replicated run type bootstrap to create 10 random partitions. Each partition consisted of 75% of the dataset used for SDM calibration (training), with the other 25% used for model evaluation (testing). After this, the result was an average model of these 10 partitions. Predictive maps were generated from a threshold of minimum presence. We first modeled the current distribution using selected current climate variables available in WorldClim v 2.0 database (Fick & Hijmans, 2017) (Table A1.7.). We calculated the Pearson correlations among the predictors variables to exclude the highly correlated ones (r > 0.75). All predictors variables have 5 minutes of resolution. After, the result of the average model developed in the current climate was projected in three paleoclimate scenarios with same bioclimatic variables, one simulating the last interglacial period (LIG) 130,000 years ago (Otto-Bliesner, 2006, available at: http://pmip2.lsce.ipsl.fr/), and two simulating the last glacial maximum period (LGM) 21,000 years ago: CCSM4 (Community Climate System Model, available at: http://www.ccsm.ucar.edu), and MIROC (Model of Interdisciplinary Research on Climate, available at: http://www.ccsr.utokyo.ac.jp/kyosei/hasumi/MIROC/tech-repo. pdf). The resulting models was evaluated with the underlying area (AUC) of Receiver Operating Characteristics Curve (ROC) for the modeling algorithm (Fielding & Bell, 1997; Manel, Williams & Ormerod, 2001). The AUC ranges from 0.5 (random accuracy) to a maximum value of 1.0 (perfect discrimination).

3 | RESULTS

3.1 | Genetic diversity and phylogeographic patterns

The genetic and phylogeographic patterns varied among that target species. We found high to moderate values of haplotype diversity (Hd) for almost species, except *Phyllomedusa iheringii*. *Physalaemus cuvieri* (mean ± standard deviation = 0.950 ± 0.012), *Scinax uruguayus* (0.931 ± 0.016), *Leptodactylus latrans* (0.850 ± 0.021), *Physalaemus gracilis* (0.877 ± 0.040), *Pseudis minuta* (0.732 ± 0.039) and *Phyllomedusa iheringii* (0.186 ± 0.110). Most target species presented low nucleotide diversity (Table 1.).

The phylogeographic patterns reported for the six nominal frogs codistributed in the *Campos* can be divided into two groups: (i) species with only one genetic group not geographycally structured throughout their distribution - *Physalaemus cuvieri*, *Pseudis minuta* and *Phyllomedusa iheringii* (Fig. 3., a to c; Fig. A2.2., Table A1.8. to 10.). (ii) species with more than one genetic group geographically coherent - *Leptodactylus latrans*, *Physalaemus gracilis* and *Scinax uruguayus* (Fig. 3., d to f; Fig. A2.2., Table A1.11 to 13).

We recovered 35 haplotypes for *Physalaemus cuvieri* and a mean genetic distance within haplotypes of 0.9% (p-distance ± standard deviation = 0.009 ± 0.002). *Pseudis minuta* presented 10 haplotypes and a genetic distance of 0.2% (0.002 ± 0.001) among haplotypes (Fig. 3a, b, respectively). In contrast, *P. iheringii* showed only three haplotypes and low differentiation, that is, only 0.004% (0.0004 ± 0.0003) (Fig. 3c.).

We recovered two well-structured genetic groups for *Leptodactylus latrans*, separated by thirteen mutational steps: one composed by individuals from the northern boundary of the Highland grasslands and another composed by individuals from localities in Southwest and Southern Brazil. Of the 28 haplotypes reported, 18 of them comes from the South Clade (Southern region of South America), and 10 comes from the Northwest Clade (Fig. 3d.). In the *Campos* occurs only the South Clade *L. latrans*. The mean genetic distances between haploclades of *L. latrans* was 4.8% (0.048 ± 0.009). The mean p-distance within *L. latrans* was 2.5% (0.025 ± 0.004) for North Clade+South Clade, 1.2% (0.012 ± 0.003) for South Clade and 2.1% (0.021 ± 0.005) for North Clade.

We recovered a geographically coherent genetic structure for two frog species in the *Campos*, *Physalaemus gracilis* and *Scinax uruguayus*. The evolutionary history of *Physalaemus gracilis* showed strong structure, with high divergence between genetic groups. We found three genetic populations for this species: a population of the North Clade (comprised by samples of the Highland grasslands), which is separated by 31 mutational steps from a genetic population that we named South Clade1 (composed entirely by samples from the Pampa grasslands). South Clade1 is separated by 20 mutational steps from a third genetic population named South Clade2 (composed by samples from the Brazilian Pampa coastal region) (Fig. 3e.). The mean genetic distance within *P. gracilis* was 2.7% (0.027 ± 0.003) for all samples. Distances within each genetic group were: 0.9% for North Clade (0.009 ± 0.003), 0.3% for South Clade1 (0.003 ± 0.002) and 0.5% for South Clade2 (0.005 ± 0.001). The genetic distance between the *P. gracilis* haploclades were: 5.7% for South Clade1 x South Clade2 (0.057 ± 0.011), 8.3% for South Clade1 x North Clade (0.083 ± 0.012) and 9.9% South Clade2 x North Clade (0.099 ± 0.014).

We found for *Scinax uruguayus* at least two well-structured genetic groups, separated by thirteen mutational steps. One clade was composed entirely by individuals from the Pampa grasslands (except for samples from the Alegrete locality, in Western of the Brazilian Pampa) and all samples from the Uruguayan Pampa; and a second clade composed by individuals from the Highland grasslands of the Atlantic Forest along the Brazilian states of Paraná, Santa Catarina and Rio Grande do Sul, plus Alegrete individuals, respectively (Fig. 3f.). The mean genetic distance within *S. uruguayus* was 2.7% (0.027 ± 0.005) for North Clade + South Clade, 1.2% (0.012 ± 0.003) for North clade, and 0.7% (0.007 ± 0.002) for South clade. The mean distance between the two haploclades reported for *S. uruguayus* was 4.5% (0.045 ± 0.008).

3.2 | Demographic histories

We recovered similar demographic patterns for most genetic groups of the six nominal frogs codistributed in the *Campos*. The neutrality tests were all statistically negative for *Leptodactylus latrans* (South Clade) (Fig. 4a.), *Physalaemus cuvieri* (Fig. 4b.) and *Pseudis minuta* (Fig. 4e.). The neutrality values for *Scinax uruguayus* (South and North Clade) and *Physalaemus gracilis* were not significant (Fig. 4c., d. and f.). The demographic pattern recorded for the *Phyllomedusa iheringii* was not well defined, since the neutrality tests were negative, but not significant, and BSP presented a population of constant size, but with large confidence intervals (Fig. 4g.). The BSP showed population expansion for *Leptodactylus* *latrans* (South Clade) (~250 kya, Fig. 4a.), *Physalaemus cuvieri* (~260 kya, Fig.4b.), *Scinax uruguayus* (South Clade) (~350 kya, Fig. 4c.), and *Pseudis minuta* (~100 kya, Fig. 4d), besides an expansion signal for *Physalaemus gracilis* (South Clade1) (~100 kya, Fig. 4e). In contrast, BSP for *Scinax uruguayus* (North Clade) showed signal of population bottleneck, starting at ~120 ka (Fig. 4f.). Besides these evidences, a star-like pattern was recorded in haplotype networks of the *P. cuvieri*, *P. minuta*, *L. latrans* (South Clade), *P. gracilis* (South Clade 1), and *S. uruguayus* (South Clade).

The algorithm used in the coexpansion hABC analysis support a hyperparameter Z value of 0.8, indicating that four of the five genetic groups tested demographically expanded in a temporally clustered pulse (i.e., a partially synchronic coexpansion) (Fig. A2.3). Species that probably coexpanded at the same time pulse were *P. cuvieri*, *L. latrans* South Clade, *S. uruguayus* South Clade and *P. gracilis* South Clade1 (Mean = 1.878,132 CI = 1.803,836 – 1.964,574 generations before the present) and *P. minuta* the species that expanded randomly, that is, in an independently expanded time pulse (Mean = 892,435 CI = 624,882 – 1.246,970 generations before the present) (Fig. 5a). These values in generations correspond to approx. 3.756 Mya (CI = 3.617-3.929) and 1.784 Mya (CI = 1.249-2.493), respectively. The PCA (based on discrete summary statistics) helped us to identify species that probably did not coexpand with the other four, and although these are not so obvious, due to the adjustments of the model, it is possible to observe that the orange circles (*P. minuta*) overlap other species (Fig. 5b). Our partially synchronous expansion model was summarized (Fig. 6.) following the scheme of Chan et al. (2014).

3.3 | Ecological niche modeling

The current potential distribution model presented better performance than a random model and this is observed by AUC mean values ranging from 0.98 to 0.99 (Appendice Fig. A2.4 to A2.9). The current models provided an area compatible with the current distribution of target species. It is observed a superprediction of the distribution of *Leptodactylus latrans* and *Physalaemus cuvieri* to the northernmost region of the LGM, with less distribution in the LIG and now more restricted to the south (Fig.A2.4 and A2.6). The other species analyzed did not showed remarkable changes of distributions, but presented a better distribution during the LGM (Fig.A2.7 to A2.9).

4 | DISCUSSION

Exploring the phylogeographic pattern of the six nominal frogs codistributed in the *Campos*, we found that the diversification processes in this region are complex and that, in fact, species associated with open vegetation domains present variable responses to historical events (Turchetto-Zolet et al., 2013). We expected to find phylogeographic patterns directly related to distribution patterns and species specificities (generalists *versus* specialist), but we recovered genetic diversity that varied from high to low levels, species with and without genetic structuring and demographic histories with some peculiarities, but congruent in their majority. Demographic analyzes recovered population expansions during a glacial period for most species, with partially synchronized expansion pulses. In addition, our models of paleodistribution have detected the effects of historic climate change on the distribution dynamics of target species. In general, the grassland anuran species seem to have been favored by colder periods (Abreu-Jardim 2018; this study), except for a typical grassland frog that was favored by warmer periods (Langone et al., 2016). Thus, it appears that different ecologies and life histories influence the process of species diversification and that there is no single pattern for specialist and generalist species in open environments. Thus, we reinforce that the interpretation of diversification patterns needs to consider the interaction of biology, geography, and climatic changes (Hewitt, 2000).

Most frog species codistributed in the *Campos* showed high haplotype diversity, as found in previous studies from this region (Barraso, 2014; Langone et al., 2016; Abreu-Jardim, 2018; de Oliveira Miranda et al., 2019). The high genetic diversity seems to be related to the abundance of species throughout their distribution, since according to Pabijan, Wollenberg & Vences (2012), high levels of genetic diversity within populations are associated with high effective population size during long periods of time. Most of our target species presented low nucleotide diversity, similar to reported for *Physalaemus fernandezae* and *Physalaemus cuvieri* (Barraso, 2014; de Oliveira Miranda et al., 2019), but opposite of the recorded for *Physalaemus henselii, Pseudopaludicola falcipes*, and *Scinax squalirostris* (Barraso, 2014; Langone et al., 2016; Abreu-Jardim, 2018). High haplotype diversity and low nucleotide diversity also refer to signatures of population expansion (Arenas, Ray, Currat & Excoffier, 2011), as reported for *Physalaemus cuvieri*, *Leptodactylus latrans* (clade that occurs in the *Campos* – South Clade*), Physalaemus gracilis* (South Clade1), *Scinax uruguayus* (South Clade), and *Pseudis minuta*. Some frogs in our study fit Frankham's (1996) prediction that species with larger distribution ranges are expected to show greater genetic diversity than restricted species and thus have a low risk of extinction. In fact, none of the target species in this study are in threat categories (IUCN, 2019). In addition, *Phyllomedusa iheringii* also fitted to Frakkham' predictions, since this frog represented the case of restricted distribution and an extremely low genetic diversity, for haplotype and nucleotides. Although Brunes, Sequeira, Haddad & Alexandrino (2010) reported a high haplotype diversity and low nucleotide diversity for *P. iheringii*, they used a larger and more variable marker (ND2) and only six individuals, reporting only four haplotypes for this marker. In our study, we analyzed 21 individuals throughout the know distribuition of *P. iheringii* and reported only three haplotypes. Therefore, *P. iheringii* seems to have actually low mitochondrial genetic variation but more markers should be investigated. According to Galtier, Depaulis & Barton (2000), low genetic diversity may be the result of a recent demographic event, for example, a "bottleneck" or "selective sweep". In fact, loss of genetic variability can also indicate high degree of inbreeding and species that exhibit this pattern may be more threatened by environmental variations. Genetic variability allows populations to maintain *fitness* and also the ability to respond to environmental changes (Moritz, 2002). *Phyllomedusa iheringii* has very specific ecological characteristics, since this species is endemic to the Pampa grasslands (Santos et al., 2014) and it is strongly associated to rocky outcrops, and presents specific requirements for reproduction (Maneyro & Langone, 2001; Dias et al., 2017). Besides that, it has low abundance in Pampa grasslands of Brazil (Iop, 2015; Bolzan, Saccol & Santos, 2016), where, in addition, it was mostly found in areas with a higher percentage of grassland remaining (Iop, 2015). This situation is worrying when we consider that Pampa grasslands have been historically modified by human action (Bilenca & Miñarro, 2004). Studies have documented that agricultural development (such as crops) can destroy the habitats (Joly, Miaud, Lehmann & Grolet, 2001; Benton, Vickery & Wilson, 2003; Biedrzycka & Konopinski, 2008; Santos et al., 2015), resulting in population bottlenecks that may cause genetic diversity loss (Wang et al., 2014). Therefore, we recommend that an approach using a larger number of molecular markers, mitochondrial and nuclear, is necessary to better clarify the evolutionary history of this endemic Pampa' species.

In relation to the population structure, we recovered: a first group composed by species that showed only one genetic group and not geographycally structured (i.e., *Physalaemus cuvieri*, *Pseudis minuta,* and *Phyllomedusa iheringii*); and a second group composed by species that showed more than one genetic group geographically coherent (i.e., *Leptodactylus latrans*, *Physalaemus gracilis,* and *Scinax uruguayus*). Species with distribution restricted to the Pampa grasslands, *Pseudis minuta* and *Phyllomedusa iheringii*, presented a genetic group throughout this ecosystem, as well as reported for another pampean species *Pseudopaludicola* *falcipes* (Langone et al., 2016). Although endemic to the Pampas, these two species differ markedly in natural history and habitat requirements. As previously mentioned, *Phyllomedusa iheringii* is specialist and has low abundance in the Pampa grasslands. The record of only a single genetic population may be related to a higher dispersion rate of males in the search for females during the reproductive period (TGS, SSAS pers. Comm.). *Pseudis minuta* presents high abundance in different types of aquatic environments, it is considered a highly plastic species regarding habitat use (i.e., may be present in anthropogenic environments) and it is associated with permanent waterbodies with floating or rooted vegetation (Huckembeck et al., 2012; Maneyro & Carreira, 2012). These characteristics certainly contributed to the maintenance of a single genetic population along the grasslands. In addition to *Pseudis minuta*, *Phyllomedusa iheringii*, and *Physalaemus cuvieri*, the Clade South of *Leptodactylus latrans* also showed a unic genetic population along the *Campos*. Although we have reported genetic structuring in this species, it is known that the *L. latrans* group is a species complex (De Sá et al., 2014). Moreover, a recent study has recovered a deep phylogenetic structure between lineages of *Physalaemus cuvieri* (de Oliveira Miranda et al., 2019), previously reported in Conte, Targueta, Zucchi, Souza & Recco-Pimentel (2014) and Lourenço et al. (2015). The final elevation of the Brazilian Central Plateau in Miocene-Pliocene transition was mentioned as having an important role in the initial divergence of the lineages of *P. cuvieri* and watersheds must have had an important effect on the spatial pattern of secondary diversification in the genetic diversity of these lineages under the name of *P. cuvieri* (de Oliveira Miranda et al. 2019). Abreu-Jardim (2018) also reported structuring in a widely distributed frog (*Scinax squalirostris*), but with only one clade codistributed in the *Campos*. The deeper divergence in *S. squalirostris* dating from the late Miocene and most of the lineage divergence in this species occurred during the Pliocene-Pleistocene transition. Currently, *Leptodactylus latrans* is a large-sized terrestrial anuran, widely distributed in South America and it has a wide range of habitat usage, living from natural grasslands as well as highly modified agroecosystems (Maneyro & Carreira, 2012). *Physalaemus cuvieri* is also a widely distributed species (from the North of Brazil to Northern Uruguay where is the Southern limit of the geographical distribution). There are records of this species in Bolivia, Paraguay, Misiones province and Northern Argentina (Maneyro & Carreira, 2012). *P. cuvieri* is a generalist species commonly found in human-disturbed areas. It breeds in cattle ponds or temporary waterbodies such as puddles, often in water-filled hoof tracks (Mijares et al., 2010).

Physlaemus gracilis and *Scinax uruguayus* presented a concordant geographic structure with strongly divergent genetic groups in the *Campos*. For the North Clade of *Physalaemus gracilis*, there is strong evidence that it is a genetically distinct group, specially because of some differences previously reported in the advertisement calls and morphology, there is the stimulus to use in the literature the name *P.* aff. *gracilis* (e.g. Kwet, Lingnau & Di-Bernardo, 2010). We suspected that this genetic group represents *P. carrizorum*, a species recently described for part of Argentina (Cardozo & Pereyra, 2018), but unfortunately, there are no molecular analyzes incluinding samples of this species to support our hypothesis. Still for *P. gracilis*, we recovered a strongly divergent structure within the Pampa grasslands, evidenced by two groups South Clade 1 and South Clade 2. The last clade mentioned was restricted to the coastal region of the Pampean grasslands. The coastal region is considered prone to present endemic species due to successive events of sea advancement and retraction during the Quaternary, which may have isolated populations of species promoting genetic differentiation over time (Quintela et al., 2015). Perhaps these events may not have been a barrier of dispersion to *Pseudis minuta* e and *Leptodactylus latrans*, but may have been a barrier to *P. gracilis*. The frogs *P. minuta* and *L. latrans* may be favored by passive dispersion via hydrochoria, a common transport mechanism in adult frogs and larvae already reported for these and other grassland frogs (Achaval, Gonzalez, Meneghel & Melgarejo, 1979; Prigione & Langone, 1983). Already, for *P. gracilis* (South Clade 2) is possible that it has remained isolated for a long period of time accumulating genetic differences and thus generating the highly divergent pattern that we recorded in relation to other pampean population (South Clade 1). Barraso (2014) also reported to other pampean frogs, *Physalaemus fernandezae* and *P. henselii*, diverged as a result of events of a marine advance. In this case, it is probable that marine transgression events were fundamental in the process of diversification of *Physalaemus gracilis* in the coastal region, but a future denser sampling of individuals in this region would help to better elucidate their evolutionary history. The efficiency of dispersion barriers depends not only on the nature of the environment but also on the characteristics of the organism itself, which vary from one taxonomic group to another, so that specific barriers may not affect all residents of a habitat in the same way (Brown & Lomolino, 2006).

For *Scinax uruguayus*, we also recovered two highly divergent clades (North Clade and South Clade) and highlighted that the Western region of the Brazilian Pampa (municipality of Alegrete) needs special attention in future studies, since all individuals of this region were grouped with North Clade (surprisingly not with the South Clade). The pattern of divergence found for *Scinax uruguayus* and for *Physalaemus gracilis* shows a similar phylogeographic break division in South and North Clades. Thus, these genetic groups associated with the break could have been influenced by the same geomorphological processes that occurred in this region, for example, the uplift of the Brazilian Meridional Plateau, subdividing the landscape into higher lands and a topographically lower portion (Basaltic Plateau and Peripheral Depression) (Verdum et al., 2019). The action of ridges and mountain uplifts as mechanism promoving gene flow disruption and divergence between populations is well-known (Turchetto-Zolet et al., 2013). *Scinax uruguayus* is recorded in the Southeastern range (Lipinski & Santos, 2014), but not is found in lowlands (such as the Central Depression or Coastal Plain of the Brazilian Pampa). *Physalaemus gracilis*, according to Maneyro & Carreira, (2012), can be found from the State of Paraná (Brazil) to the South of Uruguay, as well as in the Province of Misiones (Argentina) (but see discussion above). As reported for *Scinax squalirostris* (Abreu-Jardim, 2018), these two species presented high genetic diversity and well-structured genetic groups throughout their geographic distributions. We reported high percentages of genetic divergence between the clades for both species and according to Fouquet et al. (2007), divergences above 3%, generally indicate the occurrence of distinct species.

Although the species presented different phylogeographic patterns, we recovered a partially congruent demographic history for most frog species. Neutrality tests indicate an excess of rare polymorphisms in a population, which is consistent with demographic events of population expansion and the star-like pattern recorded in haplotype networks reflecte a recent increase in population sizes from a small population source (Slatkin & Hudson, 1991). When we evaluate demographic histories independently, i.e., through the BSPs, we observed genetic populations with expansions beginning in ~ 100 ka, ~ 250 ka, ~ 260 ka and ~ 350 ka. These times correspond to the colder periods during the Quaternary (de Oliveira, Carneiro, Vecchia & de Mello Baptista, 2019). When we evaluate demographic history through methods that integrate population-level sampling from multiple taxa into a single community-level analysis, we reported demographic pulses synchronized. *Physalaemus cuvieri*, *Leptodactylus latrans* (South Clade), *Scinax uruguayus* (South Clade), and *P. gracilis* (South Clade 1) expanded in a temporally clustered pulse, i.e., ~ 3.756 Mya, while *Pseudis minuta* expanded time independently, i.e., ~ 1.784 Mya. These pulses of coexpansion and random expansion are congruent with the transitions in the dynamics of the climatic system during Plio-Pleistocene (Neogene-Quaternary). The main glaciations of Plio-Pleistocene in South America include: (i) the generalized glaciations of the Pliocene medium (~ 3.5 Mya); (ii) the largest Patagonian glaciation (~1.1 Mya); (iii) the colder Pleistocene glaciation (~ 0.7 Mya); (iv) the last

Southern Patagonian glaciation to reach the Atlantic coast (~ 180 ka); and (v) the last glacial maximum (LGM, 23-25,000 years ago) (Rabassa & Clapperton, 1990; Singer, Ackert & Guillou, 2004; Ruzzante et al., 2008). In addition, a later expansion of *P. minuta* may be related to its ecology strongly related to the aquatic environment. Therefore, from a more favorable climate in the Quaternary, it is plausible that this species presented a later demographic pulse. According to Ortiz-Jaureguizar & Cladera (2006), during the Quaternary, and as a consequence of the glacial cycles, the cold and dry climates were interrupted by hotter and humid periods and several expansion and retraction pulses of grasslands were recorded. These dynamics of expansion and contraction of the grasslands may have influenced evolutionarily the species that inhabit these environments to adapt or to follow the dynamics of the grasslands (Abreu-Jardim, 2018). Other species responded to the climatic dynamics of the Quaternary: i) *Hypsiboas albopunctatus* underwent a significant population expansion associated with the invasion of open habitats formed during the Pleistocene in Central Cerrado and Southern Brazil (Prado, Haddad & Zamudio, 2012); ii) *Scinax squalirostris* although showing demographic stability, in colder periods, the dispersal events of this species reached more distant areas (Abreu-Jardim, 2018); *Physalaemus cuvieri* also experienced population expansion (Clade A) (de Oliveira Miranda et al., 2019), iii) *Physalaemus fernandezae, P. henselii,* and *Pseudopaludicola falcipes* do not show evidences of population reduction associated with climatic changes of the Pleistocene (Barrasso, 2014; Langone et al., 2016). The North Clade of *Scinax uruguayus* showed signs of population retraction starting at ~120 ka, corresponding to an interglacial period, but one must investigate whether there is no small internal structuring in this population. Here, signs of a likely reduction of the most recent population may be artifacts of using samples with structured populations, which mimic the coalescence behavior of lineages in a shrinking population. Unfortunatelly, it was not possible to recover the demographic history of *Phyllomedusa iheringii,* probably due to the low genetic diversity reported.

Our results indicate that frog species codistributed, although presenting distinct life histories and ecological requirements, had the demography influenced in a similar way by the climatic fluctuations and, consequently, by the cycles of contraction/expansion of the *Campos*. In this sense, we confirm the premise that species that share the same range and have congruent phylogeographic patterns have been historically codistributed and/or differentiated in response to the same geological or environmental events (Zink, 1996). Moreover, our paleodistribution results indicate that *Leptodactylus latrans* and *Physalaemus cuvieri* had a much larger geographic distribution to the North of their distribution in the latter glacial, as they followed the expansion of the grasslands in colder periods. During the Pleistocene glacial periods, with the reduction of temperature, grasslands expanded towards the North of South America and, with increasing temperature and humidity (during the interglacial periods), grasslands retract their distribution (Behling, 2002; Ortiz-Jaureguizar & Cladera 2006). Current, the *L. latrans* and *P. cuvieri* distribution are more restricted to the South, with signals expansion in this region. The signal that we reported is certainly of "range expansion". The other species, grassland frogs, do not increase their distributions very much, but look a little better in the glacial period, suggesting habitat persistence (Thomé et al., 2010). In addition, the analised species followed a pattern of mountainous forests dwellers, which are significantly less affected by LGM conditions and more tolerant to cold (Amaro, Rodrigues, Yonenaga-Yassuda & Carnaval, 2012).

Variation in the phylogeographic patterns may indicate an adaptive potential in the face of environment changes (Frankham, 2005). However, we found that groups of frog species with remarkadle distinction in life histories and in geographical distribution presented a congruent demographic pattern. This suggests that ecological and life history of species did not influence the demographic responses of species, contrary to expected under the trait-based phylogeographic perspective (Papadopoulou & Knowles, 2016; Sullivan et al., 2019) which postulate that species-specific traits should result in idiosyncratic responses to the same environmental processes. Yet, physiological data on thermal preferences and species tolerances also promotes a perception of processes related to the distribution of diversity (Amaro et al., 2012). A recent study reported that the thermal tolerance ranges of subtropical frogs increased with latitude due to the drop in minimum temperatures, promoting the adaptation to cold resistance of frogs inhabiting these regions (Madalozzo, 2018). Understanding how species responded to climate changes in the past is crucial, specially considering the most endangered vertebrate group (Wake, 1991; Stuart et al., 2004) pointed as highly threated by future (Loyola et al., 2014; Vasconcelos et al., 2018; Vilela et al., 2018).

Indeed, more studies using a comparative quantitative, instead of qualitative/descriptive approaches are necessary to shed light on the recent biogeographic processes that occurred in the *Campos*. We recommend that research focusing especially on to assess the congruence/discordance of phylogeographic (demographic and divergence) patterns based on additional taxa and genome-wide data will be necessary to understand how the fauna diversified and responded to past environmental changes. This is the first comparative phylogeographic study of amphibians from the subtropical grasslands of Southeastern South America and our discoveries highlighted the presence of enigmatic lineages under the

Physalaemus gracilis and *Scinax uruguayus*. New approaches should assess whether these genetic groups are evolutionary lineages of new species and if the phylogenetic break events were congruent and were generated by the same evolutionary events. In addition, we strongly suggest that an integrative taxonomy approach must be undertaken in the near future for validation of these lineages, if applicable. In our study it was possible to verify that phylogeographic patterns recovered so far for typical species of open areas are complex and reinforce the idea that there is no single pattern of diversification. Moreover, climatic fluctuations continue to be one of the main mechanisms to promote the fluctuation of population distributions over time in South American frog species.

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Biosketch

The authors work with communities' ecology and population genetics, with particular emphasis on phylogeography and of genetic diversity in Neotropical Grasslands species.

Conflict of Interest Statement

The authors declare no conflict of interest

Authorship

S.S.A.S, M.L.L, A.C., R.M., T.G.S., S.T.Z.C. and C.F.B.H. conceived the overall study; S.S.A.S and M.L.L. generated the genetic data; S.S.A.S, M.L.L and A.C. performed the phylogeographical analyses; J.G. performed ENMs. S.S.A.S., M.L.L., A.C. and T.G.S. wrote the manuscript**.**

TABLE 1. Values of genetic diversity of gene 16S in six nominal frogs codistributed in the *Campos*. Values for the species and for genetic groups within each species.

