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MATÍAS MAXIMILIANO MALLERET

DIVERSIDADE, FILOGEOGRAFIA E DELIMITAÇÃO DE ESPÉCIES DE ANFÍBIOS ANUROS ATRAVÉS DE BIOMAS DO SUL DE AMÉRICA DO SUL

Porto Alegre, RS, Brasil
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Tese apresentada ao Programa de Pós-Graduação em Biologia Animal, Instituto de Biociências da Universidade Federal do Rio Grande do Sul, como requisito parcial à obtenção do título de Doutor em Biologia Animal.

Área de concentração: Biodiversidade

Orientador(a): Profa. Dra. Laura Verrastro
Co-orientador(a): Prof. Dr. Arley Camargo

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SUMÁRIO

RESUMO

Na presente tese, realizei análises moleculares e de filogeografia em anuros neotropicais, que possuem ampla distribuição ao longo de biomas de América do Sul. No primeiro capitulo, uso abordagens de delimitação coalescente de múltiplas espécies e filogeografia para explorar a diversidade, padrões de diversificação e os limites de espécies dentro do complexo *Scinax granulatus*. Esta espécie presenta uma distribuição ampla no bioma Pampa da Argentina, Brasil e Uruguai, ocorrendo também no sudeste da floresta atlântica brasileira. Neste estudo, é demostrado que *S. granulatus* atualmente constitui um complexo de espécies com ao menos duas espécies. Utilizo dados multilocus e implemento diferentes métodos para a descoberta e validação da especie candidata, para confirmar o status do nível de especies da linhagen divergente. Além disso, uso modelos de distribuição de espécies para inferir as áreas climáticamente estaveis para cada linhagem. Adicionalmente, implemento um método de difusão filogeográfica para inferir os centros de origem e rotas de colonização de cada especie do complexo *S. granulatus.* Este é o primeiro estudo que utiliza dados genéticos para explorar a diversidade, estrutura genética e divergência de linhagens dentro do complexo *S. granulatus*

No segundo capítulo, uso dados de sequências mitocondriales e de RADseq *loci* de genoma amplo para investigar que processos históricos seriam os principais inpulsionadores da história evolutiva e diversificação de *Leptodactylus latinasus*. Esta rã é amplamente distribuida atraves das planícies Chaquenha e Pampeana da Argentina, Brasil, Uruguai, Paraguai e Bolivia. Neste capítulo, utilizo análises de asignação de indivíduos às populações, modelos de distribuição de espécies, demografia histórica e modelos de difusão. Se identificou cinco linhagens mitocondriais e dois conglomerados nucleares, observando inconguencias entre ambos conjuntos de dados, detectando sinais de intercâmbio de genes entre os clusters genômicos. Os dados mitocondriais indicam que população ancestral de *L. latinasus* diversificou faz aproximadamente 1.93 milhões de anos, e os subsequentes eventos cladogenêticos que originaram cada linhagem aconteceram no Pleistoceno médio. A reconstrução da difusão filogeográfica coincide com as previsões dos paleomodelos, recuperando áreas climaticamente estáveis para o último máximo glacial e interglacial próximas às regiões ancestrais inferidas para cada linhagem. Este estudo, ressalta o papel das mudanças climáticas e ambientais sobre a diversidade genética, padrão filogeográfico e diversificação de linhagens. Também sugere que os rios Paraná e Uruguai poderiam ter funcionado como barreiras ou corredores biogeográficos,

evitando ou permitindo o intercâmbio de genes entre linhagens de *L. latinasus* em diferentes periodos. Este é o primeiro estudo que investiga a diversidade genética, demografia histórica, biogeografia e história evolutiva de populações de *L. latinasus*. Esta tese destaca a importância da realização de estudos multidisciplinares para compreender os padrões da diversidade biológica e de investigar quais eventos históricos modelaram e definiram a história evolutiva da fauna neotropical.

Palavras-chave: Filogeografia, Anura, Chaco, Pampa, Mata Atlântica, Neogeno-Quaternário, Mudanças climáticas, Delimitação de Espécies, Taxonomia Integrativa

ABSTRACT

In this thesis, I applied molecular and phylogeographic analyses in Neotropical frogs widely distributed across biomes of southern South America. In the first chapter, I use multispecies coalescent delimitation and integrative taxonomy approaches for exploring the lineage diversity and species boundaries within the *Scinax granulatus* complex. This species has a widespread distribution range in the Pampa biome of Argentina, Brazil, and Uruguay, also occurring in the southernmost portion of the Brazilian Atlantic Forest. Herein, I show that *S. granulatus* is a species complex composed of a minimum of two separate species. I used multilocus data and applied several methods of species discovery and validation of candidate species, to corroborate the specific status of the candidate species. I also used species distribution models for predicting climatic suitability areas for each lineage. Moreover, I implemented the phylogeographic diffusion approach to infer the origin centers and colonization vies of each species of the *S. granulatus* complex. This is the first study that uses genetic data to access the diversity, genetic structure, and lineages divergence inside the *S. granulatus* complex.