Species	**Localities**	**Sequence number**	**Sequence length**	**Haplotype number (h)**	**Ploymorphic sites (s)**	**Haplotype diversity(Hh/SD)**	**Nucleotide diversity(π/SD)**
Scinaxs uruguayus (Nort Clade and South Clade)	13	52	423	20	34	0.931±0.016	0.02704±0.00074
S. uruguayus (North Clade)	6	29	426	11	16	0.877±0.037	0.01114±0.00055
S. uruguayus (South Clade)	7	23	472	10	12	0.842±0.052	0.00663±0.00069
Phyllomedusa iheringii	8	21	435	3	2	0.186±0.110	0.00044±0.00091
Pseudis minuta	18	60	448	10	10	0.732±0.039	0.00232±0.00025
Leptodactylus latrans (Nort Clade and South Clade)	68	121	363	28	43	0.850±0.021	0.02301±0.00190
L. latrans (Notth Clade)	34	30	367	11	30	0.816±0.0028	0.02026±0.00230
L. latrans (South Clade)	35*	91	364	18	36	0.755±0.032	0.00906±0.00104
Physalaemus cuvieri	56	84	488	35	30	0.95±0.012	0.00859±0.00039
Physalaemus gracilis (North Clade and South Clade1+2)	14	33	470	13	60	0.877±0.040	0.04061±0.00608
P. gracilis (North Clade)	3	6	534	5	8	0.933±0.122	0.00678±0.00128
P. gracilis (South Clade1)	9	21	467	6	10	0.729±0.080	0.00455±0.00117
P. gracilis (South Clade2)	2	6	531	2	3	0.533±0.172	0.00302±0.00097

* One of the locations (Ilha Comprida, SP, Brazil) has two individuals, one belongs to the North Clade and the other to the South Clade.

FIGURES

FIGURE 1. The original distribution of natural subtropical grasslands in southeastern South America (*Campos*). Adapted from Andrade et al., 2015.

FIGURE 2. Geographic distribution of the six nominal frogs according to IUCN (gray shading) and localities sampled within their respective distributions (black spots). See Tables A1-A6 for each location. (a) *Leptodactylus latrans*; (b) *Physalaemus cuvieri*; (c) *Physalaemus gracilis*; (d) *Scinax uruguayus*; (e) *Pseudis minuta* and (f) *Phyllomedusa iheringii*.

FIGURE 3. Left side of the board: 16S haplotype network for the nominal frogs analysed that presented a genetic group in the *Campos*: (a) *Physalaemus cuvieri* (N=84); (b) *Pseudis minuta* (N= 60) and (c) *Phyllomedusa iheringii* (N=21). Right side of the board: 16S haplotype network for the nominal frogs analysed that presented more than one genetic group in the *Campos*: (d) *Leptodactylus latrans* (N=121); (e) *Physalaemus gracilis* (N=33) and (f) *Scinax uruguayus* (N=52). The areas of the circles are proportional to the frequency of the haplotypes and the points in the map are localities sampled within their respective distributions. Traces between haplotypes represent the number of mutations. White circles refer to hypothetical haplotypes (unsampled or extinct).

FIGURE 4. Reconstruction of the effective population size over time based on BSP analyses for each of genetic group of the nominal frogs in the *Campos*. Demographic events of population expansion for: (a) *Leptodactylus latrans* - South Clade; (b) *Physalaemus cuvieri*; (c) *Physalaemus gracilis* – South Clade1; (d) *Scinax uruguayus* – South Clade and (e) *Pseudis minuta*. Demographic events of population bottlenecks for: (f) *Scinax uruguayus* – North Clade. Demographic events of population of stability for: (g) *Phyllomedusa iheringii*. Gray lines above and below the continuous line (average value) enclose the 95% highst posterior density. Neutrality test: Fs*=Fu's Fs; F*=Fu and Li's F*; D*=Fu and Li's D*, p value with "*" indicate significative.

FIGURE 5. (a) Estimation hABC for the "Random Time" and "Congruent Time" parameters of coexpansion of the nominal frogs in the *Campos*. Congruent time (black line) is for nominal frogs that coexpanded and Random time (red line) for one nominal frog that randomy expanded. (b) Principal components analysis (PCA) following by an hBayeSSC for identify

which of the five nominal frogs coexpanded in the *Campos*. The genetic groups of the *Leptodactylus latrans* (South Clade); *Physalaemus gracilis* (South Clade1); *Scinax uruguayus* (South Clade) and *Pseudis minuta* coexpanded. Already, *Physalaemus cuvieri* expanded in different time.

FIGURE 6. Depiction of model of partially synchronous coexpansion (z=0.8), all involving populations the five nominal frogs codistributed in the *Campos*. Each population has a contemporary effective population size of (N_i) from prior $U(N_{min}, N_{máx})$ and expands instantaneously from a population (ε_i) the current size chosen from expansion magnitudes $U(\varepsilon_{min}, \varepsilon_{máx})$ at times (t_i) or (t_s) from the uniform prior $U(t_{min}, t_{máx})$.

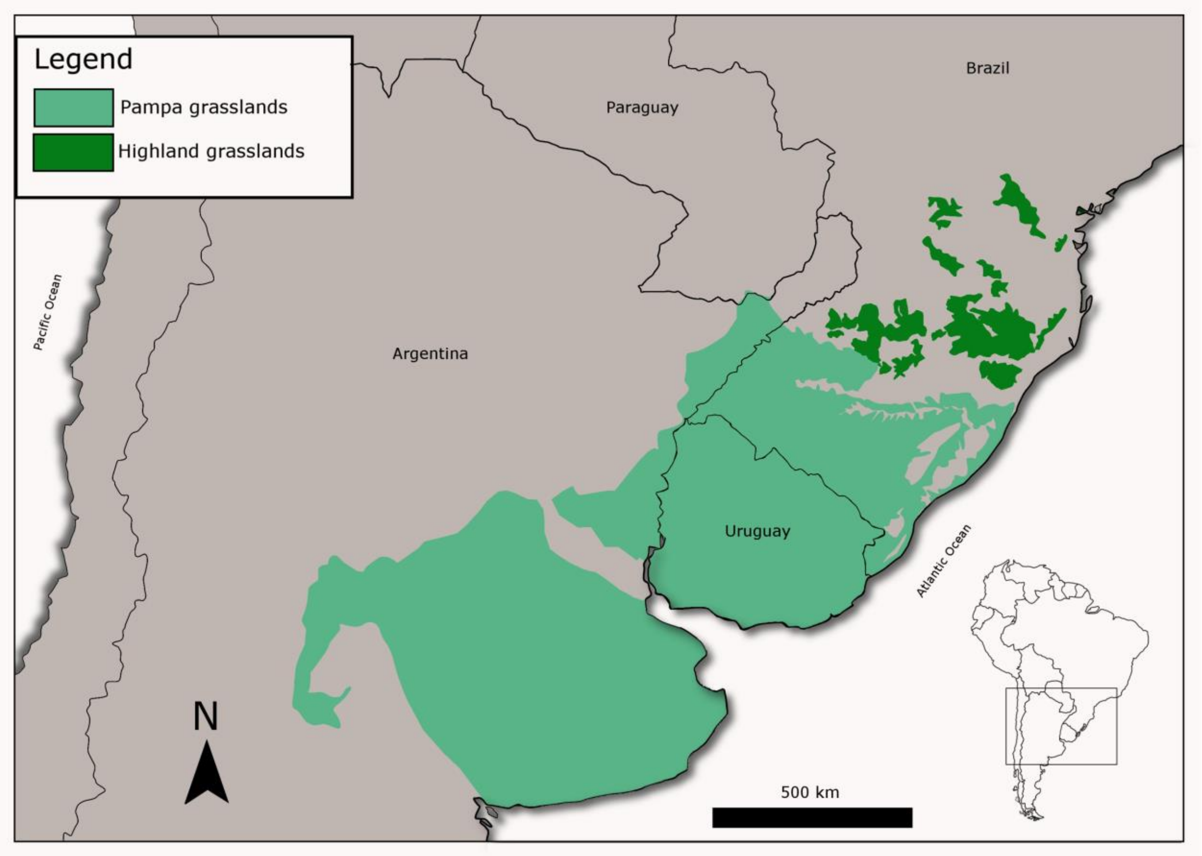
Legend
Pampa grasslands
Highland grasslands
Brazil
Paraguay
Argentina
Uruguay
Pacific Ocean
Atlantic Ocean
N
500 km

Figure 1

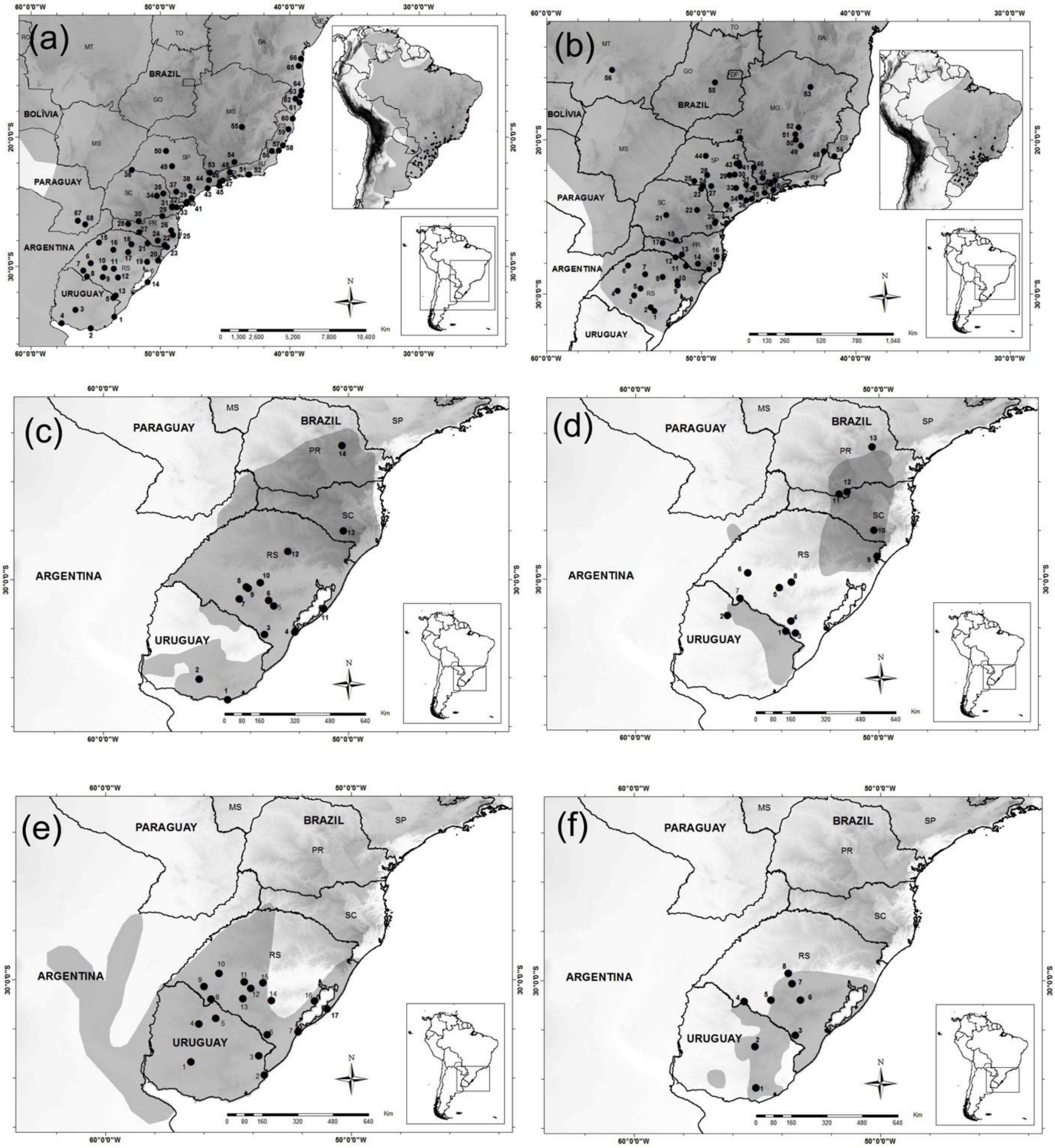
(a)
(b)
(c)
(d)
(e)
(f)
60°0'0"W
50°0'0"W
40°0'0"W
30°0'0"W
20°0'0"S
30°0'0"S
BRAZIL
BOLÍVIA
PARAGUAY
ARGENTINA
URUGUAY
MT
TO
BA
GO
DF
MG
MS
SP
ES
RJ
PR
SC
RS
RO
N
Km
0 1,300 2,600 5,200 7,800 10,400
0 130 260 520 780 1,040
0 80 160 320 480 640

Figure 2

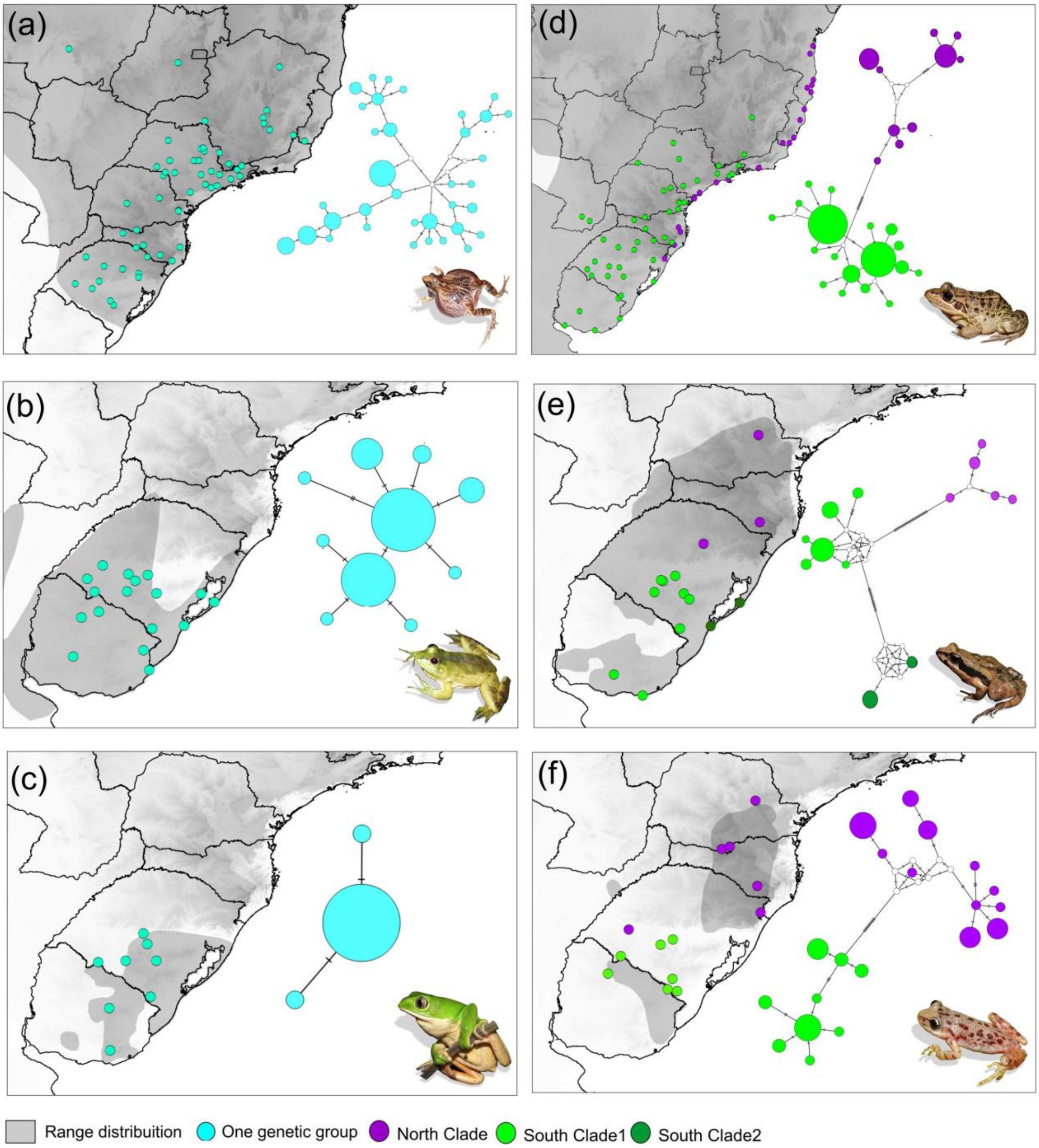
(a)
(b)
(c)
(d)
(e)
(f)
Range distribuition
One genetic group
North Clade
South Clade1
South Clade2

Figure 3

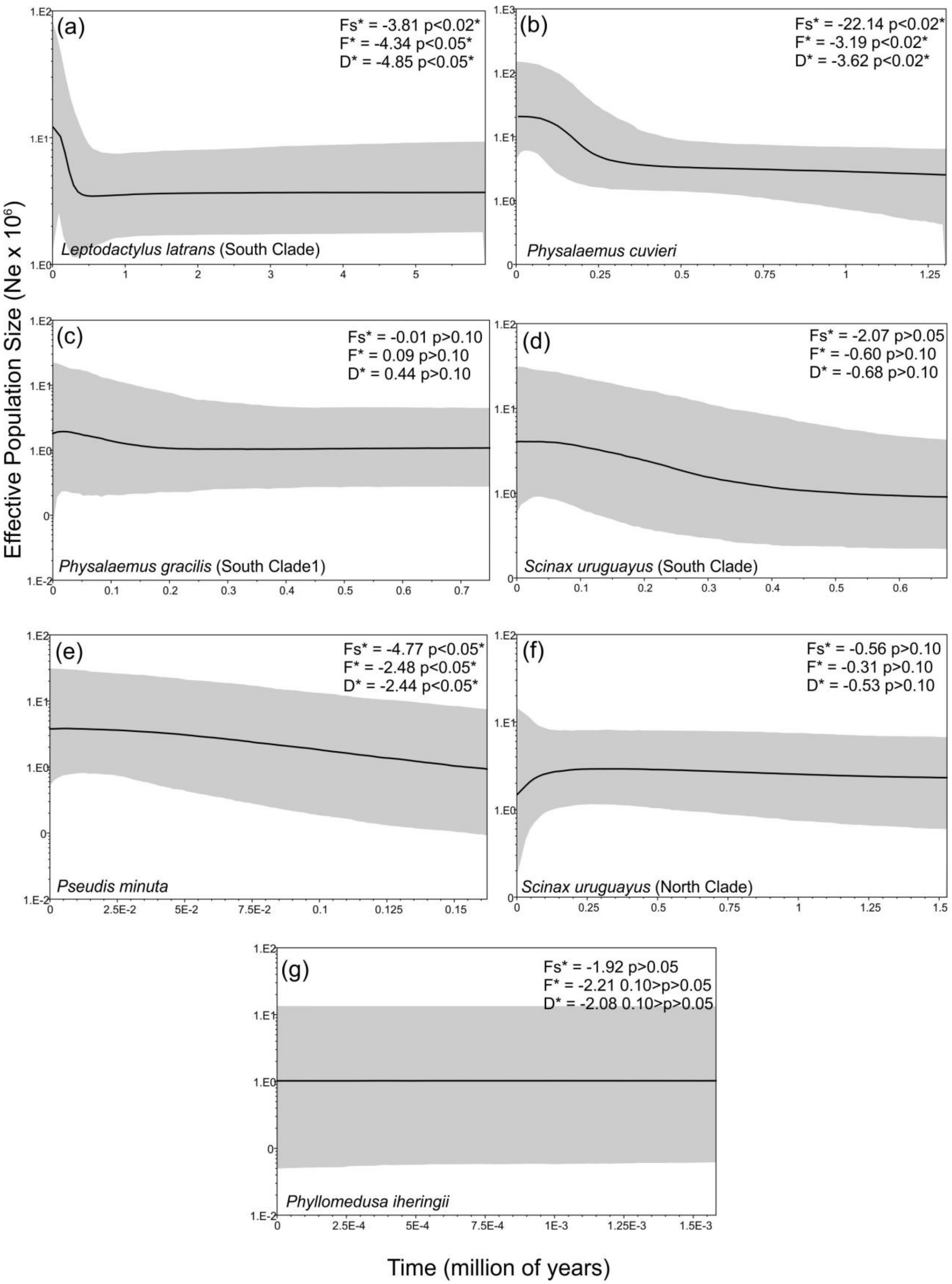
Effective Population Size (Ne x 10⁶)
(a)
Fs* = -3.81 p<0.02*
F* = -4.34 p<0.05*
D* = -4.85 p<0.05*
Leptodactylus latrans (South Clade)
1.E2
1.E1
1.E0
0
1
2
3
4
5
(b)
Fs* = -22.14 p<0.02*
F* = -3.19 p<0.02*
D* = -3.62 p<0.02*
Physalaemus cuvieri
1.E3
1.E2
1.E1
1.E0
0
0.25
0.5
0.75
1
1.25
(c)
Fs* = -0.01 p>0.10
F* = 0.09 p>0.10
D* = 0.44 p>0.10
Physalaemus gracilis (South Clade1)
1.E2
1.E1
1.E0
0
1.E-2
0.1
0.2
0.3
0.4
0.5
0.6
0.7
(d)
Fs* = -2.07 p>0.05
F* = -0.60 p>0.10
D* = -0.68 p>0.10
Scinax uruguayus (South Clade)
1.E2
1.E1
1.E0
0
0.1
0.2
0.3
0.4
0.5
0.6
(e)
Fs* = -4.77 p<0.05*
F* = -2.48 p<0.05*
D* = -2.44 p<0.05*
Pseudis minuta
1.E2
1.E1
1.E0
0
1.E-2
2.5E-2
5E-2
7.5E-2
0.1
0.125
0.15
(f)
Fs* = -0.56 p>0.10
F* = -0.31 p>0.10
D* = -0.53 p>0.10
Scinax uruguayus (North Clade)
1.E2
1.E1
1.E0
0
0.25
0.5
0.75
1
1.25
1.5
(g)
Fs* = -1.92 p>0.05
F* = -2.21 0.10>p>0.05
D* = -2.08 0.10>p>0.05
Phyllomedusa iheringii
1.E2
1.E1
1.E0
0
1.E-2
2.5E-4
5E-4
7.5E-4
1E-3
1.25E-3
1.5E-3
Time (million of years)

Figure 4

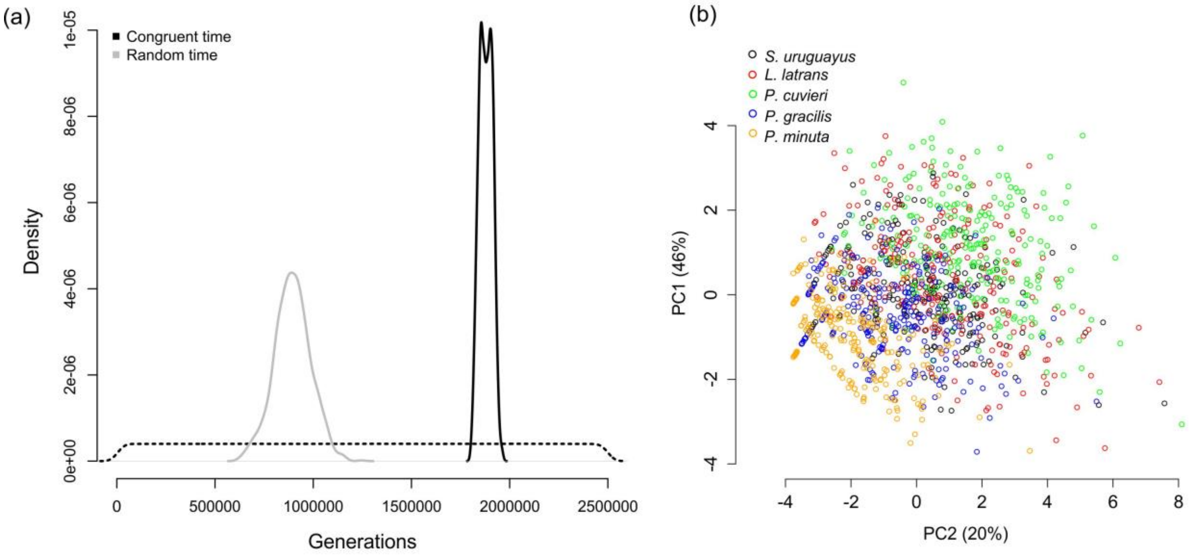
(a)
Congruent time
Random time
Density
1e-05
8e-06
6e-06
4e-06
2e-06
0e+00
0
500000
1000000
1500000
2000000
2500000
Generations
(b)
S. uruguayus
L. latrans
P. cuvieri
P. gracilis
P. minuta
PC1 (46%)
4
2
0
-2
-4
-4
-2
0
2
4
6
8
PC2 (20%)

Figure 5

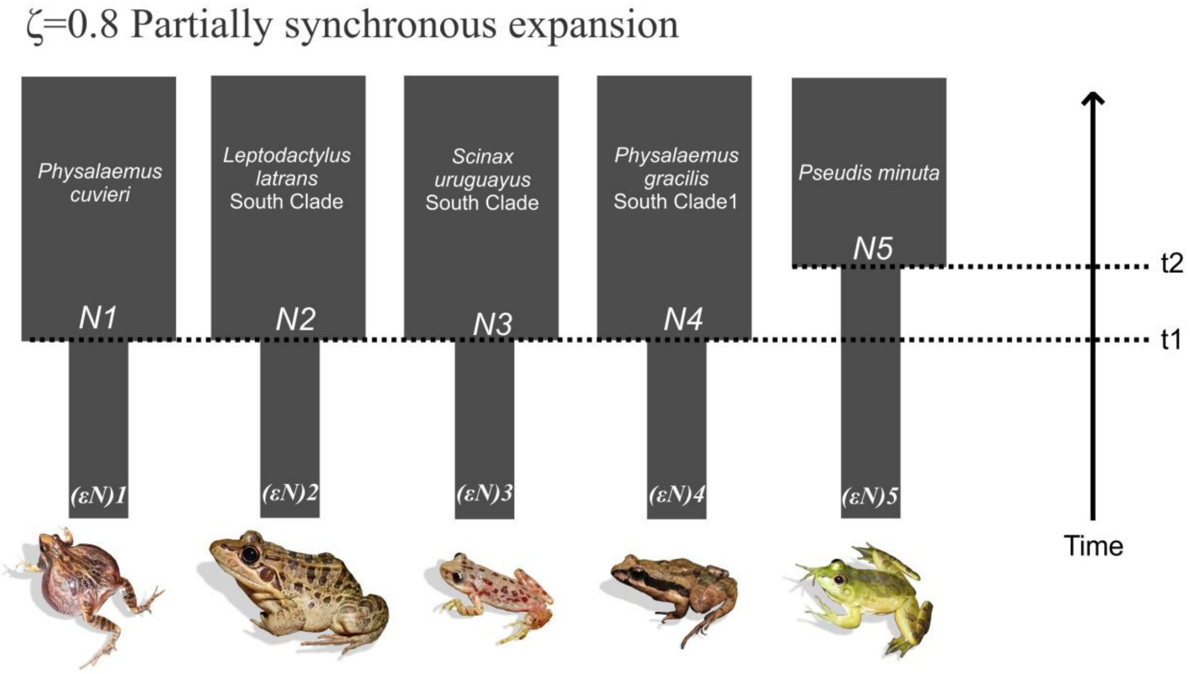
ζ=0.8 Partially synchronous expansion
Physalaemus cuvieri
Leptodactylus latrans South Clade
Scinax uruguayus South Clade
Physalaemus gracilis South Clade1
Pseudis minuta
N1
N2
N3
N4
N5
t2
t1
(εN)1
(εN)2
(εN)3
(εN)4
(εN)5
Time

Figure 6

To be submmited to Journal of Biogeography

RESEARCH PAPER

Partially Synchronous Response to Neogene-Quaternary Climatic Dynamics in Codistributed Frogs from the Southeastern American Subtropical Grasslands.

Comparative phylogeography of frogs

Suelen S. A. Saccol[1,*] | Mariana L. Lyra[2] | Arley Camargo[3] | João G. R. Giovanelli[2]| Raul Maneyro[4] | Sonia T. Z. Cechin[1] | Célio F. B. Haddad[2]| Tiago G. Santos[5]

Appendice A1 (Tables A1.1- A1.14) code numbers, locality information and sample size for, *Leptodactylus latrans, Physalaemus cuvieri, Physalaemus gracilis, Scinax uruguayus, Pseudis minuta, Phyllomedusa iheringii*; predictors variables; list of haplotypes and frequency of 16S gene sequences individuals of the species codistributed in the *Campos;* and list of abbreviations used in the code of the individuals.

Appendice A2 (Figures A2.1- A2.9) anuran species codistributed in the *Campos: Leptodactylus latrans*; *Physalaemus cuvieri*; *Physalaemus gracilis*; *Scinax uruguayus*; *Pseudis minuta* and *Phyllomedusa iheringii*. A network of haplotypes (16S) for the six nominal frogs codistributed in the *Campos*. Estimation hABC of the parameter Z and PCA of the statistical summary of the prior, posterior and the observed data. Models of habitat distribution for current time, last glacial maximum period (LGM) and last interglacial period (LIG).

Appendice A3 (Priors list A3.1) priors list used for the coexpansion analysis for codistributed species in the *Campos*.