In the second chapter, I use mitochondrial sequences and genome-wide RADseq loci data to investigate what historical processes could have been the main drivers of the evolutionary history and diversification of *Leptodactylus latinasus*. This frog is widely distributed across the Chaco and Pampa plains across Argentina, Brazil, Uruguay, Paraguay, and Bolivia. In this chapter, I used population assignment analysis, species distribution models, historical demography, ancestral area reconstruction, and diffusion models to assess the evolutionary history of *L. latinasus*. Five mitochondrial lineages and two nuclear clusters were identified, observing inconsistencies between both sets of data, detecting signs of exchange of genes between the genomic clusters. Mitochondrial data indicate that the ancestral population of *L. latinasus* diversified about 1.93 million years ago, and the subsequent cladogenetic events that gave rise to each lineage took place in the Middle Pleistocene. The diffusion reconstruction coincides with the predictions of the paleomodels, recovering climatically stable areas for the last glacial and interglacial maximum close to the ancestral regions inferred for each lineage. This study highlights the role that climatic and paleoenvironmental changes have had on genetic diversity, phylogeographic patterns, and lineages diversification. It also suggests that the Paraná and Uruguay Rivers could have operated as biogeographic barriers or corridors, avoiding or allowing the gene flow among the lineages of *L. latinasus* at different times. This is the first

study that investigates the genetic diversity, historical demography, biogeography e evolutionary history of *L. latinasus* populations. This thesis highlights the importance to carry out multidisciplinary studies to comprehend the biological diversity patterns, investigating what historical events modeled and defined the evolutionary history of the Neotropical fauna.

Keywords: Phylogeography, Anura, Chaco, Pampa, Atlantic Forest, Neogene-Quaternary, Climatic Changes, Species Delimitation, Integrative Taxonomy

INTRODUÇÃO GERAL

A América do Sul (AS) se estende entre as latitudes 12°N e 56°S, sendo o quarto maior continente em termos de superfície, cobrindo aproximadamente o 12% da superfície terrestre atual da Terra (Orme, 2007). Esta grande variação latitudinal, a Cordilheira dos Andes, e os oceanos Pacífico e Atlântico que o circundam, fazem da América do Sul um continente mega diverso com uma grande variedade de climas que caracterizam as diversas formações fitogeográficas que definem os diferentes biomas e ecorregiões (Olson et al., 2001; Orme, 2007) (Fig. 1). Os padrões biogeográficos e diversificação da flora e fauna da AS, seria uma consequência de processos geomorfológicos do Neógeno, seguido por subsequentes reorganizações paleogeográficas e ambientais, e mantida pela ação das mudanças climáticas do Pleistoceno (Rull, 2011; Meseguer et al., 2021). Os principais eventos que teriam modificado a paisagem de diferentes regiões da AS, impulsando a diversificação e/ou moldando a diversidade da biota seriam: a) as sucessivas fases de elevação da Cordilheira dos Andes, b) os eventos de incursão marinha e c) os ciclos glaciais do Pleistoceno. Além disso, a menor continentalidade da América do Sul, em particular do Cone Sul, sugere que esta região em comparação com outros continentes, teria sido afetada de maneira diferencial durante os ciclos glaciais do Quaternário (Fraser et al., 2012).

O sistema orogênico andino foi iniciado no final do período mesozoico e atingiu o pico com uma elevação massiva nos últimos 30 milhões (Mi) de anos (Orme, 2007). A Cordilheira dos Andes gerou uma barreira para os ventos alísios provenientes do Oceano Pacífico, e a sombra de chuva oriental resultante deste bloqueio, gerou os ambientes semiáridos e áridos que constituem a Diagonal Árida de América do Sul. O Neógeno tardio se caracterizou por ser um período no qual prevaleceram os eventos geomorfológicos de elevação dos Andes e houve um aumento na frequência das mudanças no nível do mar global. Por volta do Mioceno Médio e Tardio as planícies do sul da América do Sul, nos planos Chaquenhos e Pampeanos (Fig. 1) foram cobertas pelo “Mar Paranaense” (Donato, 2006; Hernández et al., 2005), esta incursão marinha foi temporalmente concordante com a fase Quéchua de diastrofismo andino (~14-10 Mi; Ortiz-Jaureguizar & Cladera, 2006; Ramos, 1988). O Mar Paranaense teria tido um papel proeminente na fragmentação de linhagens e diversificação em diferentes grupos de vertebrados (p. ex., Brusquetti et al., 2018; Morando et al., 2014). O final deste período, coincide com o