Table A1.1. Locality information, code numbers and sample size for the nominal frog *Leptodactylus latrans.*

Code	**Locality**	**Sample Size**	**Latitude**	**Longitude**
1	Uruguay, Rocha	1	-33.900000	-53.516667
2	Uruguay, Maldonado	5	-34.783360	-55.366661
3	Uruguay, Durazno	1	-33.366769	-56.516673
4	Uruguay, Colonia	1	-34.400151	-57.616649
5	Uruguay, Cerro Largo	1	-32.433426	-53.616649
6	Brazil, Rio Grande do Sul, Alegrete	4	-29.751301	-55.360465
7	Brazil, Rio Grande do Sul, Quaraí	7	-30.311528	-55.935987
8	Brazil, Rio Grande do Sul, Santana Livramento	1	-30.781710	-55.643156
9	Brazil, Rio Grande do Sul, Lavras do Sul	5	-30.826356	-54.424579
10	Brazil, Rio Grande do Sul, São Gabriel	4	-30.109535	-54.298178
11	Brazil, Rio Grande do Sul, São Sepé	1	-30.163989	-53.571821
12	Brazil, Rio Grande do Sul, Santana Boa Vista	4	-30.851365	-53.236955
13	Brazil, Rio Grande do Sul, Jaguarão	5	-32.236603	-53.410742
14	Brazil, Rio Grande do Sul, Tavares	2	-31.211586	-50.977345
15	Brazil, Rio Grande do Sul, Cerro Largo	1	-28.143747	-54.719226
16	Brazil, Rio Grande do Sul, Cruz Alta	2	-28.712423	-53.612689
17	Brazil, Rio Grande do Sul, Soledade	5	-28.871875	-52.451428
18	Brazil, Rio Grande do Sul, Mato Castelhano	1	-28.296992	-52.212389
19	Brazil, Rio Grande do Sul, Sapiranga	1	-29.635379	-51.006948
20	Brazil, Rio Grande do Sul, Itati	2	-29.533536	-50.151508
21	Brazil, Rio Grande do Sul, Vacaria	5	-28.195446	-50.97502
22	Brazil, Santa Catarina, Treviso	2	-28.513817	-49.457133
23	Brazil, Santa Catarina, Bom Jardim da Serra	1	-28.340535	-49.626536
24	Brazil, Santa Catarina, Painel	3	-27.985402	-50.168304

25	Brazil, Santa Catarina, Angelina	1	-27.573195	-48.984210
26	Brazil, Santa Catarina, Botuvera	1	-27.221988	-49.141872
27	Brazil, Santa Catarina, Capinzal	1	-27.348192	-51.604351
28	Brazil, Santa Catarina, Ipuaçu	1	-26.695283	-52.467853
29	Brazil, Santa Catarina, Mafra	1	-26.112950	-49.806792
30	Brazil, Paraná, Palmas	3	-26.499936	-51.639489
31	Brazil, Paraná, Quatro Barras	1	-25.367645	-49.076735
32	Brazil, Paraná, Antonina	1	-25.432696	-48.715970
33	Brazil, Paraná, Piraquara	1	-25.441799	-49.063045
34	Brazil, Paraná, Jaguariaíva	1	-24.344057	-49.731987
35	Brazil, Paraná, Tibagi	5	-24.545027	-50.247578
36	Brazil, São Paulo, Teodoro Sampaio	1	-22.531440	-52.173362
37	Brazil, São Paulo, Ribeirao Branco	1	-24.221069	-48.764012
38	Brazil, São Paulo, Pilar do Sul	1	-23.807723	-47.723057
39	Brazil, São Paulo, Ilha do Cardoso	1	-25.135468	-47.962775
40	Brazil, São Paulo, Iguape	2	-24.709763	-47.556221
41	Brazil, São Paulo, Cananeia	2	-25.012621	-47.933869
42	Brazil, São Paulo, Ilha Comprida	2	-24.745983	-47.550640
43	Brazil, São Paulo, Santos	1	-23.956128	-46.326407
44	Brazil, São Paulo, Santa Isabel	1	-23.319570	-46.226786
45	Brazil, São Paulo, São Sebastião	1	-23.748000	-45.413000
46	Brazil, São Paulo, Bairro Alto	1	-23.477244	-45.351496
47	Brazil, São Paulo, São Luis do Paratinga	1	-23.344974	-45.136050
48	Brazil, São Paulo, São José do Barreiro	1	-22.719000	-44.618000
49	Brazil, São Paulo, Bauru	1	-22.235001	-49.080987
50	Brazil, São Paulo, Nova Itapirema	1	-21.074000	-49.540000
51	Brazil, Rio de Janeiro, Rio de Janeiro	3	-22.906847	-43.172897
52	Brazil, Rio de Janeiro, Niteroi	1	-22.885897	-43.115241

53	Brazil, Minas Gerais, Camanducaia	1	-22.748629	-46.135133
54	Brazil, Minas Gerais, Arantina	1	-21.910662	-44.255940
55	Brazil, Minas Gerais, Santana do Riacho	1	-19.200138	-43.675958
56	Brazil, Espírito Santo, Mimoso do Sul	1	-21.063270	-41.361923
57	Brazil, Espírito Santo, Marataizes	1	-21.045508	-40.831777
58	Brazil, Espírito Santo, Guarapari	1	-20.654005	-40.511008
59	Brazil, Espírito Santo, Linhares	1	-19.395367	-40.066467
60	Brazil, Espírito Santo, Conceicao da Barra	1	-18.587792	-39.736268
61	Brazil, Bahia, Prado	1	-17.335730	-39.225324
62	Brazil, Bahia, Itamaraju	1	-17.037717	-39.538913
63	Brazil, Bahia, Caraiva	1	-16.796432	-39.152337
64	Brazil, Bahia, Porto Seguro	1	-16.444688	-39.065590
65	Brazil, Bahia, Uruçuca	1	-14.515498	-39.299866
66	Brazil, Bahia, Camamu	1	-13.944322	-39.104560
67	Paraguay, Yegros, Caazapa	1	-26.450000	-56.345999
68	Paraguay, Alto Vera, Itapuã	2	-26.760000	-55.771000
Total		**121**		

Table A1.2. Locality information, code numbers and sample size for the nominal frog *Physalaemus cuvieri*.

Code	**Locality**	**Sample Size**	**Latitude**	**Longitude**
1	Brazil, Rio Grande do Sul, Piratini	2	-31.107181	-53.029743
2	Brazil, Rio Grande do Sul, Santana da Boa Vista	4	-30.843324	-53.245506
3	Brazil, Rio Grande do Sul, São Gabriel	9	-30.084938	-54.319688
4	Brazil, Rio Grande do Sul, Alegrete	3	-29.787652	-55.386457
5	Brazil, Rio Grande do Sul, Santa Maria	1	-29.648617	-53.921106
6	Brazil, Rio Grande do Sul, Cerro Largo	2	-28.152012	-54.718251
7	Brazil, Rio Grande do Sul, Cruz Alta	2	-28.712423	-53.612689
8	Brazil, Rio Grande do Sul, Soledade	5	-28.897824	-52.476168
9	Brazil, Rio Grande do Sul, São Pedro da Serra	1	-29.416326	-51.509749
10	Brazil, Rio Grande do Sul, Bento Gonçalves	1	-29.166640	-51.516906
11	Brazil, Rio Grande do Sul, Vacaria	5	-28.172029	-50.976876
12	Brazil, Rio Grande do Sul, Machadinho	1	-27.600832	-51.662342
13	Brazil, Santa Catarina, Campos Novos	2	-27.435137	-51.220839
14	Brail, Santa Catarina, Painel	1	-28.027500	-50.187144
15	Brazil, Santa Catarina, Lauro Muller	1	-28.385427	-49.466934
16	Brazil, Santa Catarina, Angelina	1	-27.573339	-48.984365
17	Brazil, Santa Catarina, Ipuaçu	1	-26.695895	-52.468078
18	Brazil, Paraná, Palmas	2	-26.513276	-51.614816
19	Brazil, Paraná, Quatro Barras	1	-25.367805	-49.076776
20	Brazil, Paraná, Campina Grande do Sul	1	-25.304891	-49.055570
21	Brazil, Paraná, Palmital/Guaruva	1	-24.872497	-52.234700
22	Brazil, Paraná, Tibagi	3	-24.545027	-50.247578
23	Brazil, Paraná, Jacarezinho	1	-23.173433	-49.978172
24	Brazil, São Paulo, Ourinhos	1	-22.980741	-49.867050

25	Brazil, São Paulo, Assis	1	-22.660501	-50.418569
26	Brazil, São Paulo, Ribeirao Grande	1	-24.210166	-48.358891
27	Brazil, São Paulo, Manduri	1	-23.005476	-49.317028
28	Brazil, São Paulo, Gália	1	-22.274501	-49.593385
29	Brazil, São Paulo, Brotas	1	-22.279958	-48.125570
30	Brazil, São Paulo, Itirapina	1	-22.254781	-47.815925
31	Brazil, São Paulo, Campinas	1	-22.909938	-47.062633
32	Brazil, São Paulo, Tietê	1	-23.115640	-47.705086
33	Brazil, São Paulo, Atibaia	1	-23.117535	-46.556728
34	Brazil, São Paulo, Piedade	1	-23.714470	-47.426021
35	Brazil, São Paulo, Parelheiros	1	-23.821174	-46.702699
36	Brazil, São Paulo, Juquitiba	1	-23.928886	-47.071390
37	Brazil, São Paulo, Santa Branca	1	-23.407970	-45.887415
38	Brazil, São Paulo, São Luis do Paraitinga	1	-23.222869	-45.311807
39	Brazil, São Paulo, Pindamonhangaba	1	-22.845142	-45.489200
40	Brazil, São Paulo, Piquete	1	-22.607488	-45.186617
41	Brazil, São Paulo, Santa Rita do Passa Quatro	1	-21.687047	-47.490226
42	Brazil, São Paulo, Luis Antonio	1	-21.551006	-47.701280
43	Brazil, São Paulo, São Simão	1	-21.479571	-47.554774
44	Brazil, São Paulo, José Bonifácio	1	-21.049343	-49.686929
45	Brazil, Minas Gerais, Estiva	1	-22.475772	-45.999425
46	Brazil, Minas Gerais, Poços de Caldas	1	-21.785553	-46.562051
47	Brazil, Minas Gerais, Sacramento	1	-19.862645	-47.451265
48	Brazil, Minas Gerais, Carangola	1	-20.731475	-42.029923
49	Brazil, Minhas Gerais, Ouro Preto	1	-20.379708	-43.512777
50	Brazil, Minas Gerais, Nova Lima	1	-19.992385	-43.848574
51	Brazil, Minas Gerais, Lagoa Santa	1	-19.644584	-43.886470

52	Brazil, Minas Gerais, Santana do Riacho	1	-19.200092	-43.675905
53	Brazil, Minas Gerais, Grão Mogol	1	-16.565330	-42.893236
54	Brazil, Espírito Santo, Mimoso do Sul	1	-21.063267	-41.361927
55	Brazil, Goiás, Campo Limpo de Goias	1	-16.274279	-49.101926
56	Brazil, Mato Grosso, Chapada dos Guimaraes	1	-15.458540	-55.752439
Total		**84**		

Table A1.3. Locality information, code numbers, and sample size for the nominal frog *Physalaemus gracilis*.

Code	**Locality**	**Sample Size**	**Latitude**	**Longitude**
1	Uruguay, Maldonado	1	-34.916764	-54.916641
2	Uruguay, Florida	1	-34.083333	-56.066667
3	Brazil, Rio Grande do Sul, Jaguarão	4	-32.25285	-53.406275
4	Brazil, Rio Grande do Sul, Rio Grande, Bairro Bolaxa	2	-32.158257	-52.169474
5	Brazil, Rio Grande do Sul, Piratini	2	-31.093114	-53.029895
6	Brazil, Rio Grande do Sul, Santana da Boa Vista	2	-30.855869	-53.241207
7	Brazil, Rio Grande do Sul, Lavras do Sul	3	-30.795847	-54.429181
8	Brazil, Rio Grande do Sul, São Gabriel	1	-30.318750	-54.157323
9	Brazil, Rio Grande do Sul, Santa Margarida do Sul	3	-30.366920	-54.079522
10	Brazil, Rio Grande do Sul, São Sepé	4	-30.144151	-53.584747
11	Brazil, Rio Grande do Sul, Tavares	4	-31.211346	-50.979794
12	Brazil, Rio Grande do Sul, Soledade	2	-28.866669	-52.456296
13	Brazil, Santa Catarina, Painel	1	-28.025900	-50.183771
14	Brazil, Paraná, Tibagi	3	-24.545027	-50.247578
Total		**33**		

Table A1.4. Locality information, code numbers and sample size for the nominal frog *Scinax uruguayus.*

Code	**Locality**	**Sample Size**	**Latitude**	**Longitude**
1	Uruguay, Cerro Largo	2	-32.150166	-53.800008
2	Uruguay, Tacuarembó	2	-31.516667	-56.200000
3	Brazil, Rio Grande do Sul, Jaguarão	5	-32.235869	-53.40996
4	Brazil, Rio Grande do Sul, Pedras Altas	4	-31.736874	-53.604956
5	Brazil, Rio Grande do Sul, Santa Margarida do Sul	4	-30.366920	-54.079522
6	Brazil, Rio Grande do Sul, Alegrete	16	-29.751534	-55.360514
7	Brazil, Rio Grande do Sul, Santana do Livramento	2	-30.810076	-55.685990
8	Brazil, Rio Grande do Sul, São Sepé	4	-30.144151	-53.584747
9	Brazil, Rio Grande do Sul, Cambará do Sul	1	-29.064007	-50.055000
10	Brazil, Santa Catarina, Painel	4	-28.003726	-50.181786
11	Brazil, Paraná, Palmas	5	-26.509966	-51.615470
12	Brazil, Paraná, General Carneiro	1	-26.421000	-51.299000
13	Brazil, Paraná, Tibagi	2	-24.575830	-50.265289
Total		**52**		

Table A1.5. Locality information, code numbers and sample size for the nominal frog *Pseudis minuta*.

Code	Locality	Sample Size	Latitude	Longitude
1	Uruguay, Durazno	2	-33.366750	-56.516692
2	Uruguay, Rocha	1	-33.899945	-53.516625
3	Uruguay, Treinta y Tres	2	-33.116701	-53.749994
4	Uruguay, Tacuarembó	1	-31.816777	-56.183325
5	Uruguay, Rivera	3	-31.583474	-55.499994
6	Brazil, Rio Grande do Sul, Jaguarão	7	-32.247485	-53.410489
7	Brazil, Rio Grande do Sul, Rio Grande	2	-32.130000	-52.154444
8	Brazil, Rio Grande do Sul, Santana do Livramento	1	-30.810076	-55.685990
9	Brazil, Rio Grande do Sul, Quaraí	4	-30.29164	-55.969909
10	Brazil, Rio Grande do Sul, Alegrete	4	-29.751301	-55.360465
11	Brazil, Rio Grande do Sul, São Gabriel	10	-30.105845	-54.337846
12	Brazil, Rio Grande do Sul, Santa Margarida do Sul	4	-30.366920	-54.079522
13	Brazil, Rio Grande do Sul, Lavras do Sul	5	-30.781627	-54.396725
14	Brazil, Rio Grande do Sul, Santana da Boa Vista	7	-30.861916	-53.232136
15	Brazil, Rio Grande do Sul, São Sepé	3	-30.144151	-53.584747
16	Brazil, Rio Grande do Sul, Arambaré	2	-30.874556	-51.490253
17	Brazil, Rio Grande do Sul, Tavares	2	-31.211586	-50.977345
Total		**60**		

Table A1.6. Locality information, code numbers and sample size for the nominal frog *Phyllomedusa iheringii*.

Code	**Locality**	**Sample Size**	**Latitude**	**Longitude**
1	Uruguay, Lavalleja	1	-34.366722	-55.049992
2	Uruguay, Cerro Largo	4	-32.700138	-55.083309
3	Brazil, Rio Grande do Sul, Jaguarão	3	-32.241787	-53.443911
4	Brazil, Rio Grande do Sul, Santana do Livramento	1	-30.855452	-55.526142
5	Brazil, Rio Grande do Sul, Lavras do Sul	3	-30.802439	-54.430176
6	Brazil, Rio Grande do Sul, Santana da Boa Vista	3	-30.810771	-53.228999
7	Brazil, Rio Grande do Sul, São Sepé	4	-30.144151	-53.584747
8	Brazil, Rio Grande do Sul, Santa Maria	2	-29.721117	-53.726823
Total		**21**		

Table A1.7. Predictors variables used to produce a potential distribution in current climate.

Source	Dataset	Predictors variables	References
WorlClim 2.0	Bioclim	Mean Diurnal Range (Bio02) Isothermality (Bio03) Mean Temperature of Wettest Quarter (Bio08) Precipitation Seasonality (Bio15) Precipitation of Warmest Quarter (Bio18) Precipitation of Coldest Quarter (Bio19)	Fick and Hijmans (2017)

Table A1.8. List of haplotypes, frequency of 16S gene sequences and individuals of the nominal frog *Physalaemus cuvieri* that have this haplotype.

Haplotype number	Frequence Sequence	Individual Code
1	3	Pcuvi_ALE1_RS_BR Pcuvi_ALE2_RS_BR Pcuvi_CLA1_RS_BR
2	14	Pcuvi_ALE3_RS_BR Pcuvi_SMAR1_RS_BR Pcuvi_MACH1_RS_BR Pcuvi_BG1_RS_BR Pcuvi_PAI1_SC_BR Pcuvi_SGAB2_RS_BR Pcuvi_SGAB8_RS_BR Pcuvi_SOL1_RS_BR Pcuvi_SOL2_RS_BR Pcuvi_SOL3_RS_BR Pcuvi_SOL5_RS_BR Pcuvi_VAC1_RS_BR Pcuvi_VAC2_RS_BR Pcuvi_CA2_RS_BR
3	6	Pcuvi_IPU1_SC_BR Pcuvi_CNOV1_SC_BR Pcuvi_PAL1_PR_BR Pcuvi_PAL2_PR_BR Pcuvi_VAC4_RS_BR Pcuvi_CA1_RS_BR
4	1	Pcuvi_ANG1_SC_BR
5	1	Pcuvi_GMOG1_MG_BR
6	3	Pcuvi_CLIMP1_GO_BR Pcuvi_BROT1_SP_BR Pcuvi_EST1_MG_BR
7	4	Pcuvi_JAC1_PR_BR Pcuvi_OUR1_SP_BR Pcuvi_GAL1_SP_BR Pcuvi_TIE1_SP_BR
8	3	Pcuvi_NLIM1_MG_BR Pcuvi_LSAN1_MG_BR Pcuvi_SRIAC1_MG_BR
9	3	Pcuvi_QBAR1_PR_BR Pcuvi_PIED1_SP_BR Pcuvi_CGSUL1_PR_BR
10	1	Pcuvi_PCAL1_MG_BR
11	1	Pcuvi_CLA2_RS_BR
12	5	Pcuvi_CGUI1_MT_BR Pcuvi_SBRAN1_SP_BR Pcuvi_LANT1_SP_BR Pcuvi_ITIR1_SP_BR Pcuvi_PARE1_SP_BR
13	1	Pcuvi_PGUA1_PR_BR
14	1	Pcuvi_CNOV2_SC_BR
15	1	Pcuvi_RGRA1_SP_BR

16	2	Pcuvi_OP1_MG_BR Pcuvi_CARAN1_MG_BR
17	1	Pcuvi_SSIM1_SP_BR
18	1	Pcuvi_LMUL1_SC_BR
19	1	Pcuvi_SACR1_MG_BR
20	1	Pcuvi_CAMP1_SP_BR
21	3	Pcuvi_PIND1_SP_BR Pcuvi_PIQ1_SP_BR Pcuvi_SLPAR1_SP_BR
22	1	Pcuvi_SPSER1_RS_BR
23	1	Pcuvi_MAND1_SP_BR
24	1	Pcuvi_ASS1_SP_BR
25	1	Pcuvi_SRPQ1_SP_BR
26	1	Pcuvi_JUQUI1_SP_BR
27	1	Pcuvi_JBON1_SP_BR
28	1	Pcuvi_ATIB1_SP_BR
29	1	Pcuvi_MSUL1_ES_BR
30	6	Pcuvi_SBV1_RS_BR Pcuvi_SBV2_RS_BR Pcuvi_SBV3_RS_BR Pcuvi_SBV4_RS_BR Pcuvi_PIR1_RS_BR Pcuvi_PIR2_RS_BR
31	7	Pcuvi_SGAB1_RS_BR Pcuvi_SGAB3_RS_BR Pcuvi_SGAB4_RS_BR Pcuvi_SGAB5_RS_BR Pcuvi_SGAB6_RS_BR Pcuvi_SGAB7_RS_BR Pcuvi_SGAB9_RS_BR
32	1	Pcuvi_SOL4_RS_BR
33	2	Pcuvi_TIB1_PR_BR Pcuvi_TIB2_PR_BR
34	1	Pcuvi_TIB3_PR_BR
35	2	Pcuvi_VAC3_RS_BR Pcuvi_VAC5_RS_BR

Table A1.9. List of haplotypes, frequency of 16S gene sequences and individuals of the nominal frog *Pseudis minuta* that have this haplotype.

Haplotype Number	Frequency Sequence	Individual Code
1	25	Pmin_ALE1_BR Pmin_ALE2_BR Pmin_ALE4_BR Pmin_SMAR1_BR Pmin_SMAR2_BR Pmin_SMAR3_BR Pmin_JAG1_BR Pmin_JAG3_BR Pmin_JAG4_BR Pmin_JAG5_BR Pmin_JAG7_BR Pmin_LSUL4_BR Pmin_LSUL5_BR Pmin_SBV1_BR Pmin_SBV3_BR Pmin_SBV4_BR Pmin_SGAB6_BR Pmin_SGAB8_BR Pmin_SGAB10_BR Pmin_SANT1_BR Pmin_TRINT1_UY Pmin_TRINT2_UY Pmin_RIVE2_UY Pmin_DUR1_UY Pmin_DUR2_UY
2	18	Pmin_ALE3_BR Pmin_RGR1_BR Pmin_ARAM1_BR Pmin_ARAM2_BR Pmin_RGR2_BR Pmin_SEP1_BR Pmin_SEP2_BR Pmin_SEP3_BR Pmin_SMAR4_BR Pmin_SGAB1_BR Pmin_SGAB2_BR Pmin_SGAB3_BR Pmin_SGAB4_BR Pmin_SGAB7_BR Pmin_SGAB9_BR Pmin_RIVE3_UY Pmin_TACUA1_UY Pmin_TAV2_BR
3	6	Pmin_JAG2_BR Pmin_JAG6_BR Pmin_QUA1_BR Pmin_QUA2_BR Pmin_QUA3_BR Pmin_QUA4_BR
4	2	Pmin_LSUL1_BR Pmin_LSUL3_BR
5	1	Pmin_LSUL2_BR
6	4	Pmin_SBV2_BR Pmin_SBV5_BR Pmin_SBV6_BR Pmin_SBV7_BR
7	1	Pmin_SGAB5_BR
8	1	Pmin_RIVE1_UY
9	1	Pmin_ROCH1_UY
10	1	Pmin_TAV1_BR

Table A1.10. List of haplotypes, frequency of 16S gene sequences and individuals of the nominal frog *Phyllomedusa iheringii* that have this haplotype.

Haplotype Number	Frequency Sequence	Individual Code
1	1	Piher_SEP3_BR
2	1	Piher_LAV_UY
3	19	Piher_SEP1_BR Piher_LSUL1_BR Piher_SBV2_BR Piher_SEP2_BR Piher_SMAR2_BR Piher_CL4_UY Piher_JAG1_BR Piher_JAG3_BR Piher_JAG2_BR Piher_LSUL2_BR Piher_CL3_UY Piher_CL1_UY Piher_SBV1_BR Piher_SBV3_BR Piher_CL2_UY Piher_LSUL3_BR Piher_SEP4_BR Piher_SANT_BR Piher_SMAR1_BR

Table A1.11. List of haplotypes, frequency of 16S gene sequences and individuals of the nominal frog *Leptodactylus latrans* that have this haplotype.

Haplotype Number	Frequency Sequence	Individual Code
1	8	Llatr_RJAN1_RJ_BR Llatr_CAR1_BA_BR Llatr_RJAN3_RJ_BR Llatr_MARAT1_ES_BR Llatr_NIT1_RJ_BR Llatr_SSEB1_SP_BR Llatr_GUAR1_ES_BR Llatr_MSUL_ES_BR]
2	1	Llatr_CAM1_BA_BR]
3	35	Llatr_ARA1_MG_BR Llatr_JAGUA1_PR_BR Llatr_SRIA1_MG_BR Llatr_SISA1_SP_BR Llatr_PSUL1_SP_BR Llatr_SJBAR1_SP_BR Llatr_BALT1_SP_BR Llatr_BAU1_SP_BR Llatr_TIB1_PR_BR Llatr_CAPIN1_SC_BR Llatr_TSAMP1_SP_BR Llatr_NITA1_SP_BR Llatr_IPU1_SC_BR Llatr_RBRA1_SP_BR Llatr_PIRA1_PR_BR Llatr_SLPAR1__SP_BR Llatr_SOL1_RS_BR Llatr_VAC2_RS_BR Llatr_VAC3_RS_BR Llatr_VAC4_RS_BR Llatr_VAC5_RS_BR Llatr_TIB1_PR_BR_2 Llatr_TIB2_PR_BR Llatr_TIB3_PR_BR Llatr_TIB4_PR_BR Llatr_SOL2_RS_BR Llatr_SOL3_RS_BR Llatr_SOL4_RS_BR Llatr_PAL1_PR_BR Llatr_PAL2_PR_BR Llatr_PAL3_PR_BR Llatr_PAI1_SC_BR Llatr_PAI2_SC_BR Llatr_PAI3_SC_BR Llatr_CA1_RS_BR]
4	3	Llatr_PS1_BA_BR Llatr_CB1_ES_BR Llatr_URU1_BA_BR]
5	11	Llatr_ANG1_SC_BR Llatr_BOT1_SC_BR Llatr_TREV1_SC_BR Llatr_IGUA1_SP_BR Llatr_SANTO1_SP_BR Llatr_ICOM1_SP_BR Llatr_CANA2_SP_BR Llatr_IGUA2_SP_BR Llatr_ICARD1_SP_BR Llatr_ITAT1_RS_BR Llatr_ITAT2_RS_BR]
6	1	Llatr_RJAN2_RJ_BR]
7	28	Llatr_MCAST1_RS_BR Llatr_CLA1_RS_BR Llatr_SEP1_RS_BR Llatr_YEG1_PY Llatr_AVER1_PY Llatr_ROCH1_UY Llatr_MALD1_UY Llatr_COL1_UY Llatr_SANT1_RS_BR Llatr_SGAB2_RS_BR Llatr_SGAB4_RS_BR Llatr_SBV1_RS_BR Llatr_SBV3_RS_BR Llatr_SBV4_RS_BR Llatr_QUA6_RS_BR Llatr_QUA7_RS_BR Llatr_LS1_RS_BR Llatr_LS2_RS_BR Llatr_LS3_RS_BR Llatr_LS4_RS_BR Llatr_LS5_RS_BR Llatr_JAG1_RS_BR Llatr_JAG2_RS_BR Llatr_JAG4_RS_BR Llatr_JAG5_RS_BR Llatr_ALE2_RS_BR Llatr_TAV1_RS_BR Llatr_CA2_RS_BR
8	1	Llatr_BJS1_SC_BR
9	3	Llatr_SAP1_RS_BR Llatr_SGAB1_RS_BR Llatr_QUA1_RS_BR

10	1	Llatr_CANA1_SP_BR
11	2	Llatr_PRAD1_BA_BR Llatr_ITAM1_BA_BR
12	1	Llatr_ANTO1_PR_BR
13	1	Llatr_LINH1_ES_BR
14	7	Llatr_AVER2_PY Llatr_DURAZ1_UY Llatr_MALD5_UY Llatr_VAC1_RS_BR Llatr_QUA3_RS_BR Llatr_ALE3_RS_BR Llatr_ALE4_RS_BR
15	1	Llatr_MAF1_SC_BR
16	1	Llatr_ICOM2_SP_BR
17	1	Llatr_CAMAN1_MG_BR
18	1	Llatr_QBAR1_PR_BR
19	1	Llatr_TREV2_SC_BR
20	4	Llatr_CL2_UY Llatr_MALD2_UY Llatr_MALD3_UY Llatr_ALE1_RS_BR
21	1	Llatr_MALD4_UY
22	1	Llatr_SOL5_RS_BR
23	1	Llatr_SGAB3_RS_BR
24	1	Llatr_SBV2_RS_BR
25	1	Llatr_QUA2_RS_BR
26	2	Llatr_QUA4_RS_BR Llatr_QUA5_RS_BR
27	1	Llatr_JAG3_RS_BR
28	1	Llatr_TAV2_RS_BR

Table A1.12. List of haplotypes, frequency of 16S gene sequences and individuals of the nominal frog *Physalaemus gracilis* that have this haplotype.