desenvolvimento dos habitats de áreas abertas do sul da AS, período chamado de “Idade das Planícies do Sul”, no qual o clima foi predominantemente frio com acentuada sazonalidade e variada subdivisão ambiental (Ortiz-Jaureguizar & Cladera, 2006). Posteriormente, durante o Plio-Pleistoceno, na fase Diaguita (~3-1.7 Mi) do diastrofismo dos Andes, ocorreu a elevação dos Andes centrais, elevação das *Sierras Pampeanas* e região da Mesopotâmia da Argentina, o que ocasionou mais mudanças paleogeográficas e ambientais na região sul da América do Sul (Chernicoff et al., 2002; Ortiz-Jaureguizar & Cladera, 2006).

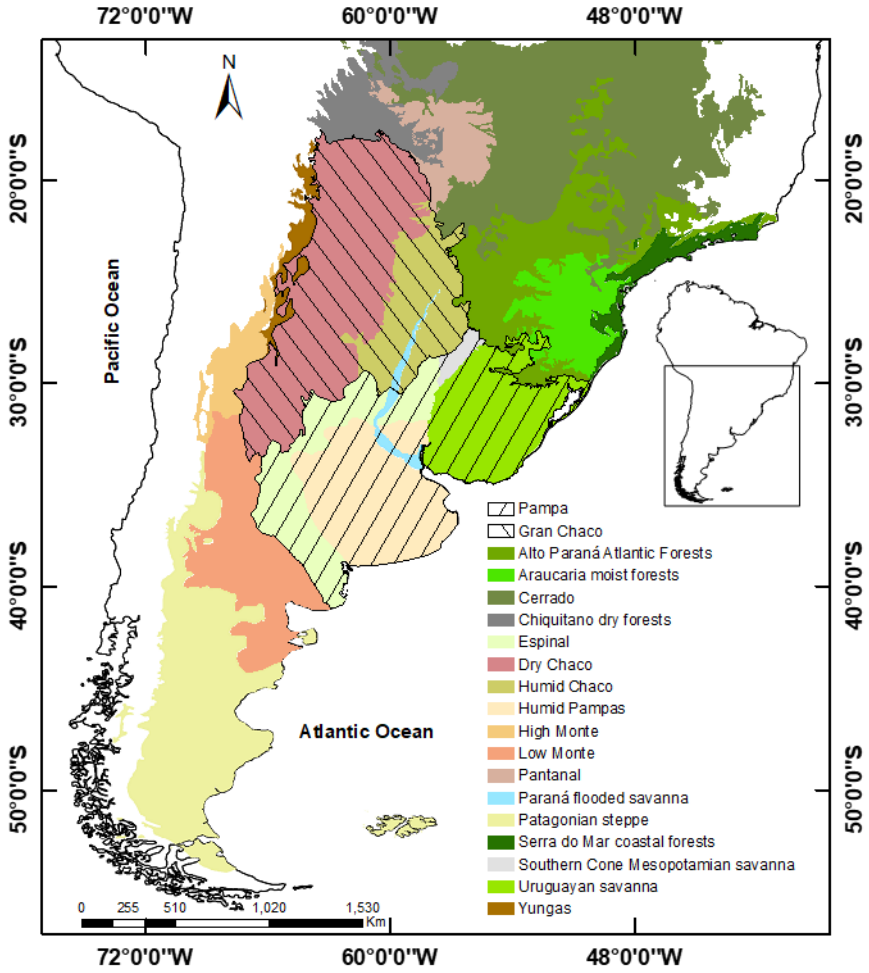

Figura 1. Ecoregiões do sul de América do Sul, delimitando os límites do Gran Chaco e do Pampa e as formações fitogeográficas que circumdam ambos biomas

Por último, os ciclos glaciais do Pleistoceno influíram na dinâmica de diferentes formações fitogeográficas, impactando na biogeografia de diferentes regiões da América do Sul. Os Andes da Patagônia foram cobertos por um manto de gelo de montanha contínuo, que se estendeu das

latitudes 37 °S até 56 °S e que se formou em, pelo menos, cinco grandes glaciações ao longo do último milhão de anos (Rabassa et al., 2005; 2011). Dentre estes eventos glaciais, os principais foram a *Greatest Patagonian Glaciation* (GPG, ~1.2-1 Ma) e o *Last Glacial Maximum* (LGM, ~23-18 Ma). O avanço e retrocesso de glaciais, além de afetar os habitats patagônicos, teriam influenciado de forma indireta na paleogeografia e na dinâmica da paisagem de outras ecorregiões e biomas, tais como o Monte, Chaco e Pampa. Em outras regiões fora da Patagônia, os mudanças climáticas associadas às glaciações teriam ocasionado a expansão de áreas abertas e retração de florestas durante os ciclos glaciais enquanto durante os períodos mais quente (interglaciais), as florestas teriam estendido sua distribuição para o sul (Behling, 2002; Clapperton, 1993; Spichiger et al., 2004). Estas mudanças na paisagem de áreas abertas (p. ex., Monte e Pampa) ou formações de floresta sazonalmente seca (p. ex., Gran Chaco) teriam influenciado na divergência de linhagens e na dinâmica populacional de plantas (Bartoleti et al., 2017; Cosacov et al., 2010; Moreno et al., 2018; Turchetto et al., 2014) e animais (Brusquetti et al., 2019; Langone et al., 2016; Rocha et al., 2020), sendo os processos responsáveis na determinação dos limites de distribuição e definido os padrões biogeográficos destas espécies.

Filogeografia

Os indivíduos geralmente agrupam-se em populações geográficas —isso é, organismos de uma mesma espécie que habitam em uma região geográfica particular e que por sua proximidade tendem a ter maior probabilidade de se intercruzarem entre eles que com indivíduos de populações mais distantes. Tais agrupamentos geográficos forman-se como resultado de processos intrínsecos e/ou extrínsecos que possibilitam a reprodução e/ou intercambio de genes, dando forma à diversidade e composição de linhagens genéticas. Assim, os estudos dos princípios e processos que determinam a distribuição geográfica de linhagens genealógicas, intra e interespecíficas, é chamado de Filogeografía (Avise et al., 1987; Avise, 2000). Os estudos filogeográficos exercem um papel fundamental na compreensão de quais processos históricos modificaram a história demográfica das populações em ampla escala (de espaço e tempo), deixando sinais no material genético da biota de diferentes regiões. Tais estudos podem ajudar a entender os modos de dispersão, tempo de diversificação, extinções, áreas de refúgios, expansão espacial, demográfica e processos microevolutivos, tais como deriva genética, seleção e fluxo gênico (Manel et al., 2003; Turchetto-Zolet et al., 2013). Em estudos filogeográficos

pioneiros, os pesquisadores usaram fragmentos de DNA mitocondrial (mDNA) resultantes da digestão com enzimas de restrição (isto é, RFLP, *Restriction fragment length polymorphism*) para detectar polimorfismos dentro de populações (ver Avise, 2000). No entanto, o crescimento da filogeografia não teria sido possível sem os grandes avanços na tecnologia de sequenciamento de DNA, que aconteceu durante a décadas de 1970 e 1980 (Carstens et al, 2012) e fez com que os marcadores citoplasmáticos, o mtDNA em animais e plastidial DNA (cpDNA) em plantas começaram a ser os mais utilizados nesse tipo de estudos (Beheregaray, 2008).

Os dados de sequencias de DNA (componente temporal) em conjunto com a informação de pontos georreferenciados (componente espacial), possibilitaram uma abordagem espacialmente explícita que permite conhecer a composição genética e relações filogenéticas entre linhagens geográficas intimamente relacionadas. No entanto, devido aos marcadores mitocondriais constituírem uma pequena fração do genoma de um organismo e apenas representarem linhagens maternas, as genealogias baseadas somente neste marcador podem não representar a verdadeira história da população ou de linhagens a nível de espécies. Por isso, a utilização de múltiplos *loci*, empregando sequências mitocondriais e nucleares, são fundamentais para compreender a história de populações e espécies. Por outro lado, na última década metodologias de sequenciamento massivo do nível genômico têm revolucionado diferentes áreas da biologia evolutiva (p. ex. filogeografia, sistemática filogenética, genética de populações, etc.), devido a permitirem obter dados de milhares de *loci* a partir de centenas de indivíduos, diminuíndo o custo por base e aumentando a quantidade de dados que podem ser sequenciados (Carstens et al., 2012). Porém, estas metodológias de sequenciamento, oferecem novos desafios relacionados à preparação das amostras (geração de bibliotecas), necessidade de *clusters* para a análises e armazenar grandes volumes de dados e desafios intrínsecos ao uso de bioinformática (McCormack et al., 2013).