Haplotype Number	**Frequency Sequence**	**Individual Code**
1	2	Pgrac_RGR1_BR Pgrac_RGR2_BR
2	10	Pgrac_SEP1_BR Pgrac_SMARG1_BR Pgrac_SEP2_BR Pgrac_SMARG2_BR Pgrac_SMARG3_BR Pgrac_LSUL3_BR Pgrac_SEP3_BR Pgrac_JAG2_BR Pgrac_SEP4_BR Pgrac_PIR2_BR
3	1	Pgrac_JAG1_BR
4	2	Pgrac_FLOR1_UY Pgrac_MAL1_UY
5	5	Pgrac_SBV1_BR Pgrac_SBV2_BR Pgrac_JAG3_BR Pgrac_JAG4_BR Pgrac_PIR1_BR
6	2	Pgrac_LSUL1_BR Pgrac_LSUL2_BR
7	1	Pgrac_SOL1_BR
8	1	Pgrac_SOL2_BR
9	1	Pgrac_SGA1_BR
10	1	Pgrac_TIB1_BR
11	2	Pgrac_TIB2_BR Pgrac_TIB3_BR
12	4	Pgrac_TAV1_BR Pgrac_TAV2_BR Pgrac_TAV3_BR Pgrac_TAV4_BR
13	1	Pgrac_PAI1_BR

Table A1.13. List of haplotypes, frequency of 16S gene sequences and individuals of the nominal frog *Scinax uruguayus* that have this haplotype.

Haplotype Number	Frequency Sequence	Individual Code
1	8	Suru_ALE1_BR Suru_ALE2_BR Suru_ALE3_BR Suru_ALE7_BR Suru_ALE10_BR Suru_ALE11_BR Suru_ALE13_BR Suru_ALE14_BR
2	1	Suru_CAMB1_BR
3	1	Suru_GCAR1_BR
4	5	Suru_SEP1_BR Suru_SEP2_BR Suru_SEP3_BR Suru_SMAR1_BR Suru_SMAR2_BR
5	1	Suru_SEP4_BR
6	1	Suru_TIB1_BR
7	1	Suru_TIB2_BR
8	8	Suru_PALT1_BR Suru_PALT4_BR Suru_SMAR4_BR Suru_JAG5_BR Suru_TACUA1_UY Suru_TACUA2_UY Suru_CL1_UY Suru_CL2_UY
9	2	Suru_PALT2_BR Suru_SMAR3_BR
10	2	Suru_PALT3_BR Suru_JAG1_BR
11	1	Suru_JAG2_BR
12	1	Suru_JAG3_BR
13	1	Suru_JAG4_BR
14	2	Suru_SANT1_BR Suru_SANT2_BR
15	3	Suru_PAI1_BR Suru_PAI2_BR Suru_PAI4_BR
16	1	Suru_PAI3_BR
17	5	Suru_PALM1_BR Suru_PALM2_BR Suru_PALM3_BR Suru_PALM4_BR Suru_PALM5_BR
18	3	Suru_ALE4_BR Suru_ALE5_BR Suru_ALE9_BR
19	4	Suru_ALE6_BR Suru_ALE12_BR Suru_ALE15_BR Suru_ALE16_BR
20	1	Suru_ALE8_BR

Table A1.14. List of abbreviations used in the code of the individuals of the six nominal frogs codistributed in the *Campos*.

	Abreviations	**Locality**
1	ALE	Alegrete_RS_BR
2	ANTO	Antonina_PR_BR
3	ARA	Arantina_MG_BR
4	ASS	Assis_SP_BR
5	ATIB	Atibaia_SP_BR
6	AVER	Alto Vera, Itapua, PY
7	BALT	Bairro_Alto_SP_BR
8	BAU	Bauru, SP
9	BG	Bento_Gonçalves_RS_BR
10	BJS	Bom_Jardim_da_Serra_SC_BR
11	BOT	Botuvera_SC_BR
12	BROT	Brotas_SP_BR
13	CA	Cruz_Alta_RS_BR
14	CAM	Camamu_BA_BR
15	CAMAN	Camanducaia_MG_BR
16	CAMB	Cambará do Sul_RS_BR
17	CAMP	Campinas_SP_BR
18	CANA	Cananeia_SP_BR
19	CAPIN	Capinzal_SC_BR
20	CAR	Caraiva_BA_BR
21	CARAN	Carangola_MG_BR
22	CB	Conceicao_da_Barra_ES_BR
23	CGSUL	Campina_Grande_do_Sul_PR_BR
24	CGUI	Chapada_dos_Guimaraes_MT_BR
25	CL	Cerro Largo_UY_
26	CLA	Cerro_Largo_RS_BR
27	CLIMP	Campo_Limpo_de_Goias_GO_BR
28	CNOV	Campos_Novos_SC_BR
29	COL	Colonia_UY
30	DURAZ	Durazno_UY
31	EST	Estiva_MG_BR
32	FLOR	Florida_UY
33	GAL	Galia_SP_BR
34	GCAR	General Carneiro_PR_BR
35	GMOG	Grao_Mogol_MG_BR
36	GUAR	Guarapari, ES
37	ICARD	Ilha_do_Cardoso_SP_BR
38	ICOM	Ilha_Comprida_SP_BR
39	IGUA	Iguape_SP_BR
40	IPU	Ipuaçu_SC_BR

41	IPU	Ipuaçu_SC_BR
42	ITAM	Itamaraju_BA_BR
43	ITAT	Itati_RS_BR
44	ITIR	Itirapina_SP_BR
45	JAC	Jacarezinho_PR_BR
46	JAGUA	Jaguariaiva_PR_BR
47	JBON	Jose_Bonifacio_SP_BR
48	JUQUI	Juquitiba_SP_BR
49	LANT	Luis_Antonio_SP_BR
50	LAV	Lavalleja_UY_
51	LINH	Linhares_ES_BR
52	LMUL	Lauro_Muller_SC_BR
53	LS/LSUL	Lavras_do_Sul_RS_BR
54	LSAN	Lagoa_Santa_MG_BR
55	MACH	Machadinho_RS_BR
56	MAF	Mafra_SC_BR
57	MAL/MALD	Maldonado_UY
58	MAND	Manduri_SP_BR
59	MARAT	Marataizes_ES_BR
60	MCAST	Mato_Castelhano_RS_BR
61	MSUL	Mimoso_do_Sul_ES_BR
62	NIT	Niteroi_RJ_BR
63	NITA	Nova Itapirema, SP
64	NLIM	Nova_Lima_MG_BR
65	OP	Ouro_Preto_MG_BR
66	OUR	Ourinhos_SP_BR
67	PAI	Painel_SC_BR
68	PAL	Palmas_PR_BR
69	PALM	Palmas_PR_BR
70	PALT	Pedras Altas_RS_BR
71	PARE	Parelheiros_SP_BR
72	PCAL	Poços_de_Caldas_MG_BR
73	PGUA	Palmital_Guaruva_PR_BR
74	PIED	Piedade_SP_BR
75	PIND	Pindamonhangaba_SP_BR
76	PIQ	Piquete_SP_BR
77	PIR	Piratini_RS_BR
78	PIR	Piratini_RS_BR
79	PIRA	Piraquara_PR_BR
80	PRAD	Prado_BA_BR
81	PS	Porto_Seguro_BA_BR
82	PSUL	Pilar_do_Sul_SP_BR
83	QBAR	Quatro_Barras_PR_BR
84	QUA	Quarai_RS_BR

85	RBRA	Ribeirao_Branco_SP_BR
86	RGR	Rio Grande_RS_BR
87	RGRA	Ribeirao_Grande_SP_BR
88	RJAN2	Rio _de_Janeiro_RJ_BR
89	ROCH	Rocha_UY
90	SACR	Sacramento_MG_BR
91	SANT	Santana_Livramento_RS_BR
92	SANTO	Santos_SP_BR
93	SAP	Sapiranga_RS_BR
94	SBRAN	Santa_Branca_SP_BR
95	SBV	Santana_Boa_Vista_RS_BR
96	SEP	São_Sepe_RS_BR
97	SGA/SGAB	São Gabriel_RS_BR
98	SISA	Santa_Isabel_SP_BR
99	SJBAR	Sao Jose do Barreiro, SP
100	SLPAR	Sao Luis do Paraitinga, SP
101	SMAR	Santa_Maria_RS_BR
102	SMAR/SMARG	Santa Margarida do Sul_RS_BR
103	SOL	Soledade_RS_BR
104	SPSER	Sao_Pedro_da_Serra_RS_BR
105	SRIA	Santana_do_Riacho_MG_BR
106	SRIAC	Santana_do_Riacho_MG_BR
107	SRPQ	Santa_Rita_do_Passa_Quatro_SP_BR
108	SSEB	Sao Sebastiao, SP
109	SSIM	Sao_Simao_SP_BR
110	TACUA	Tacuarembó_UY
111	TAV	Tavares_RS_BR
112	TIB	Tibagi_PR_BR
113	TIE	Tiete_SP_BR
114	TREV	Treviso_SC_BR
115	TSAMP	Teodoro_Sampaio_SP_BR
116	URU	Uruçuca_BA_BR
117	VAC	Vacaria_RS_BR
118	YEG	Yegros_Caazapa_PY

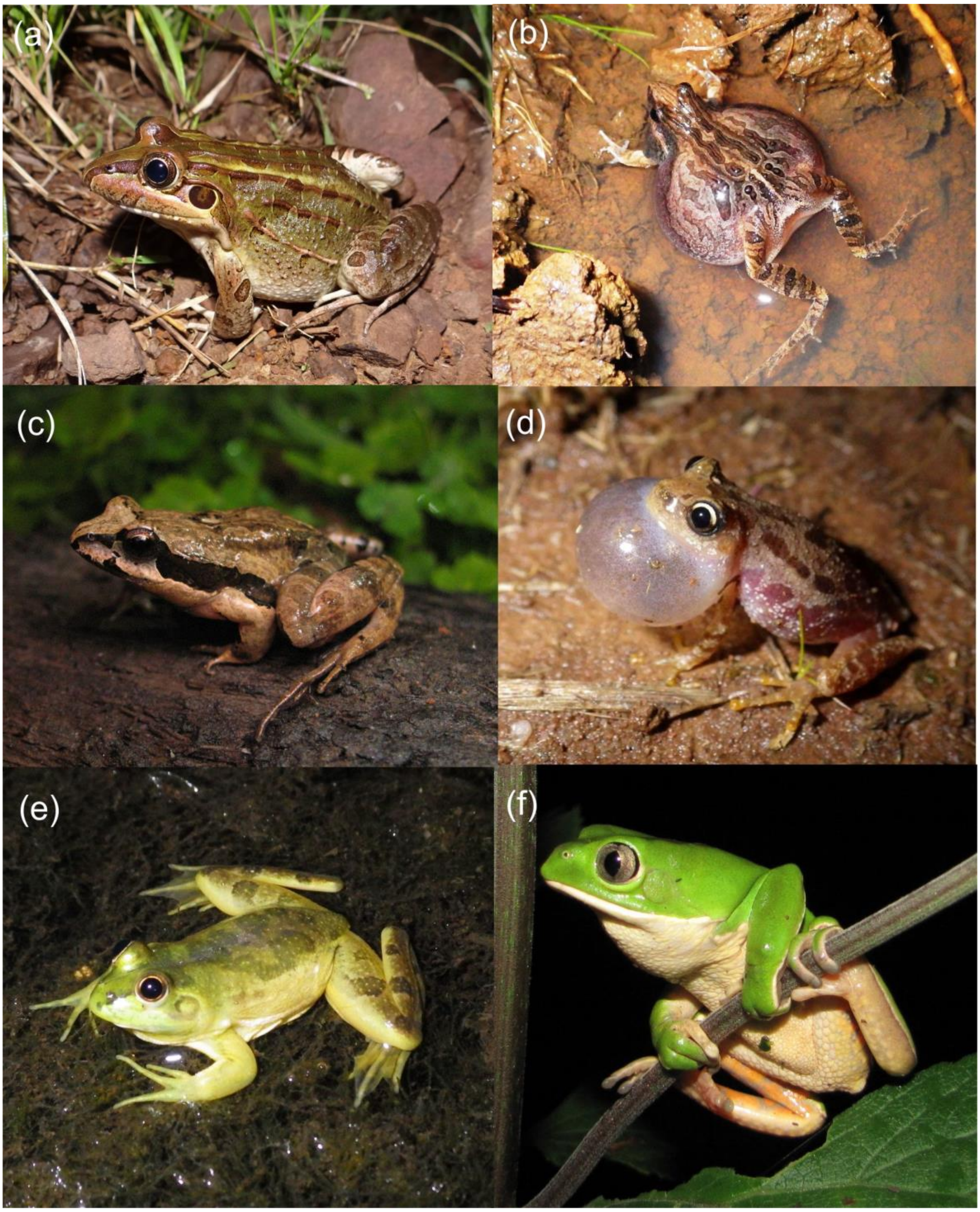

Figure A2.1 The nominal frog codistributed in the *Campos*. (a) *Leptodactylus latrans*; (b) *Physalaemus cuvieri*; (c) *Physalaemus gracilis*; (d) *Scinax uruguayus*; (e) *Pseudis minuta* and (f) *Phyllomedusa iheringii*. Photos: Saccol, S.S.A. and Santos, T.G.

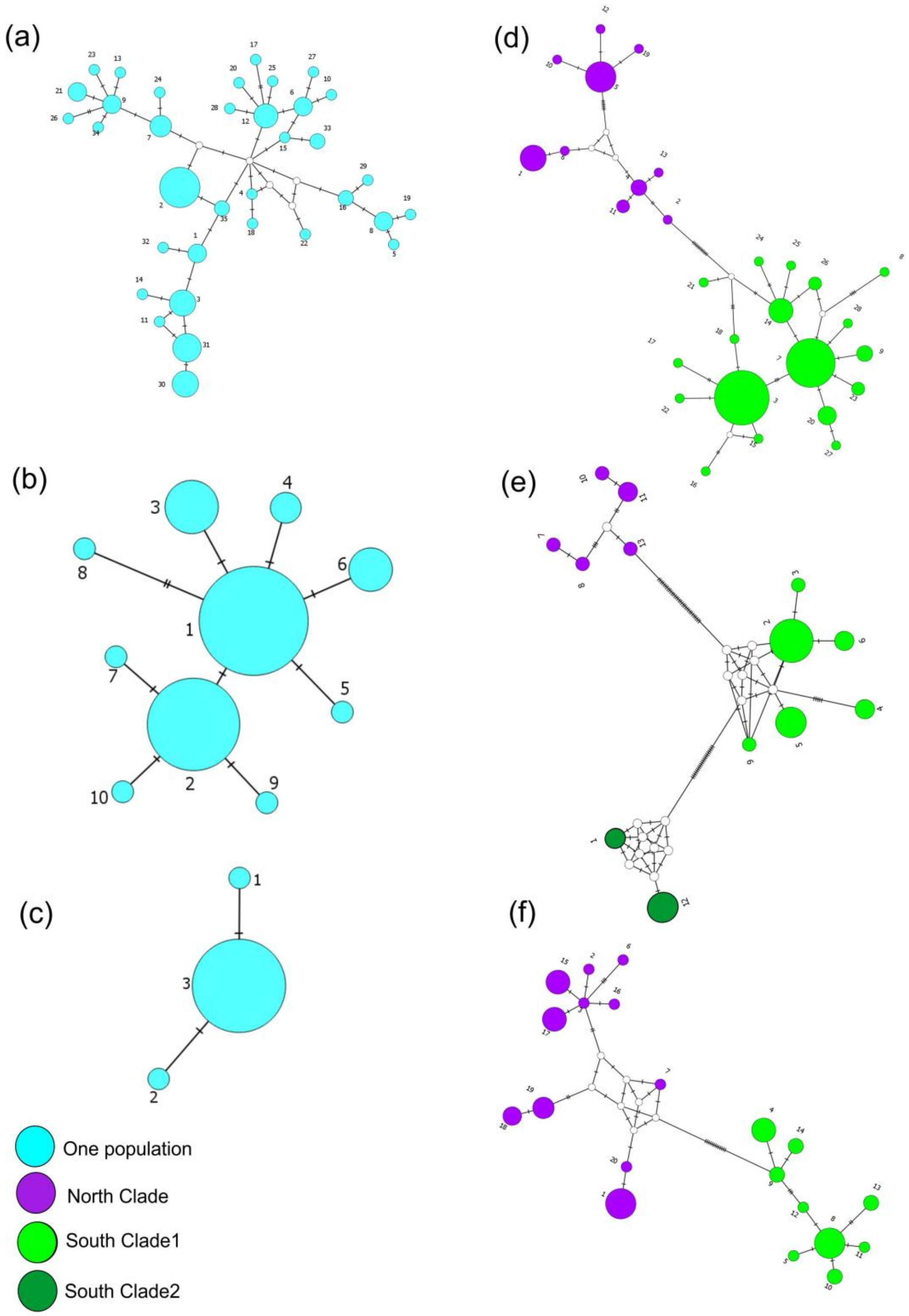

Figure A2.2 A network of haplotypes (16S) for the six nominal frogs codistributed in the *Campos*. Networks representing a single genetic population (a-c) and more than one genetic population (d-f). (a) *Physalaemus cuvieri* (N = 84); (b) *Pseudis minuta* (N = 60); (c) *Phylomedusa iheringii* (N = 21); (d) *Leptodactylus latrans* (N = 121); (e) *Physalaemus gracilis* (N = 33) and (f) *Scinax uruguayus* (N = 52). The size of the circles is proportional to the frequency of the haplotype in the population and the colors represent different genetic populations, according to the legend. Traits between haplotypes represent the number of mutations and the numbers indicate the haplotype number. White circles refer to hypothetical haplotypes (unsampled or extinct).

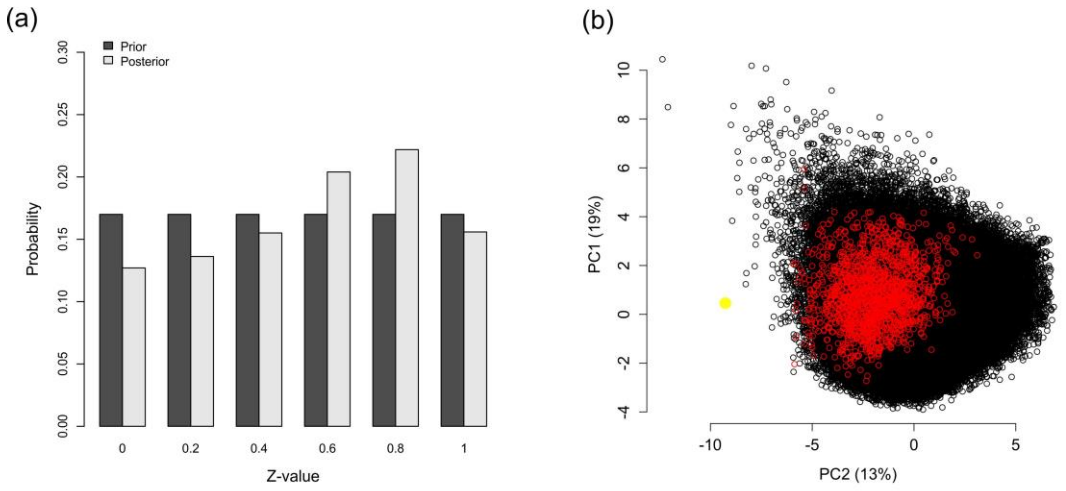

Figure A2.3 Estimation hABC of the parameter Z with the three methods generated from 500.000 simulations for model, totally three million of the simulations: (a) prior and posterior. The model points to a value of z = 0.8, i.e., four nominal frogs expanded in a synchronous pulse and one nominal frog expanded randomly; (b) prior and posterior checking using principal component analysis (PCA) of summary statistics. Statistical summary of the prior (black points), posterior (red) and the observed data (yellow).

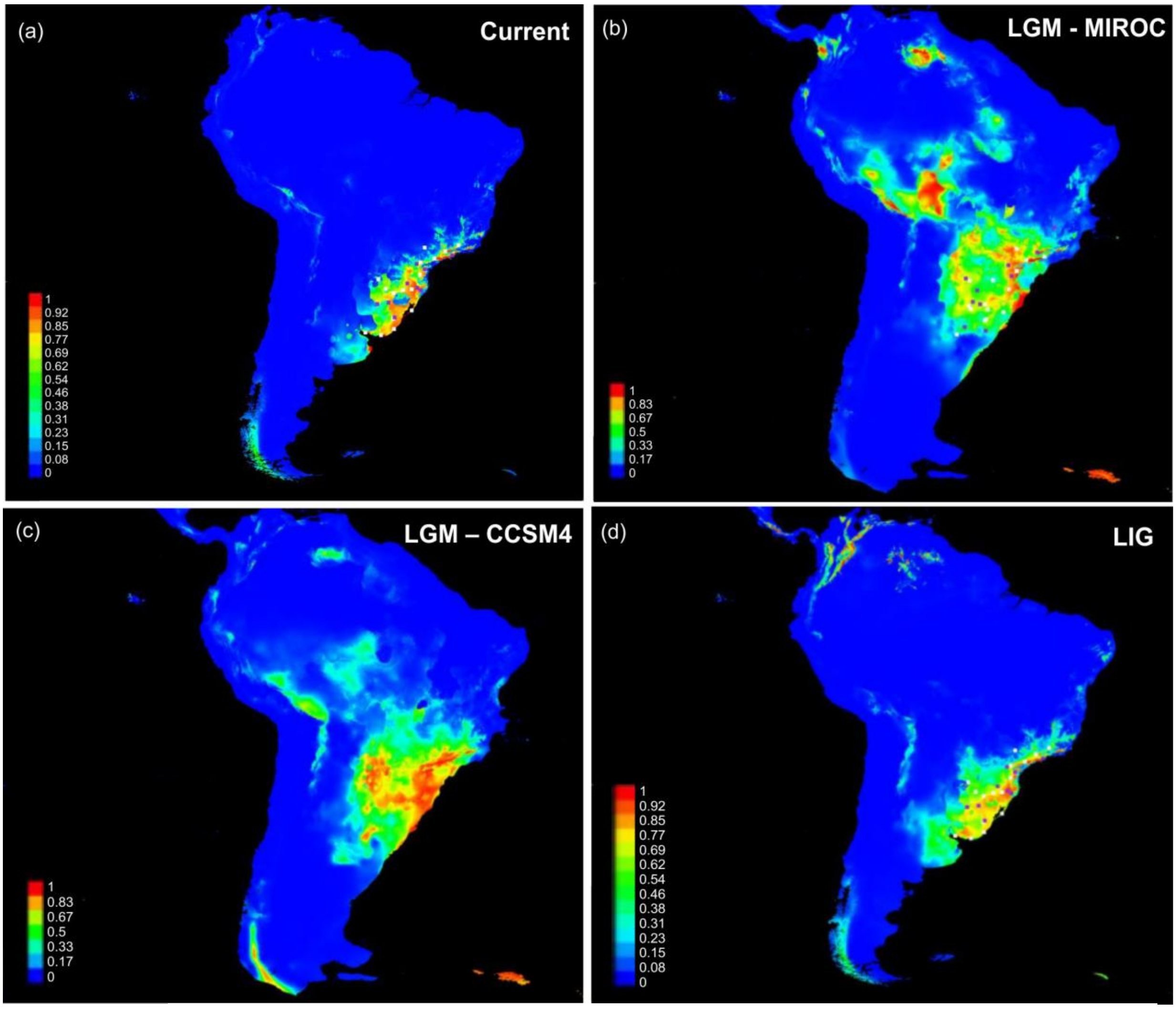

Figure A2.4 Models of habitat distribution for *Leptodactylus latrans* in (a) current time, (b) Last glacial Maximum Period - LGM MIROC, (c) Last glacial Maximum Period - LGM CCSM4 and (d) Last Interglacial Period (LIG). AUC mean value = 0.98.

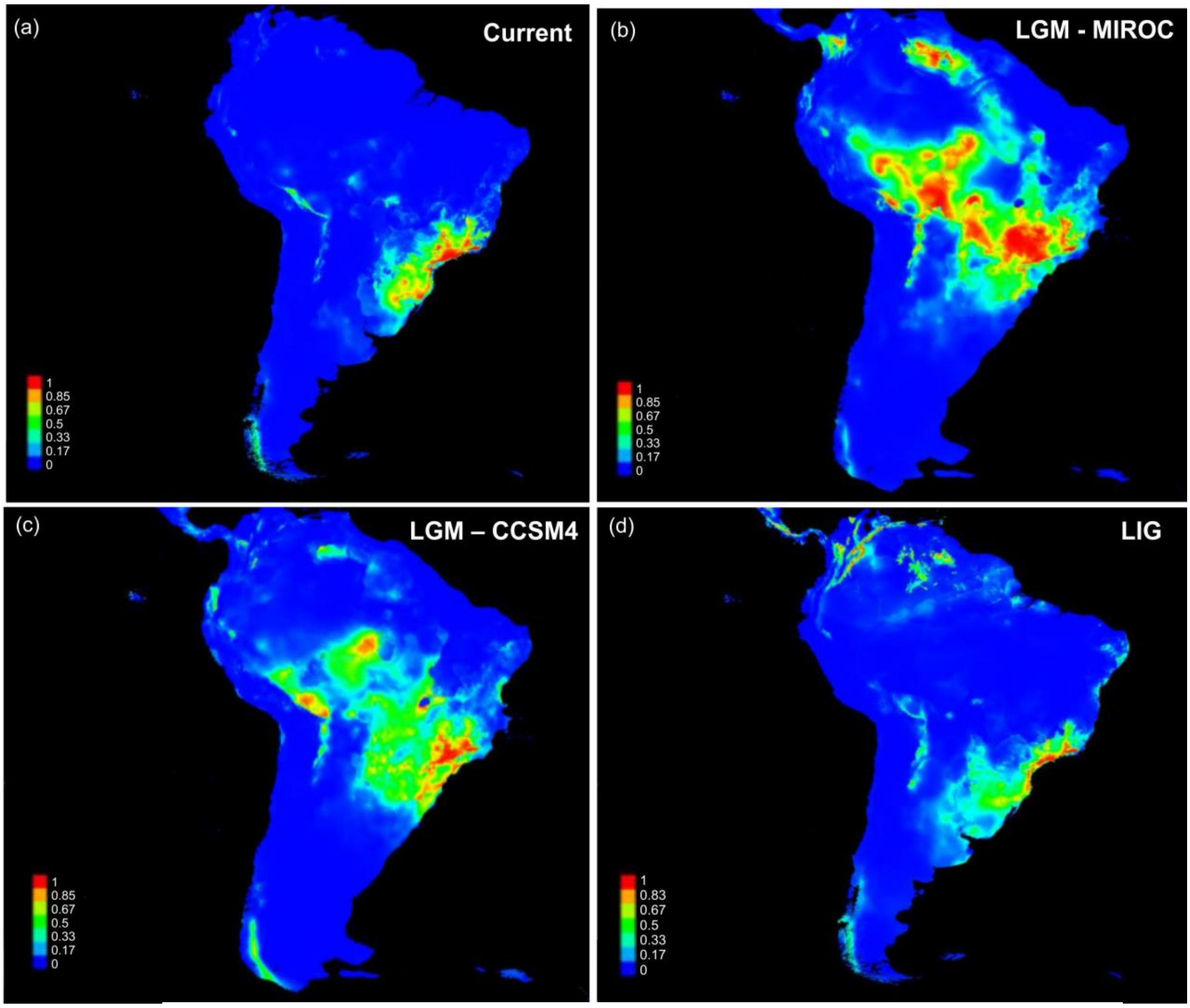

Figure A2.5 Models of habitat distribution for *Physalaemus cuvieri* in (a) current time, (b) Last glacial Maximum Period - LGM MIROC, (c) Last glacial Maximum Period - LGM CCSM4 and (d) Last Interglacial Period (LIG). AUC mean value = 0.99.

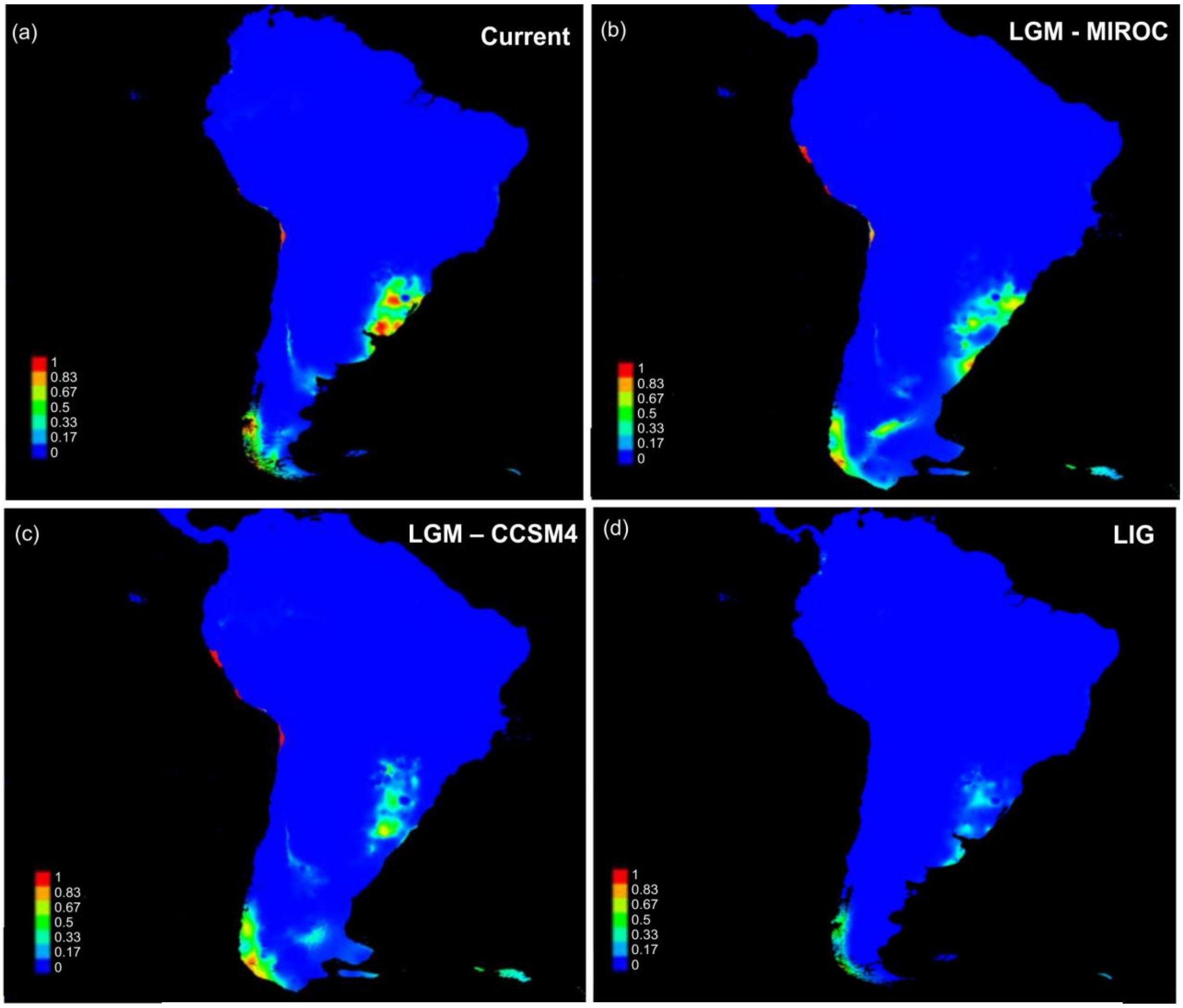

Figure A2.6 Models of habitat distribution for *Physalaemus gracilis* in (a) current time, (b) Last glacial Maximum Period - LGM MIROC, (c) Last glacial Maximum Period - LGM CCSM4 and (d) Last Interglacial Period (LIG). AUC mean value = 0.99.

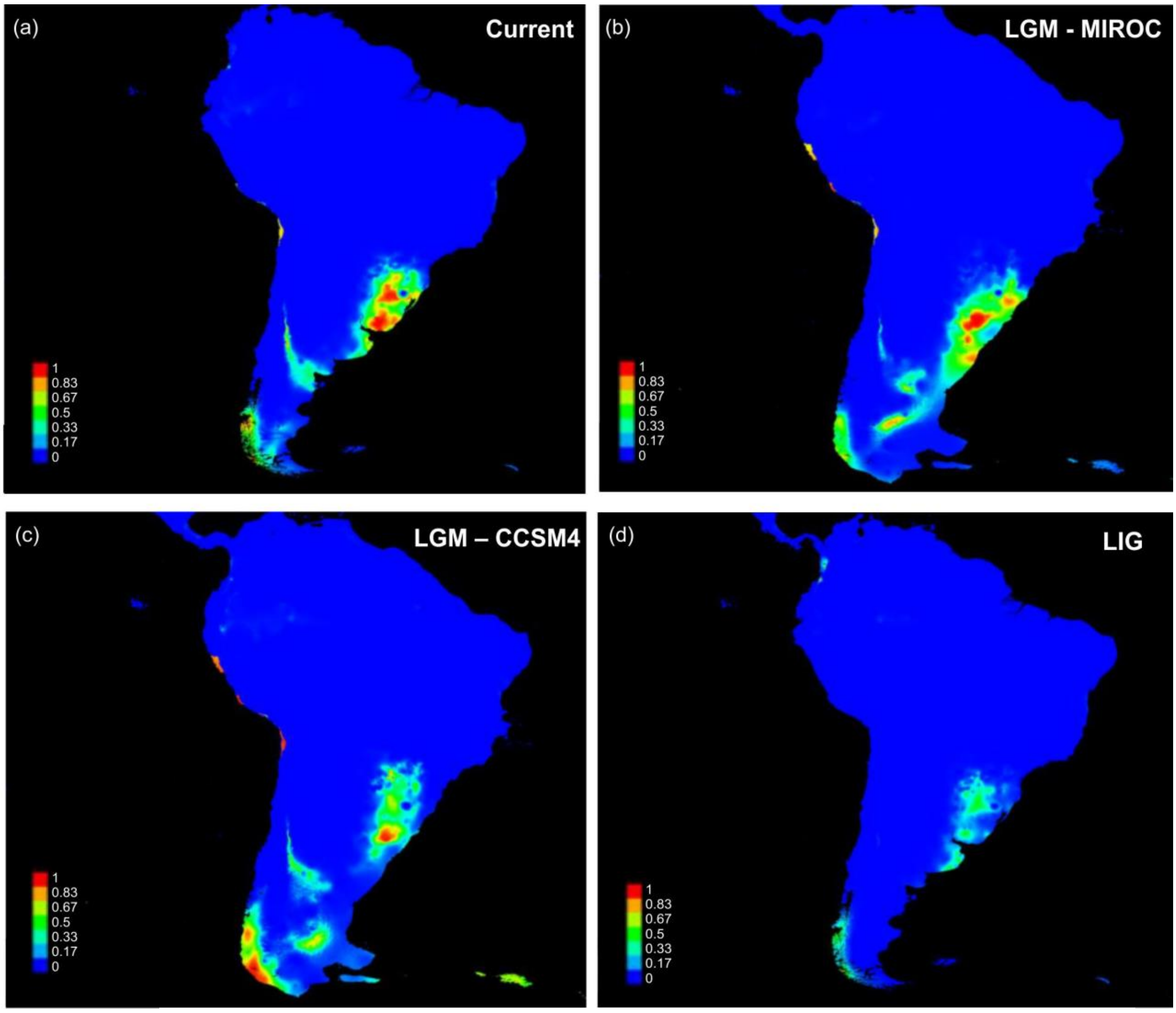

Figure A2.7 Models of habitat distribution for *Scinax uruguayus* in (a) current time, (b) Last glacial Maximum Period - LGM MIROC, (c) Last glacial Maximum Period - LGM CCSM4 and (d) Last Interglacial Period (LIG). AUC mean value = 0.98.

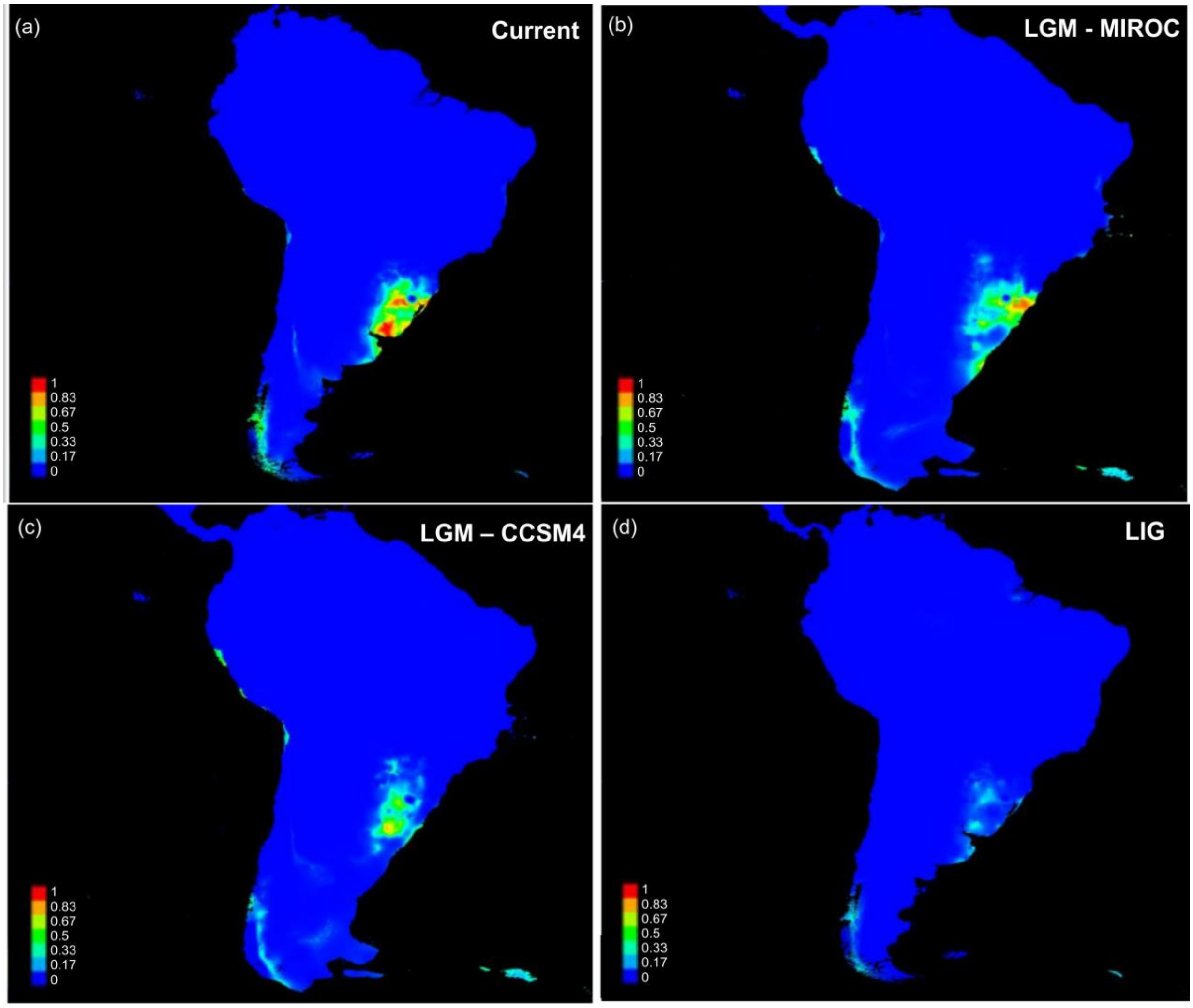

Figure A2.8 Models of habitat distribution for *Pseudis minuta* in (a) current time, (b) Last glacial Maximum Period - LGM MIROC, (c) Last glacial Maximum Period - LGM CCSM4 and (d) Last Interglacial Period (LIG). AUC mean value = 0.99.

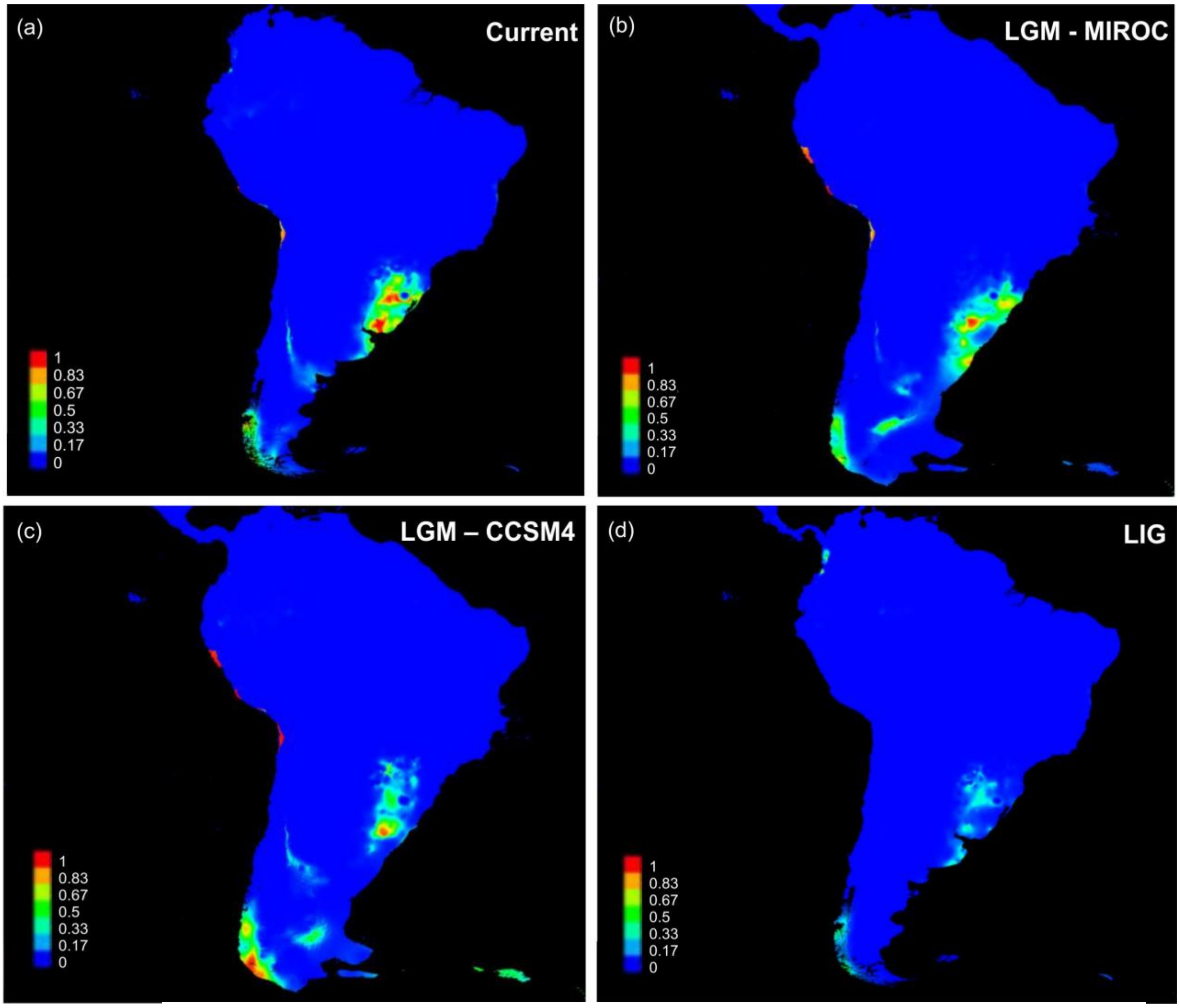

Figure A2.9 Models of habitat distribution for *Phyllomedusa iheringii* in (a) current time, (b) Last glacial Maximum Period - LGM MIROC, (c) Last glacial Maximum Period - LGM CCSM4 and (d) Last Interglacial Period (LIG). AUC mean value = 0.99.

Appendice A3.1 Supplementary file with the priors used with the coexpansão analysis for the five nominal frogs that had population expansion in the *Campos*.

```
//Number of population samples - random expansion
1
//Population sizes
{U:50000,1000000}
//Sample sizes
56
//Growth rates
0
//Number of migration matrices : If 0 : No migration between demes
0
//Historical event format:
1 historical event
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//mutation rate
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//Number of independent loci
1
//Data type, num loci, rec.rate, mut rate, gamma, shape
DNA 4.532
//
0.4 10
```

CAPÍTULO 2: CRYPTIC DIVERSITY IN CODISTRIBUTED FROGS FROM THE SOUTH AMERICAN SUBTROPICAL GRASSLANDS REVEALED BY STRONG MITOCHONDRIAL DIVERGENCE

Campos Arbustivos, município de Santana da Boa Vista, RS, Brasil.
Foto: Suelen da Silva Alves Saccol.

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RESEARCH PAPER

Cryptic Diversity in Codistributed Frogs from the South American Subtropical Grasslands Revealed by Strong Mitochondrial Divergence

Cryptic diversity in grassland frogs

Suelen S. A. Saccol[1,*] | Arley Camargo[2] | Mariana L. Lyra[3] | Raul Maneyro[4] | Sonia T. Z. Cechin[1] | Célio F. B. Haddad[3]| Tiago G. Santos[5]

[1]Departmento de Ecologia e Evolução, UFSM – Universidade Federal de Santa Maria, Centro de Ciências Naturais e Exatas, Prédio 17, sala 1140 - D, Cidade Universitária Camobi, Km 29 CEP 97105-900, Santa Maria, RS, Brazil

[2]Centro Universitario de Rivera, Universidad de la República, Ituzaingó 667, Código Postal 40.000, Rivera, Uruguay

[3]Departamento de Zoologia, Instituto de Biociências, UNESP – Universidade Estadual Paulista, Campus Rio Claro, Av. 24 A, No. 1515, Bela Vista, CEP 13506-970 Rio Claro, SP, Brazil

[4]Universidad de la República de Uruguay, Faculty of Sciences, Montevideo, Uruguay

[5]Universidade Federal do Pampa, Campus São Gabriel, Av. Antônio Trilha, 1847 CEP 97300-350, São Gabriel, RS, Brazil

**Correspondence: Departamento de Ecologia e Evolução, UFSM – Universidade Federal de Santa Maria, Centro de Ciências Naturais e Exatas, Prédio 17, sala 1140 - D, Cidade Universitária Camobi, Km 29 CEP 97105-900, Santa Maria, RS, Brazil. Phone: 0xx55 3220-8465 sub-branch 20 or branch 21.*

E-mail: suelenbio.ufsm@gmail.com

ABSTRACT

A recent phylogeographic study in the subtropical grasslands of Southeastern South America (*Campos*) found a strong divergence between genetic groups of two codistributed frogs: *Physalaemus gracilis* and *Scinax uruguayus*. Herein, we use comparative phylogeography to reconstruct patterns and process of diversification in these taxa. Using genetic polymorphisms from a mitochondrial region (16S), we reconstructed phylogenetic relationships and phylogeographic diffusion, assessed species boundaries with a bayesian coalescent-based approach, and tested to shared divergence times between the genetic groups of both species. The geographically concordant divergence events were asynchronous with divergence events taking place in the Miocene period and along to the Pliocene-Pleistocene. The phylogeographic diffusion analysis of lineages showed that probable ancestors of *Physalaemus gracilis* and *Scinax uruguayus* occurred in the Brazilian Pampas, and presented a dispersal rates of 54 and 111 km per million years, respectively. The species delimitation analyses strongly suggest that there is more than one evolutionarily independent unit within each species, representing possible candidates for new species. The two species show incongruent phylogeographic divergence, that means, their genetic groups diverged at different evolutionary times. The Miocene events were important in the initial process of diversification of these lineages that continued to diversify throughout the Pliocene-Pleistocene. We emphasize the importance of the Pampas grasslands as center of dispersal and *in situ* diversification, revealing the occurrence of cryptic species within this subtropical ecosystem.

Keywords

anuran diversification, South America’ campos, grassland conservation, comparative phylogeography, species delimitation, speciation.

1 | INTRODUCTION

In the last decades, the advance in molecular biology mainly in phylogenetic methods has revolutionized the study of biological diversification (Rull, 2018) with direct implications for biodiversity conservation. Despite this breakthrough, crucial issues remain unanswered. Hortal et al. (2015) suggested seven main shortfalls of biodiversity knowledge: of systematics and taxonomy (the Linnean shortfalls), biogeography and distribution (Wallacean), population biology and abundance (Prestonian), evolutionary patterns (Darwinian), functional (trait-based) ecology (Raunkiæran), abiotic tolerances (Hutchinsonian), and ecological interactions (Eltonian). For instance, according to the Linnean shortfalls, there are species that have not yet been sampled, and species that have already been sampled but have not yet been described. The Wallacean shortfalls refers to the incomplete knowledge about geographical distribution of most species (Hortal et al., 2015). The taxonomic challenge presented by cryptic species (two or more distinct species classified as a single taxonomic unit) is ancient, but the advent of DNA sequencing provided researchers with a new tool to detect and differentiate morphologically similar species (Bickford et al., 2007). The ever-growing availability of genetic data and a wider geographic sampling led to a rapid increase in the number of cryptic species discovered, comprising a significant proportion of the diversity of many region (Bickford et al., 2007; Singhal, Hoskin, Couper, Potter & Moritz, 2018). The frequency in which cryptic species are discovered using DNA sequences suggests that molecular data should always be considered in taxonomic studies (Bickford et al., 2007). Because phylogeography is a field of study concerned with the principles and processes that govern the geographical distribution of family lineages, especially those within and between closely related species (Avise et al., 1987; Avise, 2000), this conceptual and methodological bridge between systematics and population genetics has a crucial role in filling these biodiversity gaps.

Speciation is not always accompanied by morphological changes (Bickford et al., 2007) and the relationship between genotypic and phenotypic differentiation in the early stages of speciation is still poorly understood (Kaefer, Tsuji-Nishikido, Mota, Farias & Lima, 2013). Thus, cryptic species, or taxa that are morphologically similar but genetically divergent, are the major challenge for species delimitation (Singhal et al., 2018). However, species delimitation is a complex topic, in part because there is no universally accepted definition of species (Mallet, 2013; Rannala, 2015). Among complementary methods for delimitation of candidate lineages of species, selection of Bayesian models are considered suitable for identifying morphologically cryptic species (Leaché, Zhu, Rannala & Zang, 2018). Reconstruction of phylogeographic related patterns of cryptic species could reveal sites of endemism and diversity still unknown that could justify a reconsideration of specific conservation habitats or sites (Bickford et al., 2007).

Amphibians are considered a widely diversified group with more than 8,000 species currently known (Frost, 2019), presenting an average of 150 new species described per year worldwide (AmphibiaWeb: https://amphibiaweb.org). In fact, amphibians have presented a high rate of discovery and species description (Köhler et al., 2005) and, with the advancement in molecular techniques, several taxonomic groups underwent extensive systematic and phylogenetic revision (Loebman et al., 2015). Biodiversity inventories of frogs based only on morphology may be misleading since the conservative morphological evolution of amphibians might obscure divergent evolutionary lineages that deserve recognition and protection (Stuart, Inger & Voris, 2006). Despite the increase in amphibian biodiversity, studies indicate that amphibian populations have been seriously reduced worldwide (Wake, 1991; Gibbons et al., 2000; Schlaepfer, Hoover & Dodd, 2005; Beebee, Wilkinson & Buckley, 2009) and currently the loss and fragmentation of habitat have been identified as one of the major causes of global population declines (Stuart et al., 2004; Ficetola, Rondinini, Bonardi, Baisero & Padoa-Schioppa, 2015). Avoiding habitat loss is perhaps the greatest challenge for preserving global biodiversity, and prioritization of habitats for conservation often depend on estimating species richness and endemism (Bickford et al., 2007). However, there is still a considerable amount of unrecognized diversity, requiring an acceleration of taxonomic exploration (Vences, Thomas, Bonett & Vieites, 2005).

The subtropical grasslands in the Southeastern region of South American (latitudinal range between 25°S and 35°S), referred hereafter as *Campos*, are commonly divided into the Pampa grasslands (present at Uruguay, part of Argentina and Southern Brazil) and the Highland grasslands (forming a natural mosaic associated to the Araucarian forest in Southern Brazil) (Andrade et al., 2015; Overbeck et al., 2015; Andrade et al., 2019). Despite the immense biodiversity found in several groups of plants and animals, and the environmental diversity provided by the grasslands, little is known about the processes that have influenced the diversification of species at the grassland of South America (Turchetto-Zolet, Pinheiro, Salgueiro & Palma-Silva, 2013). In recent years, the few studies about the phylogeographic pattern of taxa occurring in these subtropical grasslands have shown a complex diversification process (see refs in Saccol et al., in prep.). In this study, the authors used a statistical, coalescent-based approach compared the phylogeographic patterns of six nominal frog species codistributed in the *Campos* (Saccol et al., in prep.), and highlighted that climatic dynamics of the Neogene-Quaternary were important to the demographic history of frog species. For Ortiz-Jaureguizar & Cladera (2006), Neogene climatic and geological events, as well as Quaternary climate fluctuations have been pointed out as the main causes of the biota diversification in this region.

In this work, we investigated the phylogeographic pattern recovered by Saccol et al. (in prep.) for two nominal frogs codistributed in the Pampa and Highland grasslands: *Physalaemus gracilis* (Boulenger, 1883) and *Scinax uruguayus* (Schmidt, 1944). We investigated these diversification processes using the 16S gene, which in amphibians is considered as sufficiently variable to unambiguously identify most species (Vences, Thomas, van der Meijden, Chiari & Vieites, 2005) and is sensitive to recent evolutionary history (Kaefer et al., 2013). We used an approximate and hierarchical approximate bayesian computation (hABC) approach to test for temporal congruence in divergence events. Following a new perspective in the field towards trait-based predictive phylogeography (Papadopoulou & Knowles, 2016; Sullivan et al., 2019), we expected that species with similar life histories and geographical distributions will tend to show congruent phylogeographic patterns, and will respond in a similar fashion to environmental changes. In addition, we reconstructed the phylogeographic diffusion pattern in space and the time, and we used BPP (Bayesian Phylogenetics and Phylogeography) for delimiting species, considering the suggested threshold of 3% of divergence to identify candidate species of Neotropical anurans (Fouquet et al., 2007).

2 | MATERIAL AND METHODS

2.1 | Sampling

The selected species *Physalaemus gracilis* and *Scinax uruguayus* are distributed along both Pampa and Highland grasslands (Santos, Iop & Alves., 2014), and in our recent studies they presented high genetic diversity and strong genetic structure, with geographically concordant phylogeographic breaks (Saccol et al., in prep.). *P. gracilis* (Leptodactylidae) is a medium size frog belonging to a complex species group under same name (Lavilla, Kwet, Segalla, Langone & Baldo, 2010) and occupies a wide variety of environments, from meadows and plains to more anthropized places (Maneyro & Carreira, 2012). The *S. uruguayus* (Hylidae) is a small to medium size frog living exclusively in grasslands of rocky hills (Santos et al., 2014) and occurs in small bodies of water, such as ponds or temporary puddles where it reproduces explosively (Silvano, García, Kwet, Lavilla & Langone, 2004; Kwet, Lingnau & Di-Bernardo, 2010; Haddad & Prado, 2005). The species were sampled according distribution maps, the availability of tissues in the biological collections and the nomenclature used follows Jetz & Pyron, (2018). Currently these species are included in the least concern category (LC) by the International Union for Conservation of Nature (IUCN 2019).

We sampled individuals across the distribution of each species based on distribution maps of the IUCN report (Lavilla et al., 2010; Silvano et al., 2004) created using ArcGIS® software, version 10.1 (ArcGIS 2011). Our sampling effort ranged from one to 16 individuals per locality and per species. We obtained tissue samples from 85 individuals (33 of the 14 localities for *P. gracilis* and 52 of the 13 localities for *S. uruguayus*), considering the material available in herpetological collections and in field efforts. The number of individuals and localities sampled per species are available at Table A1.1 and A1.2 in Appendices. Voucher specimens of most samples are deposited in the following collections: 'Célio F. B. Haddad' amphibian collection (CFBH) at the Departamento de Zoologia, Instituto de Biociências, Universidade Estadual Paulista – UNESP, Rio Claro, SP, Brazil; Herpetological collection of the Universidade Federal de Santa Maria (UFSM) and Herpetological collection of the Universidad de la República (ZVC-B, Facultad de Ciencias, Udelar, Uruguay).

2.2 | Laboratory protocols

We isolated the total genomic DNA using ammonium acetate precipitation method (Maniatis, Fritsch & Sambrook, 1982) or glass fiber extraction protocol (CCDB - Canadian Center for DNA Barcoding, Ivanova, Dewaard & Hebert, 2006). We PCR amplified a fragment of the 16S ribosomal RNA gene (16S, ~543 bp) using primers 16SAR 'CGCCTGTTTATCAAAAACAT' and 16SBR 'CCGGTCTGAACTCAGATCACGT' described in Kessing et al. (1989). We used Taq DNA Mastex mix (Ampliqon II) to perform PCR amplifications with a combination of standard and touchdown thermal-cycling profiles used in frogs (Lyra, Haddad & De Azeredo–Espin, 2017). We purified the PCR products with enzymatic reactions and sent to Macrogen Inc. (Korea) for sequencing. We visualized the resulting chromatograms, edited and cleaned using Geneious® 9.1.4 program (www.geneious.com, Biomatters; Kearse et al., 2012), and subsequently aligned the sequences of each species using MUSCLE software (Edgar, 2004) implemented in MEGA 7.0 (Kumar, Stecher & Tamura, 2016). We searched each sequence against the NCBI database using BLAST (Basic Local Alignment Search Tool, blast.ncbi.nlm.nih.gov/Blast.cgi; Altschul, Gish, Miller, Myers & Lipman., 1990) to confirm identity. We submitted the sequences to GenBank (accession numbers will be provided upon manuscript acceptance).