Delimitação de espécies

O uso de dados de múltiplos *loci* e o acesso a dados de genoma amplo foi favorecido pelos métodos disponíveis e o advento de novas abordagens que permitem detectar divergência e outros processos demográficos á nível de populações e espécies. Dentre estes enfoques, o modelo coalescente de mútilplas espécies que aplica a teoria coalescente (Kingman, 2000) sobre múltiplas espécies, permitindo modelar a distribuição das árvores genicas contidas na

história de una espécie, e permite estimar os processos coalescentes conectados por uma árvore de espécies (Degnan & Rosenberg, 2009; Rannala & Yang, 2003). O modelo coalescente leva em consideração a separação incompleta de linhagens (ILS), tentando superar as incertezas das genealogias genicas, o qual muitas vezes pode confundir as relações genealógicas, produzir discordância mito-nuclear e obscurecer os limites das espécies (p. ex., Funk & Omland, 2003; Liu et al., 2009). Métodos de descoberta e delimitação de espécies têm como objetivo identificar linhagens divergentes a nível de espécies, e baseados na coalescencia, usam modelos probabilísticos que não requerem de monofilia recíproca ou caracteres diagnósticos (ou seja, fixados) para o teste de hipótese. Estes métodos utilizam os dados de múltiplos *loci* considerando as incertezas e discordâncias entre hipóteses de árvores de genes resultantes da estocasticidade do modelo coalescente (Fujita et al., 2012). Dentre os métodos baseados no coalescente de múltiplas espécies, o mais amplamente usado com dados de sequências é o *Bayesian Phylogenetics and Phylogeography* (BPP, Yang & Rannala, 2010), contudo, para um elevado número de *loci* como com dados de genoma amplo, os metodos coalescentes tornam-se intratáveis e muito lentos (Pei et al., 2018). Por isso, pelo incremento nas matrizes de dados, recentemente, foram desenvolvidas novas metodologias que utilizam a abordagem de *Machine learning* (p. ex., Derkarabetian et al., 2019; Pei et al., 2018; Smith & Carstens, 2020). Estes métodos podem ser supervisionados onde o usuário deve configurar o modelo proporcionando informação *a priori* —por exemplo, parâmetros populacionais; ou não supervisionados nos quais o modelo é conduzido sem hipóteses *a priori* (Derkarabetian et al., 2019).

Por outro lado, os limites das espécies geralmente são corroborados com base em múltiplas linhas de evidência, esta etapa de validação permitira assumir futuras hipóteses filogenéticas (Carstens et al., 2013; Fujita et al., 2012; Jackson et al., 2017). Além disto, as abordagens multidisciplinarias com diferentes conjuntos de caracteres podem auxiliar na compreensão de que mecanismos evolutivos poderiam ter participado no processo de especiação. A divergência evolutiva nem sempre vem acompanhada de mudanças fenotípicas, por exemplo, a diversificação de linhagens pode ocorrer com pouca mudança morfológica (Adams et al., 2009; Fišer et al., 2018; Struck et al., 2018), por isso a delimitação e classificação das espécies deve ser feita por uma abordagem integrativa (Bickford et al., 2007; Dayrat, 2005; Padial et al., 2010). Neste sentido, a identificação e a descrição de espécies não devem ser baseadas apenas em características morfológicas como realizada na taxonomia tradicional, devido que a composição das linhagens pode ser subestimada (Bickford et al., 2007; Fišer et al., 2018; Struck et al., 2018).

Diversidade críptica, erros de identificação e conservadorismo morfológico são frequentes entre espécies de anfíbios (Colombo et al., 2008; Fouquet et al., 2007; Pombal et al., 1995a; Stuart et al., 2006), levando a hipóteses taxonômicas incorretas as quais somente podem ser resolvidas utilizando múltiplos conjuntos de dados —por exemplo, de sequências, fenotípicos, comportamentais, ecológicos (Brusquetti et al., 2014; Vacher et al., 2017). Por isso, uma abordagem multidisciplinar com múltiplas linhas de evidência são recomendadas para compreender a real diversidade biológica, identificando com robustez o verdadeiro número de espécies (Carstens et al., 2013; Edwards & Knowles, 2014).