2.3 | Phylogenetic relationships

We inferred the genealogies of the nominal frogs *Physalaemus gracilis* and *Scinax uruguayus* using Bayesian analyses implemented in the software BEAST v1.8.4 (Drummond, Suchard, Xie & Rambaut, 2012). We performed Markov chain Monte Carlo (MCMC) analyses assuming the model "Strict Clock" calibrated with the mutation rate of the 0.26% (0.0026 substitutions/million years, SD = 0.0001) (Evans, Kelley, Tinsley, Melnick & Cannatella, 2004; Lemmon, Lemmon & Cannatella, 2007), under Yule tree prior, including outgroups (*Physalaemus barrioi* Bokermann, 1967 and *Scinax fuscovarius* (Lutz, 1925)) and the best-fit substituition model chosen by jModelTest v.2.0 (Darriba, Taboada, Doallo & Posada, 2012). We performed one independent runs of 10 million generations and sampled every 1.000 steps. We analyzed convergence and stationarity using TRACER v1.6 (Rambaut, Suchard, Xie & Drummond, 2014), and results considered when Effective Sample Size (ESS) ≥ 200. We combined runs and trees after removing a 10% burn-in with LOGCOMBINER v1.8.0 (Drummond et al., 2012). We obtained the Maximum Clade Credibility (MCC) tree with TREEANNOTATOR v1.8.0 (Drummond et al., 2012). We visualized trees in FigTree v.1.3.1 (available at: http://tree.bio.ed.ac.uk/software/figtree/). Supported nodes were those with Bayesian support with posterior probability (PP) >0.95.

2.4 | Codivergence hypothesis testing

To test for synchrony in the divergence time (i.e., codivergence) between the phylogeographic breaks within both species, we used *dpp - msbayes* as implemented in the PyMsbayes python script (joaks1.github.io/PyMsBayes/) (Oaks, 2014), a modified version of the msBayes package (Huang, Takebayashi, Qi & Hickerson, 2011). We estimated the hyperparameter Psi (ψ) that represents the number of shared divergence events with two possible values: $\psi = 1$ (single event, codivergence) and $\psi = 2$ (two events, no codivergence) for a couple of species that shares a spatial break (see Results). We also estimated the hyperparameter Tau (τ), the shared divergence time parameter. We performed a total of one million coalescent simulations to sample from prior distributions assuming no migration across the break and approximated the posterior probability of ψ (simple rejection estimate and GLM adjusted) using default summary statistics. Prior distributions of parameters are described in the Appendix A3.1. The unit of time used was substitutions per site and to transform to years, we assumed a 16S substitution rate of 0.26% (= 0.0026 substitutions/million years) (Evans et al., 2004; Lemmon et al., 2007). In order to evaluate the model fit, we performed a Principal Components Analysis with the summary statistics of the prior, the posterior and the observed data using the function prcomp in R software (R development Core Team, 2018).

2.5 | Phylogeographic diffusion of lineage

We reconstructed the phylogeographic diffusion pattern in space and time using BEAST v1.8.4 (Drummond et al., 2012). In this analysis, we considered all samples of all individuals together for each species, not considering the genetic structure found. We applied a relaxed random walk (RRW) to model the variation in diffusion rates across branches of the genealogy and to estimate the geographic coordinates of ancestral nodes, considering genealogical uncertainty and a flexible demographic model (Lemey Rambaut, Welch & Suchard, 2010). We specified a Bayesian Skyride model (Minin, Bloomquist & Suchard, 2008), the best-fit substitution model chosen by jModelTest v.2.0 (Darriba, Taboada, Doallo & Posada, 2012), and a "Strict Clock" model. We applied a normal distributed prior for the substitution rate with a mean of 0.0026 (stand. dev. = 0.0001). We performed one independent runs of 10 million generations and sampled every 1.000 steps. Sampled parameters in log files were inspected with Tracer v. 1.4 (Available at: http://beast.bio.ed.ac.uk/Tracer) to check for convergence between runs, discard the burn-in, and estimate model parameters. We used TreeAnnotator in BEAST to produce a maximum a posteriori (MAP) tree, which was used in Spread v. 1.0.6 (Bielejec, Rambaut, Suchard & Lemey, 2011) to annotate diffusion-model parameters and produce an animation of the continuous diffusion process for visualization in Google Earth^{TM}.

2.6 | Species delimitation

The candidate species recovered as the main lineages in the phylogenetic analyses were subsequently tested in BPP3.4 (Bayesian Phylogenetics and Phylogeography) (Yang & Rannala, 2010; Yang, 2015; Flouri, Jiao, Rannala & Yang, 2018), a Bayesian inference program that uses a reversible jump (rjMCMC) to delimit species using DNA sequences under a coalescent model for multiple species (MSC). BPP analyses were run under the A10 configuration [species delimitation using a fixed guide tree] (Yang, 2015; Flouri et al., 2018). We used the BPP3 version that also co-estimates a tree together with the delimitation from a combination of rjMCMC and tree Nearest Neighbor Interchange (NNI) movements. The novel MCMC proposal based on the NNI algorithm for rooted trees, explores the space of species tree topology, eliminating the need for a user-specified guide tree (Yang & Rannala, 2014). Prior distributions of parameters are described in the Appendix A3.2 and A3.3, for *Physalaemus gracilis* and *Scinax uruguayus*, respectively). We used the same outgroups mentioned above.

3 | RESULTS

3.1 | Phylogenetic relationships

We recovered well supported clades (PP > 0.95) for both species by Bayesian analyses (Fig.1). For *Physalaemus gracilis,* we recovered three clades: North Clade containing individuals from the Highland grasslands; the South Clade 1 composed by individuals from most Pampa grasslands; and the South Clade 2 including just locations from the Coastal Region from Pampa grasslands. *P. gracilis* diverged at ~17.62 Mya (Miocene), and the divergence between the Pampa grasslands clades occurred ~10.92 Mya (Miocene), with divergences within major clades dating from the Pliocene and Quaternary periods. For *Scinax uruguayus,* we recovered two clades: North Clade composed by individuals from the Highland grasslands and some individuals from the Western Pampa grasslands (i.e. Brazilian municipality of Alegrete, RS); and the South Clade composed by individuals from the Pampa grasslands. *S. uruguayus* diverged at ~24.46 Mya (Oligocene), and the divergence between the main clades occurred ~8.64 Mya (Miocene), with divergences in the most internal clades dating from the late Pliocene. The main North and South clades of both species, despite the wide confidence intervals associated, began to diversify during the Miocene period.

3.2 | Codivergence hypothesis testing

The concordance in divergence times evaluated for the genetic groups (North and South Clades) of both species showed a very similar spatial phylogeographical break between Highland and Pampean grasslands. However, the *dpp-msbayes* analysis rejected the simultaneous divergence, that means these clades diverged at different evolutionary times, suggesting that distinct events caused inter-clade divergences (Table 1; Fig. 2). The North Clade and South1 Clade of *P. gracilis* diverged about ~17.907 Mya (95%CI=10.004–25.176 Mya), and the North and South Clades of *S. uruguayus* diverged from about ~11.270 Mya (95%CI=5.628–17.407 Mya), implying in an asynchronous pattern of divergence between these species. For both species, the mean divergence times corresponded to the Miocene period.

3.3 | Phylogeographic diffusion of lineages

The phylogeographic reconstruction of the spatio-temporal diffusion showed that the most probable ancestor of *Physalaemus gracilis* was in the grasslands of the Brazilian Pampa, near the metropolitan region of Porto Alegre (in surroundings of the municipality of Butiá, RS), located at the Depressão Central. Two dispersal events started from this region (between ~ 18.6 Mya and ~ 16 Mya), one in direction to the Southeast of the Rio Grande do Sul state (Serra do Sudeste or Escudo-Sul-Rio-Grandense), and another in direction to the Northeast of state (Planalto Meridional) (Fig 3a), thus colonizing the Southeastern and central regions of this state (~ 12 Mya) (Fig 3b). The Northeast of the Uruguayan Pampas and Southwest of Rio Grande do Sul were colonized later, ~ 4 Mya (Fig 3c). The Highland grasslands, i.e., the

Northeastern region of Rio Grande do Sul and central Santa Catarina, were colonized at ~ 2.79 Mya, with dispersal to Southern Paraná occurring at ~ 2 Mya (Fig 3c and d). The Coastal Region of Rio Grande do Sul state was colonized at ~ 1.24 Mya, while Southern Uruguay more recently (~ 600 ka). There is a final dispersal event from the Northeastern Rio Grande do Sul for municipality of Soledade at ~ 151 ka (Fig 3d). The mean diffusion rate throughout the phylogeographic history of *P. gracilis* was 53.135 km per million years (~ 5cm/year).

The phylogeographic diffusion analyses estimated that the ancestral location of *Scinax uruguayus* occurred in the grasslands of the Brazilian Pampa, in the central-west region of Rio Grande do Sul state, between São Gabriel, Cacequi and Rosário do Sul municipalities (all located in the Depressão Central) (Fig 4a). Two dispersal events originated from this point at ~ 5 Mya: one towards the region between the municipalities of Manoel Viana and São Vicente do Sul (region North of the Ibicuí River – situated in Planalto Meridional), and subsequently, another event that colonized the Southeast and Southwest regions of Southern Rio Grande do Sul (Fig 4b and c) (Serra do Sudeste and Cuesta do Haedo, respectively). Several other events occurred in this region since at ~ 3.16 Mya, concentrated in the region above (North of the Ibicuí River), and from there, two dispersal events departed towards the Highland grassland at ~ 2.25 Mya. One event colonized the grasslands of Santa Catarina and Paraná states at ~ 1.40Ma, and another event set off directly to the Northeast of Paraná (Tibagi municipality) (Fig 4c). Several other dispersal events occurred in the Southwest and Southeast of Rio Grande do Sul state in the last million years. However, the region of Alegrete (south of the Ibicuí River - situated in Cuesta do Haedo) was colonized more recently, aprox. ~ 800 ka via a dispersal event from the Northern side of the Ibicuí River, and a series of several dispersal events occurred in the last 400 thousands of years. The grasslands of the Uruguayan Pampa were also more recently colonized, approximately at 20 ka, from the dispersal events in the Southwestern region of Rio Grande do Sul state (Fig. 4d). The mean diffusion rate throughout the phylogeographic history of *S. uruguayus* was 110.606 km per million years (~ 11cm/year). Animations of the phylogeographic diffusion dynamics for each nominal frog are provided as supplemental files in ‘kml’ format that can be watched in Google Earth^{TM}.

3.4 | Species delimitation

Species delimitation analysis (BPP) supported three coalescent lineages as candidates for species recognition in *Physalaemus gracilis*: speciation probability for the basal node ((Sul1, Sul2), (North, Outgroup)) was supported with PP = 0.94, the node of the (South 1, South 2) clade with PP = 1, and the node of (North, Outgroup) clade also supported with PP = 0.96. The best delimitation models indicate a value PP = 0.96 for the delimitation of 4 species considering the Outgroup (Fig 1a and Fig 5a). Species delimitation analysis for *Scinax uruguayus* strongly supported three coalescent lineages as candidate species: speciation probability was PP = 0.99 for the divergence between the Outgroup and (North, South) clade, and PP = 1 for North vs. South and Out Group. The best delimitation model with PP = 1 contains 3 species, including the Outgroup (Fig 1b and Fig 5b).

4 | DISCUSSION

Herein, we found that *Scinax uruguayus* and *Physalaemus gracilis,* despite sharing the same distribution area, responded differently to historical, geological or environmental events. This result supports that the differences between natural histories can explain the population structure and demographic patterns. We reported intraspecific clades congruent with previously identified haploclades of Saccol et al. (in prep.), deep mtDNA structure typical of low-vagility species and potential cryptic species. The phylogenetic trees indicate that the initial diversification of *Physalaemus gracilis* dates back to the early Miocene, and of *Scinax uruguayus* to the Oligocene. Estimates of divergences pointed out by our comparative phylogeographic analysis and estimates of divergence in our phylogenetic trees indicate that the major clades (North and South) of *Physalaemus gracilis* and *Scinax uruguayus* started the diversification during the Miocene (~ 17.65 - 17.62 Mya; ~ 11.27 – 8.64 Mya, respectively), and the clades of *P. gracilis* and *S. uruguayus* continued to diversify during the Pliocene and the Quaternary.

Most of herpetofauna in South America suffered a division of lineages during the Miocene and Pliocene, with some amphibians also originating during the Oligocene (Turchetto-Zolett et al., 2013, see ref Kaefer et al., 2013). In South America, this period was marked by the rise of the Andes and marine transgressions. The Andean resurgence, for example, began at the end of the Oligocene and early Miocene (~ 23 Mya), and the most intense Andean peak formation occurred during the late Miocene (~ 12 Mya) and early Pliocene (~ 4-5 Mya) (Hoorn et al., 2010; Folguera et al., 2011). This mountain uprising played an important role in climate and ecological control in South America (Hoorn, Guerrero, Sarmiento & Lorente, 1995). Also, during the Middle and Upper Miocene, there were at least three marine transgressions that led to the formation of broad plains that were quickly colonized by grasslands when sea level decreased (Ortiz-Jaureguizar & Cladera, 2006). In relation to the herpetofauna in the Brazilian and Uruguayan Pampas, a study recovered the initial origin and diversification of a gecko lizard (*Homonota uruguayensis*) at the beginning of the Miocene (~ 20-15 Mya) (Felappi, Vieira, Fagundes & Verrastro, 2015). For amphibians, two species of *Physalaemus* diversify around ~ 15 Mya, but each species shows a distinct phylogeographic pattern. *Physalaemus fernandezae* is distributed in flat lands in the province of Buenos Aires and it has a weak phylogeographic structure, but *P. henselii* occurs in the undulating grasslands of Uruguay and southern Brazil, and it presents a strong phylogeographic structure (Barraso, 2014). To Chacoan anurofauna, frogs of the genus *Lepidobatrachus*, the period of lineage diversification occurred during the Middle Miocene

(Brusquetti, Netto, Baldo & Haddad, 2019). In these studies, the marine transgression events were evoked as the main responsible for the initial diversification of species. It is likely that marine transgressions events continued to influence the process of species diversification in the *Campos* after the Miocene, as reported to *Pseudopaludicola falcipes*, an endemic Pampa frog (Langone, Camargo & De Sá, 2016).

The divergence times between the main clades of *Scinax uruguayus* and *Physalaemus gracilis* are consistent with Andean orogeny and also with periods of marine transgression. Considering the relief of the subtropical grasslands in Southeastern South America and the current distribution of lineages, it is probable that the marine transgressions played a role in the initial diversification of the main clades of *P. gracilis* (North vs. South ~ 17 Mya, codivergence analysis), in the initial diversification of the clades within the Pampa grasslands (South 1 vs. South 2 ~ 10 Mya, phylogenetic tree), as well as in the division of the North and South clades of *S. uruguayus* (~ 11 Mya, codivergence analysis). Although they are not concordant in time (i.e., they did not diverge synchronously), they can be concordant in the event that caused this diversification, which occurred multiple times during the biogeographic history of the subtropical grasslands of Southeastern South America. As suggested for the lizard *Homonota uruguayensis* and frog *Pseudopalucidola falcipes*, these transgressions could have extinguished ancestral lineages allowing the diversification of new lineages of former "refuge" areas not affected by marine transgressions (Felappi et al., 2015; Langone et al., 2016).

Changes in sea level due to glacier melting in interglacial periods also played an important role in the evolutionary history of the Coastal Region biota (Westberg & Kadereit, 2009). Due to successive sea advance and retraction events during the Quaternary, species populations were isolated, promoting genetic differentiation over time, consequently the coastal region would be more likely to present endemic species (Quintela, Gonçalves, Bertuol, Gonzalez & Freitas, 2015). During the Pleistocene, for example, four transgressive-regressive cycles occurred in the Brazilian Pampa Coastal Region and were responsible for the formation of barriers by sand accumulation (Fernández-Stolz, 2007). For a rodent inhabitant of the Coastal Region of the Brazilian Pampa, marine transgression and regressions shaped their habitat and were determinants of the diversification process (Montes, Oliveira, Bonatto, Callegari-Jacques & Mattevi, 2008). Moreover, for a complex of lizards of the genus *Liolaemus*, the expansion of sand dunes in the Quaternary period may have influenced the early speciation of these lizards (Villamil et al., 2019). In this sense, it is possible that the events of advancement and retreat of the sea in the Coastal Region of the Brazilian Pampas also influenced the diversification of *Physalaemus gracilis* South Clade 2 (Coastal Plain).

In fact, barriers may not affect all species even within a group of related species (Brown & Lomolino, 2006). *Pseudis minuta*, for example, did not present phylogeographic structure and also occurs in the Coastal Region of the Brazilian Pampas (Saccol et al., In Prep.). The effectiveness of such barriers in preventing dispersal depends not only on the nature of the environment but also on the characteristics of the organisms themselves. These characteristics, of course, vary from one taxonomic group to another, resulting that several amphibians codistributed in this region of the grasslands do not show phylogeographic structure, such as *Pseudopaludicola falcipes* (Langone et al., 2016), *Scinax squalirostris* (Abreu-Jardim, 2018), *Physalaemus cuvieri* (de Oliveira Miranda et al., 2019; Saccol et al., In prep.), *Leptodactylus latrans*, and *Phyllomedusa iheringii* (Saccol et al., In prep.). Instead, most of these frogs presented population expansions associated with colder periods (Saccol et al., In Prep). Although these species are codistributed in the Campos, they have very peculiar phylogeographic patterns, suggesting that they have different population histories and/or ecological requirements, despite their long-term persistence in the grassland habitats. These results are consistent with the framework of the trait-based comparative phylogeography approach, which predicts discordant patterns across codistributed species as a result of life history differences (Papadopoulou & Knowles, 2016; Sullivan et al., 2019).

In our diffusion analysis, for both *P. gracilis* and *S. uruguayus*, the earlier dispersal events started in the Pampa grasslands, from lower altitude regions in the Central Depression to higher altitude (hilly regions). *P. gracilis* presented two initial diffusion events: the first on the Serra do Sudeste (or Escudo-Sul-Rio-Grandense), and the second towards the Northeast of the Southern Plateau. From Serra do Sudeste, next events spread the species to other regions of the Brazilian and Uruguayan Pampas. An event also departed from the Serra do Sudeste, towards the Coastal Region, colonizing this region more recently, in the Quaternary. A similar pattern was reconstructed for the nominal frog *S. uruguayus*, that presented two diffusion events from the ancestral area: one towards a region between Cuesta do Haedo and the Southern Plateau, and another towards the Serra do Sudeste. From Serra do Sudeste, other dispersal event colonized the Southernmost portion from this same region, and the Uruguayan Pampas more recently. The peculiar grouping of the Alegrete locality with the North clade is supported by a more recent colonization of this area from an area above (north) the Ibicuí River. According to Behling, Pillar & Bauermann (2005), the grasslands of Southern Brazil were exposed to cold and dry climates during the glacial periods, with shallow waterbodies and the lack of riparian forests along the rivers, as determined by palynological analyzes. These favorable characteristics would have allowed *S. uruguayus* to easily cross over large rivers such as the Ibicuí and to colonize areas to the south. The number of dispersal events of *P. gracilis* and *S. uruguayus* in Pampa and Highland grasslands also increases considerably during the Quaternary, and recent studies show that the Pampean clades of these two species had signs of population expansion associated with colder periods (Saccol et al., In Prep). According to palynological studies, grasslands were common in cold periods in the Southern highlands of Brazil, but restricted to environments at higher altitudes during interglacial periods (Behling, 1995; 1997; 2002; Behling & Lichte, 1997). For the lizard *Homonota uruguayensis*, the basaltic outcrops are used as shelters and can be considered ecological “islands” in the Pampas “ocean” (Felappi et al., 2015). For plants, considerable evidence indicates that ecological factors (climatic and geomorphological) are diversification drivers in the Pampa grasslands during the Pleistocene (Fregonezi, Turchetto, Bonatto & Freitas, 2013). This may explain the phylogeographic diffusion pattern reported for two frog species in our study. *S. uruguayus*, for example, is considered a typical grassland and mountain species that currently does not occur in the Central Depression region. Moreover, the diffusion rates of these species are considered relatively low when compared to those estimated for other species of the subtropical grasslands of Southeastern South America. *Physalaemus gracilis* dispersed ~5cm/year and *Scinax uruguayus* ~11 cm/year, while *Pseudopaludicola falcipes* (Langone et al., 2016), a much smaller frog, dispersed ~1 m/year and *Physalaemus cuvieri*, ~ 60 cm/year (de Oliveira Miranda et al., 2019). It is important to emphasize here the difference between dispersal and diffusion. Dispersal can only be accomplished by one or a few individuals within a short period of their life, but diffusion is a slower, gradual process that involves populations and takes place over many generations. However, these two expansion mechanisms are closely related, since diffusion often follows the dispersal leap of a species to a distant, but favorable habitat (Brown & Lomolino 2006). It is likely that the strong structure and high diversification of *P. gracilis* and *S. uruguayus* is related to lower mobility and higher ecological specificity. In addition, the dispersal of Pampean clades of these two species showed a strong association with the colder periods, which is interpreted as habitat persistence (Thomé et al., 2010) typical of mountain-tolerant and cold-tolerant taxa (Amaro et al., 2012). These findings are in line with those of Madalozzo (2018), showing that the thermal tolerance range was higher with increasing latitude due to the drop in minimum temperatures that promote adaptive resistance in subtropical taxa.

The nominal frog *Physalaemus gracilis* occupies a great variety of environments, from prairies and bathed to very anthropized places. In Brazil, there are records of *P. gracilis* from the states of Paraná, Santa Catarina and Rio Grande do Sul, while Uruguay is the Southern limit of its distribution (Maneyro & Carreira 2012). The presence of this species in the province of Misiones (Argentina) and Paraguay has not been confirmed yet. However, a recent study, based on external morphology, advertisement call, and 16S genetic distances, described a new species of this genus from the Atlantic Forest of Misiones, Northeastearn Argentina: *Physalaemus carrizorum* (Cardozo & Pereira, 2018). There is a consensus among taxonomists that this group is a complex of species and it has been suggested based on morphological and acoustic data the existence of another lineage from the Planalto Meridional of Rio Grande do Sul, which has been referred as *Physalaemus* sp. aff. *gracilis* (Kwet, Lingnau & Di-Bernardo, 2010). Cardozo & Pereira (2018) also pointed out that the advertisement calls attributed to *P*. sp. aff. *gracilis* (Kwet, 2001) coincides with the duration of the call of *P. carrizorum* sp. nov., but the quality of the advertisement is weak and the taxonomic status of Brazilian lineage should be reevaluated. In addition, there are specimens from the locality of Nova Teutônia and Santa Catarina, with morphological characteristics similar to those of *P. carrizorum* sp. nov. However, no taxonomic revision has been carried out considering specimens from this region of the Highland grasslands. Our species delimitation analysis (BPP) was extremely robust in considering our Northern clade of *P. gracilis* as a new candidate species (PP = 0.96). Therefore, considering the morphological, bioacoustic and now molecular evidence, these specimens of the Northern clade of *P. gracilis* from the Highland grasslands can be classified as a confirmed candidate species (CCS, Vieites et al. (2009). The CCS category is assigned to be supposedly distinct but undescribed species that clearly differ by morphological and bioacoustic traits, and are genetically divergent from known species (Vieites et al., 2009). In spite of these considerations, our suggestion is that our

Northern clade of *P. gracilis* should be compared with *Physalaemus carrizorum* (Cardozo & Pereira, 2018), whose occurrence in Brazil has not yet been confirmed. Our species delimitation analysis (BPP) strongly supported (PP = 1) that the Southern clades 1 (Pampa grasslands) and Southern clade 2 (Coastal Region of Pampa grasslands) also are evolutionarily distinct units. In this case, there are no known differences in neither morphological nor bioacoustic traits available until now, but the large genetic divergence and the BPP analysis suggest the classification of these lineages as unconfirmed candidate species (UCS) (Vieites et al., 2009). The UCS category includes deeply divergent genealogical lineages – unknown bioacoustically and morphologically, but usually representing geographically distant populations – for which there are genetics indications that they are distinct and undescribed species (Vieites et al., 2009).

The nominal frog *Scinax uruguayus* can be found in the Southern Brazil, from the state of Paraná to Rio Grande do Sul. There are also records in Corrientes in Argentina and it is present in hilly localities from Northern Uruguay. It is a species considered to be a specialist in habitat use, mainly in mountain ecosystems. Like the frog *Physalaemus henselii* and the lizard *Homonota uruguayensis* (Barraso, 2014; Felappi et al., 2015), *S. uruguayus* is also a species with high affinity for the rocky hills called 'serranias' (Maneyro & Carreira 2012). Actually, *Scinax uruguayus* is recorded in the Southeastern range (Lipinski & Santos, 2014), but it is not found in lowlands (such as the Central Depression or Coastal Plain of the Brazilian Pampa). There have been no reports of morphological or acoustic differences between specimens under the name *S. uruguayus*. After discovering the strong geographical structure and high genetic distance between these lineages (Saccol et al., In Prep.), we heard in the field a minor distinction in the calls of individuals from Alegrete and other regions of the Pampa. In addition, the individuals from Alegrete also appear smaller and with a spotting pattern different from remain individuals from the Pampa, but without clear dissimilarity to the specimens of the rest of the North clade. However, we did not perform any morphometric or bioacoustic analysis (pers. obs. Santos, T.G and Saccol, S.S.A.). What also impresses us is that this region appears to have been colonized well after the other regions of the Pampa grasslands that are more ecologically similar and geographically closer. Therefore, we suggest that future studies with specimens from this region consider these peculiarities. Our species delimitation analysis (BPP) was extremely robust in considering the North Clade and South Clade of *S. uruguayus* as two evolutionarily distinct units (PP=1). The North Clade can be considered an unconfirmed candidate species (UCS) (Vieites et al., 2009) because the type locality of *S. uruguayus* is located inside the South Clade distribution (Quebrada de Los Cuervos, UY).

For the amphibian group, the 16S gene proved to be variable enough to unequivocally identify most species (Vences et al., 2005b), and it is sensitive to recent evolutionary history (Kaefer et al., 2013). Bayesian models are considered appropriate to help identify morphologically cryptic species (Leaché et al., 2018) and in our study, BPP delimited five coalescing lineages under the name of two typically wild species. Three lineages within *Physalaemus gracilis* and two lineages within *Scinax uruguayus*, which we classified either in UCS or CCS (Vieites et al., 2009). However, it is worth noting that the most appropriate in this case is to consider these "species candidates", which have been delimited in these models, as coalescing strains and only to support biological species when there are other data that support this delimitation (i.e., morphology, behavior, etc.), which can be approached using the integrative taxonomy. But as soon as genetic differentiation is established, these coalescing strains can also be called Management Units - MUs or Evolutionary Units - ESUs (Moritz, 1994; Dantas, 2013) and be considered as a category for conservation purposes (Moritz, 2002). Usually, ESUs coincide with sub-specific species or categories that are commonly used to define current standards of biodiversity (Rull, 2018). For conservation purposes, we can infer that the nominal frogs *P. gracilis* and *S. uruguayus* previously distributed in the Pampa grasslands and Highland grasslands now have ESUs endemic to the Pampa, the Highland grasslands, and the Coastal Region of the Brazilian Pampas. It is undeniable that species design needs to use an integrative taxonomic approach, but considering the number of new candidate species detected by this study, it is clear that such analyzes would take considerable time. However, biodiversity data are urgently needed to help define conservation priorities, especially in relation to the Pampa grasslands, which have already lost 47% of their original coverage (MapBiomas Project: http://mapbiomas.org/). Molecular diversity data can be useful surrogates for assessing the biodiversity of amphibians before they disappear. Even if in the future some of the identified lineages are not confirmed as biological species, the gain in terms of reducing the Linnaean and Wallacean shortfalls should be considered.

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Biosketch

The authors work with communities ecology and population genetics, with particular emphasis on phylogeography and of genetic diversity in Neotropical Grasslands species.

Conflict of Interest Statement

The authors declare no conflict of interest

Authorship

S.S.A.S, M.L.L, A.C., R.M., T.G.S., S.T.Z.C. and C.F.B.H. conceived the overall study; S.S.A.S and M.L.L. generated the genetic data; S.S.A.S and A.C. performed the phylogeographical analyses. S.S.A.S., M.L.L., A.C. and T.G.S. wrote the manuscript

TABLE 1 Codivergence analysis for codistributed frogs in subtropical grasslands of southeastern South America, by using *dpp-msbayes* with 1,000,000 of simulations for the posterior probability (estimate and GLM adjusted) and 10,000 simulations for prior.

		Posterior	
Two species	**Prior**	**Estimated probability**	**GLM adjusted probability**
One event	0.5	0.298	0.327
Two events	0.5	0.702	0.673
Simulations	10000	1000000	1000000

FIGURES

FIGURE 1. Geographic distribution of (a) *Physalaemus gracilis* and (b) *Scinax uruguayus* according to IUCN (gray shading), and localities sampled within their respective distributions (spots). See Tables A1 and A2 for each location. The colors of the points are related to the clades: dots in blue represent the North clade, dots in dark green represent the South or South clade 1 and the points in light green represent the South clade 2. Molecular phylogenetic tree for haplotypes (mtDNA - 16S) and time estimate of divergence for (c) *P. gracilis* and (d) *S. uruguayus*. The blue bars indicate the 95% confidence interval of the divergence time. Numbers above the nodes are times of population divergence and below the nodes are later probabilities of the clade. The time scale is millions of years ago (Ma). Different colors were assigned to each clade recovered in the phylogenetic tree, as already mentioned above. Asterisks indicate evolutionarily significant lineages according to their respective Posterior Probability (PP) in the species boundary analysis (BPP). See Tables A1.3 and A1.4 for the respective individuals of each clade.

FIGURE 2. Codivergence analysis for *Physalaemus gracilis* and *Scinax uruguayus*: (a) representative plots using *dpp-msbayes* with 1,000,000 of simulations for the posterior probability (estimate and GLM adjusted) and 10,000 simulations for prior; (b) depiction of model of asynchronous pattern of divergence for the North and South clades the two nominal frogs codistributed in the *Campos*.

FIGURE 3. Reconstructed phylogeographic diffusion pattern in space and time for the nominal frog *Physalaemus gracilis*: (a) the most probable ancestor in the grasslands of the Brazilian Pampa, near the metropolitan region of Porto Alegre (near the municipality of Butiá,

RS, Brazil), situated in Depressão Central; (b) colonization of the Serra do Sudeste or Escudo-Sul-Rio-Grandense; (c) colonization of the northeast RS (Planalto Meridional), northeast of the Uruguayan pampas and southwest of Rio Grande do Sul, and (d) colonization of the southern Paraná, coastal region of the state of Rio Grande do Sul and south of Uruguay.

FIGURE 4. Reconstructed phylogeographic diffusion pattern in space and time for the nominal frog *Scinax uruguayus*: (a) the most likely ancestor in the grasslands of the Brazilian Pampa, in the central-west region of Rio Grande do Sul, between São Gabriel, Cacequi and Rosário do Sul (situated in Depressão Central); (b) colonization of the region between the municipalities of Manoel Viana and São Vicente do Sul (region above the Ibicuí river – situated between Cuesta do Haedo and Planalto Meridional); (c) colonization of the southeast and southwest regions of Rio Grande do Sul (Serra do Sudeste and Cuesta do Haedo, respectively), grasslands of Santa Catarina and Paraná, and (d) colonization of the northeast of Paraná (Tibagi municipality), southwest and southeast of Rio Grande do Sul, region of Alegrete (below the Ibicuí River) and the Uruguayan Pampa.

FIGURE 5. The Prior and Posterior Probability for species delimitation analysis (BPP): (a) the best topology of the tree considering ((Sul1, Sul2, (North, Out Group)) with a value Posterior Probability (PP) = 0.96 for delimitation of four species candidate, considering here the Out Group for *Physalaemus gracilis*; (b) the best topology of the tree considering (Out Group, (North, South)) with a value PP = 1 for delimitation of three species candidate, considering here the Out Group for *Scinax uruguayus*.

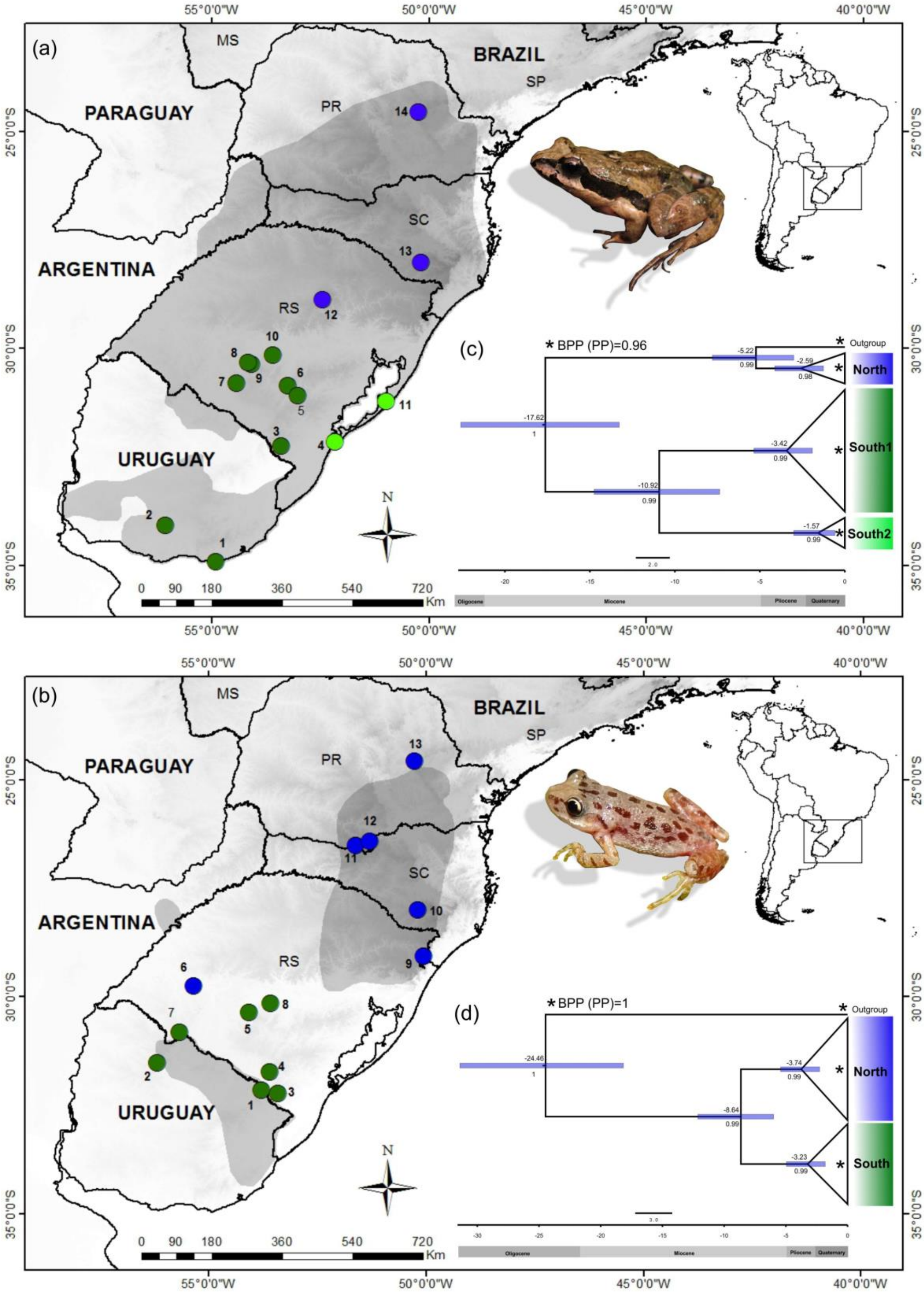
(a)
(b)
(c)
(d)
55°0'0"W
50°0'0"W
45°0'0"W
40°0'0"W
25°0'0"S
30°0'0"S
35°0'0"S
BRAZIL
PARAGUAY
ARGENTINA
URUGUAY
MS
SP
PR
SC
RS
N
0
90
180
360
540
720
Km
* BPP (PP)=0.96
-17.62
1
-10.92
0.99
-5.22
0.99
-2.59
0.98
-3.42
0.99
-1.57
0.99
Outgroup
North
South1
South2
Oligocene
Miocene
Pliocene
Quaternary
* BPP (PP)=1
-24.46
1
-8.64
0.99
-3.74
0.99
-3.23
0.99
South

Figure 1

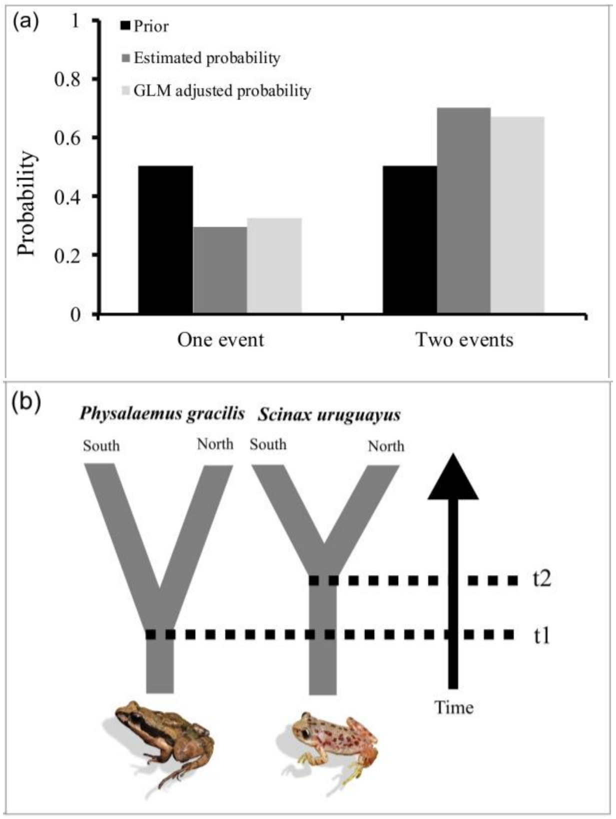
(a)
Prior
Estimated probability
GLM adjusted probability
Probability
1
0.8
0.6
0.4
0.2
0
One event
Two events
(b)
Physalaemus gracilis
Scinax uruguayus
South
North
South
North
t2
t1
Time

Figura 2

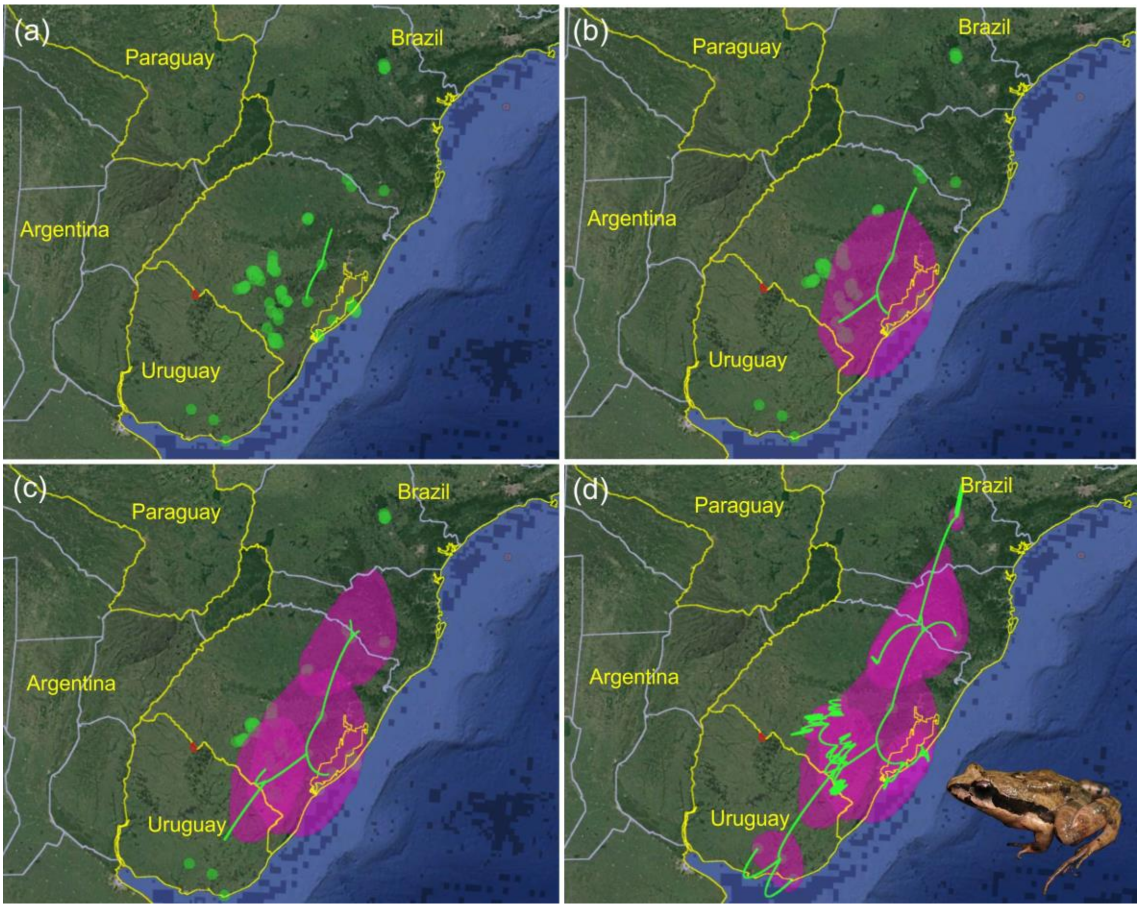
(a)
Paraguay
Brazil
Argentina
Uruguay
(b)
Paraguay
Brazil
Argentina
Uruguay
(c)
Paraguay
Brazil
Argentina
Uruguay
(d)
Paraguay
Brazil
Argentina
Uruguay

Figure 3

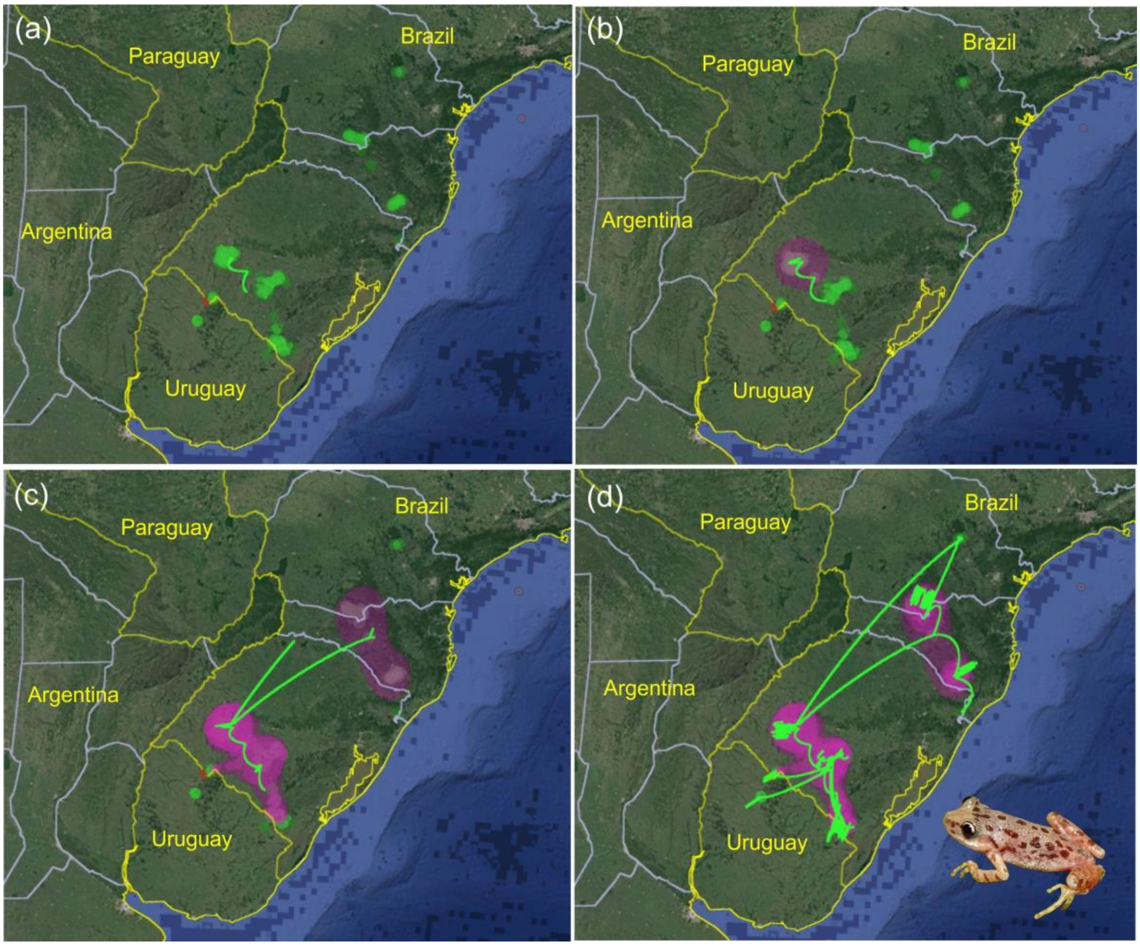
(a)
Paraguay
Brazil
Argentina
Uruguay
(b)
Paraguay
Brazil
Argentina
Uruguay
(c)
Paraguay
Brazil
Argentina
Uruguay
(d)
Paraguay
Brazil
Argentina
Uruguay

Figure 4

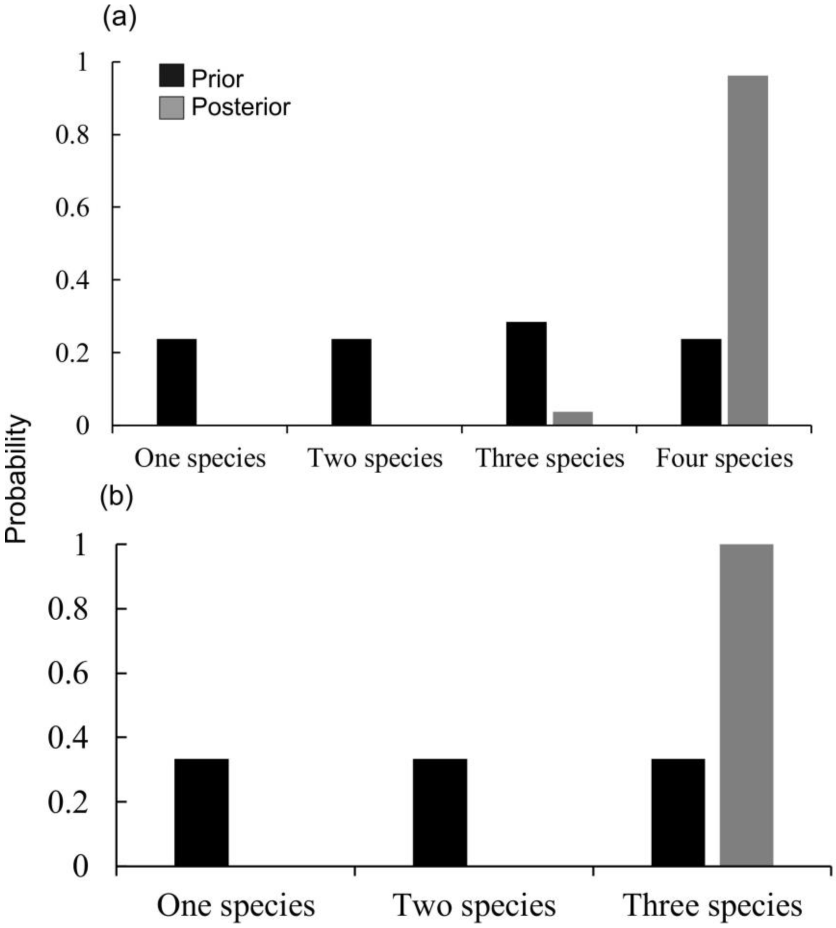
(a)
Prior
Posterior
1
0.8
0.6
0.4
0.2
0
One species
Two species
Three species
Four species
Probability
(b)
1
0.8
0.6
0.4
0.2
0
One species
Two species
Three species

Figura 5

To be submitted to Journal of Evolutionary Biology

RESEARCH PAPER

Cryptic Diversity in Codistributed Frogs from the South American Subtropical Srasslands Revealed by Strong Mitochondrial Divergence

Cryptic diversity in grassland frogs

Suelen S. A. Saccol[1,*] | Arley Camargo[2] | Mariana L. Lyra[3] | Raul Maneyro[4] | Sonia T. Z. Cechin[1] | Célio F. B. Haddad[3]| Tiago G. Santos[5]

Appendice A1 (Tables A1.1- A1.4) locality information, code numbers, and sample size for the nominal frogs. List of individual codes for each clade based on the mitochondrial 16S gene of the nominal frogs.

Appendice A2 (Figure A2.1) The nominal frogs codistributed in the Pampa grasslands and Highland grasslands.

Appendice A3 (Priors list A3.1- A3.4) priors list used for the codivergence analysis, for codistributed species in the Pampa grasslands and Highland grasslands of southeastern South America.

Table A1.1. Locality information, code numbers, and sample size for the nominal frog *Physalaemus gracilis*.

Code	**Locality**	**Sample Size**	**Latitude**	**Longitude**
1	Uruguay, Maldonado	1	-34.916764	-54.916641
2	Uruguay, Florida	1	-34.083333	-56.066667
3	Brazil, Rio Grande do Sul, Jaguarão	4	-32.25285	-53.406275
4	Brazil, Rio Grande do Sul, Rio Grande, Bairro Bolaxa	2	-32.158257	-52.169474
5	Brazil, Rio Grande do Sul, Piratini	2	-31.093114	-53.029895
6	Brazil, Rio Grande do Sul, Santana da Boa Vista	2	-30.855869	-53.241207
7	Brazil, Rio Grande do Sul, Lavras do Sul	3	-30.795847	-54.429181
8	Brazil, Rio Grande do Sul, São Gabriel	1	-30.318750	-54.157323
9	Brazil, Rio Grande do Sul, Santa Margarida do Sul	3	-30.366920	-54.079522
10	Brazil, Rio Grande do Sul, São Sepé	4	-30.144151	-53.584747
11	Brazil, Rio Grande do Sul, Tavares	4	-31.211346	-50.979794
12	Brazil, Rio Grande do Sul, Soledade	2	-28.866669	-52.456296
13	Brazil, Santa Catarina, Painel	1	-28.025900	-50.183771
14	Brazil, Paraná, Tibagi	3	-24.545027	-50.247578
Total		**33**		

Table A1.2. Locality information, code numbers and sample size for the nominal frog *Scinax uruguayus*.

Code	**Locality**	**Sample Size**	**Latitude**	**Longitude**
1	Uruguay, Cerro Largo	2	-32.150166	-53.800008
2	Uruguay, Tacuarembó	2	-31.516667	-56.200000
3	Brazil, Rio Grande do Sul, Jaguarão	5	-32.235869	-53.40996
4	Brazil, Rio Grande do Sul, Pedras Altas	4	-31.736874	-53.604956
5	Brazil, Rio Grande do Sul, Santa Margarida do Sul	4	-30.366920	-54.079522
6	Brazil, Rio Grande do Sul, Alegrete	16	-29.751534	-55.360514
7	Brazil, Rio Grande do Sul, Santana do Livramento	2	-30.810076	-55.685990
8	Brazil, Rio Grande do Sul, São Sepé	4	-30.144151	-53.584747
9	Brazil, Rio Grande do Sul, Cambará do Sul	1	-29.064007	-50.055000
10	Brazil, Santa Catarina, Painel	4	-28.003726	-50.181786
11	Brazil, Paraná, Palmas	5	-26.509966	-51.615470
12	Brazil, Paraná, General Carneiro	1	-26.421000	-51.299000
13	Brazil, Paraná, Tibagi	2	-24.575830	-50.265289
Total		**52**		

Table A1.3. List of individual codes for each clade based on the mitochondrial 16S gene of the nominal frog *Physalaemus gracilis*.

Clade	Individual Code	Locality
North	PAI1	Brazil, Santa Catarina, Painel
	SOL1	Brazil, Rio Grande do Sul, Soledade
	SOL2	Brazil, Rio Grande do Sul, Soledade
	TIB1	Brazil, Paraná, Tibagi
	TIB2	Brazil, Paraná, Tibagi
	TIB3	Brazil, Paraná, Tibagi
South 1	FLOR1	Uruguay, Flórida
	JAG1	Brazil, Rio Grande do Sul, Jaguarão
	JAG2	Brazil, Rio Grande do Sul, Jaguarão
	JAG3	Brazil, Rio Grande do Sul, Jaguarão
	JAG4	Brazil, Rio Grande do Sul, Jaguarão
	LSUL1	Brazil, Rio Grande do Sul, Lavras do Sul
	LSUL2	Brazil, Rio Grande do Sul, Lavras do Sul
	LSUL3	Brazil, Rio Grande do Sul, Lavras do Sul
	MALD1	Uruguay, Maldonado
	PIR1	Brazil, Rio Grande do Sul, Piratini
	PIR2	Brazil, Rio Grande do Sul, Piratini
	SBV1	Brazil, Rio Grande do Sul, Santana da Boa Vista
	SBV2	Brazil, Rio Grande do Sul, Santana da Boa Vista
	SEP1	Brazil, Rio Grande do Sul, São Sepé
	SEP2	Brazil, Rio Grande do Sul, São Sepé
	SEP3	Brazil, Rio Grande do Sul, São Sepé
	SEP4	Brazil, Rio Grande do Sul, São Sepé
	SGA1	Brazil, Rio Grande do Sul, São Gabriel
	SMARG1	Brazil, Rio Grande do Sul, Santa Margarida do Sul
	SMARG2	Brazil, Rio Grande do Sul, Santa Margarida do Sul
	SMARG3	Brazil, Rio Grande do Sul, Santa Margarida do Sul
South 2	TAV1	Brazil, Rio Grande do Sul, Tavares
	TAV2	Brazil, Rio Grande do Sul, Tavares
	TAV3	Brazil, Rio Grande do Sul, Tavares
	TAV4	Brazil, Rio Grande do Sul, Tavares
	RGR1	Brazil, Rio Grande do Sul, Rio Grande
	RGR2	Brazil, Rio Grande do Sul, Rio Grande

Table A1.4. List of individual codes for each clade based on the mitochondrial 16S gene of the nominal frog *Scinax uruguayus*.

Clade	Individual Code	Locality
North	ALE1	Brazil, Rio Grande do Sul, Alegrete
	ALE2	Brazil, Rio Grande do Sul, Alegrete
	ALE3	Brazil, Rio Grande do Sul, Alegrete
	ALE4	Brazil, Rio Grande do Sul, Alegrete
	ALE5	Brazil, Rio Grande do Sul, Alegrete
	ALE6	Brazil, Rio Grande do Sul, Alegrete
	ALE7	Brazil, Rio Grande do Sul, Alegrete
	ALE8	Brazil, Rio Grande do Sul, Alegrete
	ALE9	Brazil, Rio Grande do Sul, Alegrete
	ALE10	Brazil, Rio Grande do Sul, Alegrete
	ALE11	Brazil, Rio Grande do Sul, Alegrete
	ALE12	Brazil, Rio Grande do Sul, Alegrete
	ALE13	Brazil, Rio Grande do Sul, Alegrete
	ALE14	Brazil, Rio Grande do Sul, Alegrete
	ALE15	Brazil, Rio Grande do Sul, Alegrete
	ALE16	Brazil, Rio Grande do Sul, Alegrete
	CAMB1	Brazil, Rio Grande do Sul, Cambará do Sul
	GCARN1	Brazil, Rio Grande do Sul, General Carneiro
	PALM1	Brazil, Paraná, Palmas
	PALM2	Brazil, Paraná, Palmas
	PALM3	Brazil, Paraná, Palmas
	PALM4	Brazil, Paraná, Palmas
	PALM5	Brazil, Paraná, Palmas
	PAI1	Brazil, Santa Catarina, Painel
	PAI2	Brazil, Santa Catarina, Painel
	PAI3	Brazil, Santa Catarina, Painel
	PAI4	Brazil, Santa Catarina, Painel
	TIB1	Brazil, Paraná, Tibagi
	TIB2	Brazil, Paraná, Tibagi
South	CL1	Uruguay, Cerro Largo
	CL2	Uruguay, Cerro Largo
	JAG1	Brazil, Rio Grande do Sul, Jaguarão
	JAG2	Brazil, Rio Grande do Sul, Jaguarão
	JAG3	Brazil, Rio Grande do Sul, Jaguarão
	JAG4	Brazil, Rio Grande do Sul, Jaguarão
	JAG5	Brazil, Rio Grande do Sul, Jaguarão
	PALT1	Brazil, Rio Grande do Sul, Pedras Altas
	PALT2	Brazil, Rio Grande do Sul, Pedras Altas
	PALT3	Brazil, Rio Grande do Sul, Pedras Altas
	PALT4	Brazil, Rio Grande do Sul, Pedras Altas
	SANT1	Brazil, Rio Grande do Sul, Santana do Livramento
	SANT2	Brazil, Rio Grande do Sul, Santana do Livramento
	SEP1	Brazil, Rio Grande do Sul, São Sepé
	SEP2	Brazil, Rio Grande do Sul, São Sepé
	SEP3	Brazil, Rio Grande do Sul, São Sepé
	SEP4	Brazil, Rio Grande do Sul, São Sepé

	SMARG1	Brazil, Rio Grande do Sul, Santa Margarida do Sul
	SMARG2	Brazil, Rio Grande do Sul, Santa Margarida do Sul
	SMARG3	Brazil, Rio Grande do Sul, Santa Margarida do Sul
	SMARG4	Brazil, Rio Grande do Sul, Santa Margarida do Sul
	TACUA1	Uruguay, Tacuarembó
	TACUA2	Uruguay, Tacuarembó

Figure A2.1 The nominal frog codistributed in the Pampa grasslands and Highland grasslands: (a) *Physalaemus gracilis* and (b) *Scinax uruguayus*. Photos: Santos, T.G.