Os anfibios são um ótimo modelo animal para estudos filogeográficos, tendo em consideração sua distribuição global, o elevado grau de estruturação genética das populações, a facilidade de sua amostragem e baixa vagilidade (Zeisset and Beebee, 2008). Além disso, por seu ciclo de vida bifásico, sua fisiologia ectotérmica, e requerimentos de umidade, fazem deles organismos excelentes para estudos de filogeografia e ecologia, devido ao fato de sua sobrevivência e distribuição estarem intimamente associadas a seu entorno e às mudanças dele. Nesta tese utilizamos duas espécies de anfibios (*Scinax granulatus* e *Leptodactylus latinasus*) como modelos para estudos filogeográficos e a delimitação de táxons distribuidos em três biomas do sul da América do Sul: o extremo sul da Mata Atlântica, o Pampa e o Chaco.

Scinax granulatus (Peters, 1861), é uma perereca que tem hábito arborícola, e distribui-se ao longo do nordeste de Argentina, no sul de Buenos Aires, Uruguai, nos estados de Rio Grande do Sul, Santa Catarina e Paraná in Brasil e tem ocorrência potencial no sul do Paraguai (Frost, 2021) (Fig. 2a). Durante a estação reprodutiva encontram-se nas margens de corpos de água temporários, reproduzindo-se sobre gramíneas rasteiras (Iop et al., 2016; Moresco et al., 2009). Os machos vocalizam nas árvores, e os ovos são depositados sobre plantas aquáticas. *S. granulatus* tem uma história taxonômica confusa. Este táxon foi originalmente designado como *Hyla granulata* (Peters, 1871) com base na análise de uma fêmea (ZMB 7253) de Porto Alegre, Rio Grande do Sul, Brasil. Depois, Gallardo (1961) denominou uma variação coespecífica de Bella Vista, Buenos Aires - Argentina, como *Hyla strigilata eringiophila*. Posteriormente, Lutz (1973), chamou ao mesmo táxon como *Hyla eringiophila* e *Hyla x-signata eringiophila*. Uma década depois, o nome do táxon foi alterado para *Ololygon x-signata eringiophila* por Gudynas (1983), prontamente, o mesmo autor o chamou *Ololygon eringiophila* (Gudynas, 1987) e o nome *Hyla vautierii* foi proposto por Klappenbach & Langone (1992). Finalmente, Kwet

(2001b) sinonimizou todas as combinações nomenclaturais acima mencionadas como *Scinax granulatus* (rev. Frost, 2021). Kwet (2001b), reanalisou o material tipo (ZMB 7253) de *Hyla granulata* depositado em Museu für Naturkunde da Universitat Humboldt, em Berlim, Alemanha, e o comparou com espécimes de Buenos Aires, Argentina, e com a amostragem de novas populações do Rio Grande do Sul, Brasil. Kwet (2001b) também analisou a morfologia externa e características do canto do anúncio de *S. granulatus*, comparando com dados de espécies simpatricas (*S. fuscovarius* e *S. perereca*), e sugeriu que existia bastante sobreposição entre os caracteres destas três espécies.

Mais recentemente, Fonte (2010), analisou populações de *Scinax granulatus* para entender se a variação observada em caracteres morfológicos e do canto de anúncio seria intraespecífica ou interespecífica, podendo este táxon constituir um complexo de espécies. O autor analisou medidas morfológicas de 410 indivíduos, e de 20 indivíduos para comparação de parâmetros bioacústicos de *S. granulatus.* Além disso, usou este conjunto de dados para realizar comparações interespecíficas com *S. fuscovarius, S. perereca* e *S. nasicus,* espécies do clado *ruber* que ocorrem em simpatria e/ou simtopia com populações de *S. granulatus* e que comunmente são confundidas com este táxon. Finalmente, ele conclui que, apesar da alta variação morfológica e diferenças nas características bioacústicas, não havia suporte estatístico suficiente para confirmar a hipótese de que *S. granulatus* constitui-a um complexo de espécies. Adicionalmente, ele observou bastante sobreposição no morfo-espaço ao comparar a morfologia externa de *S. granulatus, S. fuscovarius, S. perereca* e *S. nasicus,* postulando que os caracteres analisados quando considerados em conjunto, e não exclusivamente, poderiam ser considerados diagnósticos (Fonte, 2010). Alguns autores já tinham sugerido sobre a necessidade de novas revisões taxonômicas devido à semelhança morfológica observada entre estas espécies, e enfatizaram a possibilidade de existência de diversidade oculta dentro destes taxons nominais (Borges-Martins et al., 2007).