Appendice A3.1 (Priors list A2.1) Supplementary file with the priors used with the codivergence analysis for the two frog species that diverged in the grasslands of southeastern South America.

```
concentrationShape = 1000.0
concentrationScale = 0.001
thetaShape = 5
thetaScale = 0.0025
ancestralThetaShape = 5
ancestralThetaScale = 0.0025
thetaParameters = 012
tauShape = 2
tauScale = 0.03
timeInSubsPerSite = 1
bottleProportionShapeA = 0
bottleProportionShapeB = 0
bottleProportionShared = 0
numTauClasses = 0
migrationShape = 0
migrationScale = 0
recombinationShape = 0
recombinationScale = 0

# taxonName   locusName     Ne_Scalar     Mut_Scalar    sampleSizeA   sampleSizeB   tstv
                  seqLen        Afreq   Cfreq   Gfreq   fastaFileName
BEGIN SAMPLE_TBL
Juruguayus              16S     0.25    1       29      23      5.4935  421     0.293   0.260   0.193
fastaFromIM/Juruguayus_16S.fasta
Pgrgracilis             16S     0.25    1       6       27      4.9594  460     0.322   0.215   0.190
fastaFromIM/Pgrgracilis_16S.fasta
END SAMPLE_TBL
```

Appendice A3.2 (Priors list A3.2) Supplementary file with the priors used with the BPP analysis for the nominal frog *Physalaemus gracilis*.

```
         seed = 123

      seqfile = Pgrac.txt
     Imapfile =Pgrac.Imap.txt
      outfile = Pgrac_out.txt
     mcmcfile = Pgrac_mcmc.txt

* speciesdelimitation = 0 * fixed species tree
 speciesdelimitation = 1 0 2    * speciesdelimitation algorithm0(e)
* speciesdelimitation = 1 1 2 1   * speciesdelimitation algorithm1(a m)
   speciestree = 1 * species tree NNI
*    speciestree = 1  0.4 0.2 0.9   * speciestree pSlider ExpandRatio ShrinkRatio

  speciesmodelprior = 1  * 0: uniform LH; 1:uniform rooted trees; 2: uniformSLH; 3: uniformSRooted

  species&tree = 4  nor  sul1  sul2  out
                 6  21  6  1
                (((sul1, sul2), nor), out);

      usedata = 1    * 0: no data (prior); 1:seq like
        nloci = 1 * 1000    * number of data sets in seqfile

    cleandata = 0    * remove sites with ambiguity data (1:yes, 0:no)?

   thetaprior = 1 220   # gamma(a, b) for theta
     tauprior = 1 22 1  # gamma(a, b) for root tau & Dirichlet(a) for other tau's

*     locusrate = 0 2.0   # (0: No variation, 1: estimate, 2: from file) & a_Dirichlet (if 1)
*      heredity = 0 4 4   # (0: No variation, 1: estimate, 2: from file) & a_gamma b_gamma (if 1)
* sequenceerror = 0 0 0 0 0 : 0.05 1   # sequencing errors: gamma(a, b) prior

      finetune = 1: 1 0.002 0.01 0.01 0.02 0.005 1.0   # finetune for GBtj, GBspr, theta, tau, mix, locusrate, seqerr

        print = 1 0 0 0   * MCMC samples, locusrate, heredityscalars Genetrees
       burnin = 4000
     sampfreq = 2
      nsample = 200000

*** Note: Make your window wider (144 columns) before running the program.
```

Appendice A3.3 (Priors list A3.3) Supplementary file with the priors used with the BPP analysis for the nominal frog *Scinax uruguayus*.

```
          seed = 123

       seqfile = Surug.txt
      Imapfile =Surug.Imap.txt
       outfile = Surug_out.txt
      mcmcfile = Jurug_mcmc.txt

* speciesdelimitation = 0 * fixed species tree
 speciesdelimitation = 1 0 2    * speciesdelimitation algorithm0(e)
* speciesdelimitation = 1 1 2 1   * speciesdelimitation algorithm1(a m)
    speciestree = 1 * species tree NNI
*    speciestree = 1  0.4 0.2 0.9   * speciestree pSlider ExpandRatio ShrinkRatio

   speciesmodelprior = 1  * 0: uniform LH; 1:uniform rooted trees; 2: uniformSLH; 3: uniformSRooted

  species&tree = 3  nor  sul out
                   29   23   1
                 ((sul, nor), out);

       usedata = 1    * 0: no data (prior); 1:seq like
         nloci = 1 * 1000    * number of data sets in seqfile

     cleandata = 0    * remove sites with ambiguity data (1:yes, 0:no)?

    thetaprior = 1 112   # gamma(a, b) for theta
      tauprior = 1 12 1  # gamma(a, b) for root tau & Dirichlet(a) for other tau's

*     locusrate = 0 2.0   # (0: No variation, 1: estimate, 2: from file) & a_Dirichlet (if 1)
*      heredity = 0 4 4   # (0: No variation, 1: estimate, 2: from file) & a_gamma b_gamma (if 1)
* sequenceerror = 0 0 0 0 0 : 0.05 1   # sequencing errors: gamma(a, b) prior

       finetune  =  1:  1  0.002  0.01  0.01  0.02  0.005  1.0   #  finetune  for  GBtj,  GBspr,  theta,  tau,  mix,
locusrate, seqerr

         print = 1 0 0 0   * MCMC samples, locusrate, heredityscalars Genetrees
        burnin = 4000
      sampfreq = 2
       nsample = 200000
```

DISCUSSÃO GERAL

Quando exploramos o padrão filogeográfico dos seis anuros nominais co-distribuídos nos campos subtropicais do sudeste da América do Sul (*Campos*), fica claro que os processos de diversificação nessa região são complexos e que, de fato, espécies associadas a domínios de vegetação aberta apresentam respostas variáveis (TURCHETTO-ZOLET et al., 2013). Nossa hipótese inicial era encontrar padrões flogeográficos relacionados a padrões de distribuição e especificidade de espécies (generalistas *versus* especialistas), entretanto recuperamos de alta a baixa diversidade genética, espécies com e sem estruturação genética, bem como histórias demográficas com algumas peculiaridades, mas congruentes em sua maioria. A maioria das nossas espécies apresentou alta diversidade haplotípica, conforme já foi reportado em estudos nessa região (BARRASO, 2014; LANGONE et al., 2016; ABREU-JARDIM, 2018; de OLIVEIRA MIRANDA et al., 2019). A alta diversidade genética parece estar relacionada à abundância dessas espécies ao longo de sua distribuição e, de acordo com PABIJAN et al. (2012), altos níveis de diversidade genética dentro de populações têm sido associados a um tamanho populacional efetivo elevado por longos períodos de tempo. No entanto, a maioria de nossas espécies apresentou baixa diversidade nucleotídica. Altos valores de diversidade de haplótipos e baixa diversidade de nucleotídeos também se referem a assinaturas de expansão populacional (ARENAS et al., 2011), conforme recuperado para *Physalaemus cuvieri*, *Leptodactylus latrans* (clado Sul), *Physalaemus gracilis* (clado Sul1), *Scinax uruguauys* (clade Sul) e *Pseudis minuta*. Quando avaliadas individualmente, observamos que o início das expansões demográficas coincide com períodos glaciais durante o Quaternário. E, quando comparamos esses clados que apresentaram sinais de expansão populacional, encontramos pulsos sincrônicos de expansões populacionais para quatro dessas espécies, com exceção de *P. minuta*, que aparentemente teve um pulso aleatório de expansão posterior às demais. Mas, ambos os pulsos de expansão parecem estar fortemente associados à dinâmica climática do Neógeno-Quaternário, principalmente durante picos glaciais. Os nossos modelos de paleodistribuição também detectaram os efeitos das mudanças climáticas ao longo do tempo na dinâmica de distribuição das espécies, evidenciando um aumento de áreas adequadas durante o Último Máximo Glacial (UMG) e uma considerável redução de áreas adequadas durante o último período interglacial. As espécies campestres parecem seguir a dinâmica dos campos, que também são favorecidos por períodos mais frios (ABREU-JARDIM, 2018 e o presente estudo), diferentemente apenas de um sapo tipicamente pampeano que foi favorecido por períodos mais quentes (LANGONE et al., 2016). Essa expansão populacional e a preferência por períodos mais frios indica persistência de habitat (THOMÉ et al., 2010) e que essas espécies co-distribuídas nos campos se comportam como espécies típicas de montanha e tolerantes ao frio (AMARO et al., 2012). Estes resultados concordam com um estudo realizado recentemente sobre fisiologia térmica de anuros, ou seja, que a faixa de tolerância térmica foi maior com o aumento da latitude devido à queda nas temperaturas mínimas que promovem a adaptação à resistência na comunidade subtropical (MADALOZZO, 2018).

Foi possível recuperar o padrão demográfico para quase todas as espécies, exceto para a perereca *Phyllomedusa iheringii,* que apresentou baixíssima variação genética para esse gene mitocondrial (16S). A baixa diversidade genética pode ser o resultado de um evento demográfico recente, por exemplo, um “gargalo” ou “varredura seletiva” (GALTIER et al., 2000). No entanto, esse padrão precisa ser confirmado com a utilização de outros marcadores moleculares, pois a perda de variabilidade genética também pode indicar alto grau de endogamia e espécies que exibem esse padrão podem estar mais ameaçadas por variações ambientais. A variabilidade genética permite que as populações mantenham sua aptidão e também sua capacidade de responder às mudanças ambientais (MORITZ, 2002). Esta espécie possui características ecológicas muito específicas, é endêmica dos campos do Pampa (SANTOS et al., 2014) e possui forte fidelidade ao habitat com afloramentos rochosos, bem como exigências específicas quanto à reprodução (MANEYRO; LANGONE, 2001; DIAS et al., 2017). Além disso, apresenta baixa abundância nos campos do Pampa brasileiro (IOP, 2015; BOLZAN et al., 2016), onde é encontrada principalmente em áreas com maior porcentagem de campo remanescente (IOP, 2015). Essa situação é preocupante quando consideramos que as pastagens do Pampa foram historicamente modificadas pela ação humana (BILENCA; MIÑARRO, 2004). Estudos documentaram que o desenvolvimento agrícola (como plantações) pode destruir os habitats (JOLY et al., 2001; BENTON et al., 2003; BIEDRZYCKA; KONOPINSKI, 2008; SANTOS et al., 2015), resultando em gargalos populacionais que podem causar perda de diversidade (WANG et al., 2014). Nesse sentido, comparando os nossos achados com outros padrões já reportados, é evidente que diferentes ecologias e histórias de vida influenciam o processo de diversificação de espécies e que não existe um padrão único para espécies especialistas e generalistas de ambientes abertos. Portanto, a interpretação dos padrões de diversidade precisa considerar a interação da biologia das espécies, da geografia e da dinâmica climática (HEWITT, 2000).

Em relação às espécies co-distribuídas nos campos do Pampa e de Altitude, reportamos uma forte estruturação geográfica, alta divergência genética com um elevado número de etapas mutacionais entre clados das espécies sob o nome *Physalaemus gracilis* e *Scinax uruguayus*. Essa forte estruturação no DNA mitocondrial é típica de espécies com baixa capacidade de dispersão e diversidade genética enigmática (WERNECK et al., 2012). A origem evolutiva e a diversificação inicial de *S. uruguayus*, por exemplo, remonta ao Oligoceno e a diversificação entre os principais clados (Norte e Sul) de *S. uruguayus* e *P. gracilis* se diversificaram no Mioceno e continuaram se diversificando durante o Plioceno-Quaternário. Devido à concordância geográfica, hipotetizamos que as quebras filogeográficas, entre os clados Norte e Sul, poderiam ter sido influenciadas pelos mesmos processos geomorfológicos que ocorreram nessa região como, por exemplo, o soerguimento do Planalto Meridional (VERDUM et al., 2019). Entretanto, aqui nossa hipótese de um padrão temporalmente congruente ou síncrono de divergência não foi confirmada. Isso significa que essas espécies foram historicamente co-distribuídas e diferenciadas em resposta a diferentes eventos geológicos ou ambientais (ZINK, 1996), sugerindo que, apesar de compartilharem a mesma área de distribuição, ambas as espécies responderam idiossincraticamente a eventos evolutivos passados. A nossa análise comparativa recuperou tempos de divergência distintos entre os principais clados Norte e Sul de *P. gracilis* e *S. uruguayus*, que remontam ao Mioceno (~17,65 e ~11,27 milhões de anos atrás - Ma, respetivamente). Na América do Sul, esse período foi marcado pela ascensão dos Andes e transgressões marinhas. O ressurgimento andino, por exemplo, começou no final do Oligoceno e início do Mioceno (~ 23 Ma) e a formação mais intensa dos picos andinos ocorreu durante o final do Mioceno (~ 12 Ma) e início do Plioceno (~ 4 - 5 Ma) (HOORN et al., 2010; FOLGUERA et al., 2011). Essa elevação desempenhou um papel importante no controle climático e ecológico na América do Sul (HOORN et al., 1995). Além disso, durante o Mioceno Médio e Superior houve pelo menos três transgressões marinhas que levaram à formação de amplas planícies que foram rapidamente colonizadas por vegetação campestre quando o nível do mar diminuía (ORTIZ-JAUREGUIZAR; CLADERA, 2006). Alguns estudos em ecossistemas campestres também mencionaram os eventos de transgressões marinhas como responsável pela diversificação inicial de linhagens (e.g., BARRASO, 2014; FELAPPI et al., 2015; BRUSQUETTI et al., 2019). Embora não sejam concordantes no tempo, podem ser concordantes no evento que causou essa diversificação. Um tipo de evento que ocorreu em vários momentos da história evolutiva dos campos subtropicais do sudeste da América do Sul.

Os outros clados de *P. gracilis* e *S. uruguayus* continuaram a diversificar durante o Plioceno e o Quaternário. Durante o Plioceno Superior, o clima se tornou mais seco e frio, a vegetação campestre se expandiu pelo continente sul-americano (ORTIZ-JAUREGUIZAR; CLADERA, 2006). Cada oscilação fria foi uma era glacial onde florestas diminuíam drasticamente em grandes altitudes e consequentemente substituídas por campos e as eras glaciais eram tempos de resfriamento em geral, em que alguns eventos quentes e úmidos ocorriam durante décadas a milênios (i.e., interglacial) (BUSH; OLIVEIRA, 2006). Essa dinâmica acabou gerando uma compartimentalização da paisagem. Para FREGONEZI et al. (2013), na região dos Pampas, um gradiente climático e diferenças significativas no solo são aparentes e fatores ecológicos foram importantes no processo de especiação das plantas da família Solanaceae. Mudanças no nível do mar, devido ao derretimento das geleiras nos períodos interglaciais, também desempenharam um papel importante na história evolutiva da biota da Região Costeira (WESTBERG; KADEREIT, 2009), sendo evocados como responsáveis pela diversificação de pequenos mamíferos e lagartos (MONTES et al., 2008; VILLAMIL et al., 2019). É possível que os eventos de avanço e recuo do mar na Região Costeira dos Pampas brasileiros também tenham influenciado a diversificação do *Physalaemus gracilis* (clado Sul 2). Portanto, é provável que as transgressões marinhas do Mioceno sejam responsáveis pela diversificação inicial das linhagens de *P. gracilis* e *S. uruguayus* e que, a dinâmica climática e campestre, juntamente com a topografia da região, tenha contribuído para moldar a atual diversidade e distribuição dessas espécies, evidenciando uma forte interação entre as forças paleogeográficas e paleoclimáticas.

Sobre a origem e a dispersão de *P. gracilis* e de *S. uruguayus*, a difusão filogeográfica aponta que os primeiros eventos de dispersão para ambas as espécies partiram dos campos do Pampa, de regiões localizadas na Depressão

Central - ou seja, de uma região de menor altitude para regiões de maior altitude - sempre em direção a regiões serranas. O número de eventos de dispersão nos campos do Pampa e de Altitude também aumentaram consideravelmente a partir do Quaternário, um achado congruente com os sinais de expansão populacional relacionados a períodos mais frios já relatados para as linhagens campestres. De acordo com estudos palinológicos, os campos eram abundantes durante os períodos frios nas terras altas do sul do Brasil, mas restritos a ambientes de maiores altitudes durante os períodos interglaciais (BEHLING, 1995; 1997; 2002; BEHLING; LICHTE, 1997). Para as plantas, evidências consideráveis indicam que os fatores ecológicos (climáticos e geomorfológicos) estão relacionados à diversificação nos campos do Pampa durante o Pleistoceno (FREGONEZI et al., 2013). *S. uruguayus*, por exemplo, é considerada uma espécie típica de ecossistemas campestres serranos e atualmente não ocorre na região da Depressão Central. Além disso, as taxas de difusão dessas duas espécies são consideradas relativamente baixas quando comparadas àquelas estimadas para outras espécies que também ocorrem nos *Campos*. O sapo *P. gracilis* dispersou ~ 5cm/ano e *S. uruguayus* dispersou ~ 11 cm/ano, enquanto *Pseudopaludicola falcipes*, um sapo muito menor, dispersou ~ 1 m/ano (LANGONE et al., 2016) e *Physalaemus cuvieri*, ~ 60 cm/ano (de OLIVEIRA MIRANDA et al., 2019). É importante enfatizar aqui, a diferença entre dispersão e difusão. Dispersão só pode ser realizada por um ou alguns indivíduos em um curto período de sua vida. A difusão é uma forma mais lenta que envolve não só indivíduos, mas também populações, e é normalmente realizada ao longo de muitas gerações onde os indivíduos se espalham gradualmente. No entanto, esses dois mecanismos de expansão de amplitude estão intimamente relacionados, uma vez que a difusão geralmente segue o salto de dispersão de uma espécie em uma região de habitat colonizada, distante, mas favorável (BROWN; LOMOLINO, 2006). É provável que a forte estruturação filogeográfica e a alta diversificação de *P. gracilis* e *S. uruguayus* estejam relacionadas à menor mobilidade e especificidade ecológica aos ambientes campestres.

Essa estruturação e diversificação, levou ou ainda segue, um processo de especiação nessas linhagens sob o nome de *P. gracilis* e *S. uruguayus*. No Brasil, registramos *P. gracilis* nos estados do Paraná, Santa Catarina e Rio Grande do Sul, sendo no Uruguai, o limite sul de sua distribuição (MANEYRO; CARREIRA, 2012). A presença desta espécie na província de Missiones (Argentina) e no Paraguai ainda não havia sido confirmada. Entretanto, um estudo recente, baseado em morfologia externa, canto de anúncio e distância genética (16S), descreveu uma nova espécie desse gênero na Mata Atlântica de Misiones, nordeste da Argentina, *Physalaemus carrizorum* (CARDOZO; PEREIRA, 2018). Existe um consenso entre os pesquisadores de que este grupo é um complexo de espécies e que já havia suspeitas (i.e., morfológicas e acústicas) de que, a partir do Planalto Central do Rio Grande do Sul, poderia ocorrer outra linhagem previamente denominada *P.* sp. aff. *gracilis* (KWET et al., 2010). Além disso, CARDOZO; PEREIRA (2018) também apontam que o canto de anúncio atribuído a *Physalaemus* sp. aff. *gracilis* (KWET, 2001) coincide com a duração do canto de *P. carrizorum* sp. nov., mas a qualidade do registro é fraca e o status taxonômico desta população deve ser reavaliado. Além disso, outros exemplares da localidade de Nova Teutônia e Santa Catarina, por exemplo, possuem características morfológicas semelhantes às de *P. carrizorum* sp. nov. No entanto, nenhuma revisão taxonômica foi realizada considerando espécimes desta região dos campos de Altitude. Nossa análise de delimitação de espécies (BPP) foi extremamente robusta ao considerar o clado Norte de *P. gracilis* como uma nova espécie candidata. Portanto, considerando que já existem evidências morfológicas, bioacústicas e agora moleculares (i.e., este estudo) de espécimes desses campos de altitude, de acordo com VIEITES et al. (2009) podemos classificar o clado Norte de *P. gracilis* como uma espécie candidata confirmada (CCS). Mas, apesar dessas considerações, nossa sugestão é que o clado norte de *P. gracilis* seja contrastado com a espécie descrita recentemente do nordeste da Argentina, *Physalaemus carrizorum* (CARDOZO; PEREIRA, 2018), cuja ocorrência no Brasil ainda não foi confirmada. Nossa análise de delimitação de espécies também apontou que o clado Sul 1 (campos do Pampa) e o clados Sul 2 (campos da Região Costeira do Pampa) de *Physalaemus gracilis* também são unidades evolutivamente distintas. Neste caso, aparentemente não foram observadas distinções, nem morfológicas nem bioacústicas, mas a grande divergência genética e a delimitação apontam para uma classificação como espécies candidatas não confirmadas (UCS) (VIEITES et al., 2009).

Scinax uruguayus pode ser encontrada na região sudeste do Brasil, do estado do Paraná ao Rio Grande do Sul. Há também registros em Corrientes na Argentina e em várias localidades do Uruguai. Essa é considerada como apresentando associação faunística denominada 'riograndense' ou 'serrana' (MANEYRO;

CARREIRA, 2012). Nossa análise de delimitação de espécies foi extremamente robusta ao considerar o clado Norte e o clado Sul de *S. uruguayus* como duas unidades evolutivamente distintas. O clado Norte pode ser considerado uma espécie candidata não confirmada (UCS) (VIEITES et al., 2009) uma vez que *S. uruguayus* foi nomeada em referência ao Uruguai, onde é a sua localidade típica (Quebrada de Los Cuervos, Departamento de Treinta y Tres) (MANEYRO; CARRERA, 2012), e a região dos Pampas uruguaios pertence ao Clado Sul.

Foi possível verificar que os padrões filogeográficos recuperados até o momento para espécies típicas de áreas abertas são complexos e reforçam a ideia de que não há um padrão único de diversificação. Os períodos Oligoceno/Mioceno também foram importantes no processo inicial de diversificação dessas duas espécies e que o Plioceno-Quaternário foi crucial para dar continuidade ao processo de diversificação de anfíbios nos *Campos*. Ainda, as flutuações climáticas continuam sendo um dos principais mecanismos para promover a flutuação das distribuições populacionais ao longo do tempo nas espécies sul-americanas (de OLIVEIRA MIRANDA et al., 2018). Compreender como essas espécies responderam à dinâmica climática no passado é crucial, especialmente considerando o grupo de vertebrados mais ameaçados (WAKE, 1991; STUART et al., 2004) e o grupo mais provável de sofrer com mudanças climáticas no futuro (LOYOLA et al., 2014; VILELA et al., 2018; VASCONCELOS et al., 2018). Além disso, a perda/fragmentação de habitat e mudanças climáticas têm sido identificadas como as principais causas de declínio dos anfíbios (STUART et al., 2004; FICETOLA et al., 2015; COHEN et al., 2018). Evitar a perda de habitat é talvez o maior desafio para a conservação global da biodiversidade, e as medidas de conservação de habitat dependem frequentemente da estimativa da riqueza e de endemismo de espécies (BICKFORD et al., 2007). Aqui, nós delimitamos cinco linhagens coalescentes sob o nome de duas espécies tipicamente campestres. *A priori*, para fins de conservação, sugerimos que essas linhagens sejam consideradas como Unidades de Manejo - MUs ou Unidades Evolutivas Significativas - ESUs (MORITZ, 1994; MORITZ, 2002; DANTAS, 2013). ESUs são uma unidade de conservação abaixo do nível de espécie que é muitas vezes definida por padrões genéticos e de distribuição geográfica único (MORITZ, 1994). Em relação ao padrão de distribuição, podemos inferir que *Physalaemus gracilis* e *Scinax uruguayus*, antes amplamente distribuídas nos campos do Pampa e nos campos de Altitude, têm ESUs endêmicas no Pampa, endêmicas dos campos de Altitude e endêmicas da Região Costeira do Pampa brasileiro e que, os campos do Pampa brasileiro foram o centro de diversificação dessas linhagens. Considerando que uma abordagem taxonômica integrativa pode levar um tempo considerável até a evidente constatação de novas “espécies biológicas”, utilizar esses dados de biodiversidade é altamente recomendável para ajudar a definir prioridades de conservação, especialmente em relação aos campos do Pampa, que já perderam 47% de sua cobertura original (Projeto MapBiomas: http://mapbiomas.org/). Os dados de diversidade molecular podem ser substitutos úteis para avaliar a biodiversidade dos anfíbios antes que eles desapareçam. Mesmo que no futuro algumas linhagens identificadas como possíveis espécies não se confirmem de fato como espécies biológicas, o ganho em termos de diversidade deve ser considerado. Ressaltamos ainda que este é o primeiro estudo com Filogeografia Comparada de Anuros nos Campos Subtropicais do Sudeste da América do Sul.

CONCLUSÕES GERAIS

Nesta Tese avaliamos como os eventos históricos evolutivos podem ter moldado e influenciado a atual diversidade de anuros dos campos subtropicais do sudeste da América do Sul. Utilizamos seis espécies nominais de sapos com diferentes histórias de vida e diferentes padrões de distribuição, porém todas co-distribuídas nos campos do Pampa e nos campos de Altitude. Avaliamos os padrões de diversidade, história demográfica e se espécies fortemente estruturadas poderiam apresentar mais de uma “espécie” sobre o mesmo nome. Para responder essas questões, usamos a filogeografia estatística sob um fragmento do gene mitocondrial 16S. Esperávamos encontrar padrões flogeográficos relacionados a padrões de distribuição e especificidade de espécies (generalistas *versus* especialistas), mas verificamos que os processos de diversificação nessa região são complexos e que, de fato, espécies associadas a domínios de vegetação aberta apresentam respostas variáveis.

<u>No capítulo 1</u>, recuperamos espécies diversidade genética variando de alta a baixa, espécies com e sem estruturação genética e histórias demográficas com algumas peculiaridades, mas congruentes em sua maioria. Análises demográficas recuperaram pulsos de expansões populacionais durante picos glaciais para a maioria das espécies, i.e., parcialmente sincronizado, ambos associados à dinâmica climática do Neógeno-Quaternário. Além disso, nossos modelos de paleodistribuição detectaram os efeitos das mudanças climáticas ao longo do tempo na dinâmica de distribuição das espécies, evidenciando um aumento de áreas adequadas durante o Último Máximo Glacial (UMG) e uma considerável redução de áreas adequadas durante o último período interglacial. Essas espécies mostraram uma forte relação com os períodos mais frios, indicando persistência de habitat e se comportando como espécies típicas de montanha e tolerantes ao frio.

<u>No capítulo 2,</u> mostramos que o padrão de estruturação filogeográfica apresentado entre linhagens de duas espécies tipicamente campestres apontam para mais de uma “espécie” sob o nome de *Physalaemus graciis* e *Scinax uruguayus*. Além disso, os eventos do Mioceno, provavelmente as transgressões marinhas, foram importantes no processo inicial de diversificação dessas linhagens, que continuaram a se diversificar ao longo do Plioceno-Pleistoceno e tiveram um

maior número de eventos de dispersão durante o Quaternário, tendo os campos de Pampas como centros de dispersão e diversificação de linhagens.

Assim, é possível considerar que a anurofauna dos campos subtropicais do sudeste da América do Sul (*Campos*) possui uma história evolutiva complexa, em que a interação de eventos paleogeográficos e paleoclimáticos influenciaram a distribuição e história demográfica das espécies, moldando a atual distribuição e diversidade genética. Além disso, sugerimos que novas abordagens utilizando múltiplos marcadores com diferentes taxas evolutivas e uma taxonomia integrativa sejam consideradas, pois fornecerão um quadro mais completo da evolução e diversificação da biota sul-americana.

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