Leptodactylus Fitzinger 1826, é um gênero composto por 83 espécies (Frost, 2021). As rãs deste gênero são caracterizadas pela deposição de ovos em ninhos de espuma autoproduzida, mostrando uma tendência evidente para um modo de vida terrestre (Heyer, 1969). O gênero é classificado em quatro clados principais: *Leptodactylus fuscus*, *L. latrans*, *L. melanonotus*, and *L. pentadactylus* (de Sá et al., 2014). Dentre estes o grupo *fuscus* é o mais diverso,

compreendendo 32 espécies (Carvalho et al., 2021; Da Silva et al., 2020). O grupo foi corroborado como monofilético baseado em dados morfológicos (Ponssa, 2008) e em evidencia total (de Sá et al., 2014). *Leptodactylus latinasus* Jiménez de la Espada 1875, é uma rã terrestre de pequeno porte (aproximadamente de 40mm Weiler et al., 2013), que é classificada como integrante do grupo *fuscus* (Ponssa, 2008; Ponssa & Barrionuevo, 2008). As fêmeas e os machos constroem ninhos de espuma subterrâneos onde ocorre a desova e o desenvolvimento de girinos (Ponssa & Barrionuevo, 2012; Weiler et al., 2013). *Leptodactylus latinasus* tem uma ampla distribuição compreendendo o Gran Chaco da Argentina, Bolivia e Paraguai, o bioma Pampa na Argentina, Uruguai e sudeste de Brasil (Frost, 2021) (Fig. 2b).

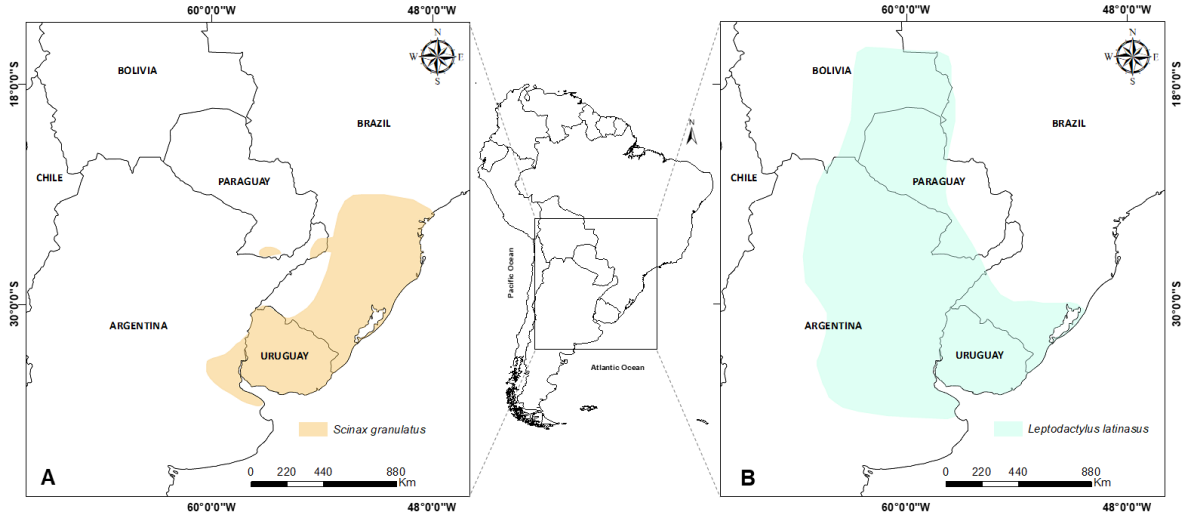

Figura 2. Distribuição geográfica de anfíbios anuros análisados neste trabalho. A) *Scinax granulatus,* B) *Leptodactylus latinasus.* Adaptado da IUCN Red List of Threatened Species.

Leptodactylus latinasus também teve uma história taxonômica complicada. Após de sua descrição por Jiménez de la Espada (1875), o nome *Leptodactylus latinasus* foi ignorado, e Boulenger (1888) propos o nome *Leptodactylus prognathus* para referir-se a este táxon. Gallardo (1964) considerou que na realidade existiam duas espécies sob o mesmo nome, *Leptodactylus prognathus* e *Leptodactylus anceps*. Poteriormente, Barrio, (1965) sinonimizou *L. anceps* com *L. prognathus*, logo Heyer (1969b) indicou que *L. latinasus* é sinonimo senior de *L. prognathus,* e Heyer (1978) confirmou que todas as combinações anteriores eram sinonimas de *Leptodactylus latinasus*. Não entanto, a confusão continuou dado que varios autores (entre eles Cei, 1980; Gallardo, 1987; Gallardo & Varela de Olmedo, 1992; Lavilla,

1994 «1992») sugeriram que *L. latinasus* era constituida por duas subespecies: *Leptodactylus latinasus latinasus* e *L. latinasus anceps*. Por fim, Ponssa & Lavilla (1998) análisaram caracteristicas morfológicas do esqueleto e demostraram que *Leptodactylus latinasus* na verdade consiste em uma única espécie (revisado em Ponssa et al., 2019; Frost, 2021).

OBJETIVOS

OBJETIVO GERAL

Investigar o padrão de diversidade genética de espécies de anuros com limites de distribuição que abrangem ambientes de áreas abertas e florestas através de ecorregiões do Chaco, Pampa e Mata Atlântica no sul de América do Sul.

OBJETIVOS ESPECÍFICOS

Capítulo 1

Acessar a diversidade genética e padrão de estruturação genética de populações de *Scinax granulatus*. Investigar se *S. granulatus* constitui um complexo de espécies, identificando linhagens divergentes, usando métodos de delimitação de espécies e múltiplas linhas de evidência para testar hipóteses de potenciais espécies candidatas. Também, investigar quais foram as prováveis areas de origem e potenciais rotas de dispersão de cada especie do complexo *S. granulatus*. Por fim, investigar as relações interespecíficas entre *S. granulatus* com espécies afins, tais como *S. fuscovarius, S. perereca, S. nasicus, S. ruber* and *S. x-signatus*, levando em conta suas relações no contexto filogenético, comparando com hipotesis topológicas disponiveis na literatura

Capítulo 2

Acessar a diversidade e estrutura genética de populações de *Leptodactylus latinasus.* Analisar a história demográfica e inferir possíveis rotas de dispersão, reconstruir potenciais áreas

ancestrais e prever os limites de distribuição de populações ancestrais, explorando como as mudanças climáticas passadas afetaram a diversidade e divergência de linhagens.

CAPÍTULO I

**Submetido na Zoologica Scripta*

Phylogeography and species delimitation of the Neotropical frog complex (Hylidae: *Scinax granulatus*)

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Phylogeography and species delimitation of the Neotropical frog complex (Hylidae: *Scinax granulatus*)

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Keywords:	Amphibians, Species boundaries, Atlantic Forest, Pampa, Species tree, Species distribution models

Phylogeography and species delimitation of the Neotropical frog complex (Hylidae: *Scinax granulatus*)

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Abstract

Understanding which factors driving the genetic structure, geographic distribution patterns, and divergence of populations/species is of great interest in ecology and evolutionary biology. Phylogeographic and species hypotheses combined with distribution models may elucidate which phenomena drove the evolutionary history of the biota of different biomes and ecoregions. Herein, we used distribution, environmental and genetic data to shed light on the evolutionary diversity of the *Scinax granulatus* complex, exploring the phylogeographic patterns, genetic structure, and species boundaries across the Pampa and Southern Atlantic Forest biomes. We recovered four mitochondrial lineages which correspond to two putative species within the *S. granulatus* complex. We used genetic data to define species hypothesis with three discovery methods: bGMYC, bPTP, and ASAP, and two delimitation approaches BPP and BPP + gdi. We validated the species boundaries, confirming the differentiation of *Scinax* sp. lineage D from Atlantic Forest lowlands habitats, based on genetic data, ecological divergence and monophyly. Lineage D exhibited niche differentiation from *S. granulatus* sensu stricto (s.s.), based on distribution and environmental data. The climatic suitability of *S.* sp. D is defined by thermal variables, while that of *S. granulatus* s.s. was associated to rainfall. The diversification of the *S. granulatus* complex began during the Pliocene, but the colonization and divergence of lineages took place more recently during the Pleistocene. This study strongly suggests species-level evolutionary divergence among lineages of *S. granulatus* complex, and highlights the need to carry out a comprehensive evaluation of diagnostic characters to confirm the lineage D as a distinct species.

1 | INTRODUCTION

The species category is considered a fundamental unit of analysis in biogeography, phylogeography, conservation biology, and ecology (Werneck et al., 2015; Gehara et al., 2017; Ward et al., 2020; Rebelato et al., 2020). For understanding the real diversity is pivotal to discern between conspecific lineages and species-level differentiation, because speciation involves a continuous process ranging from intraspecific variation and population subdivision (de Queiroz, 2007). In this sense, the species delimitation methods have allowed determining the boundaries and species hypothesis from different types of data and shedding light on the underlying processes of species formation (Sites & Marshall, 2003; Fujita et al., 2012). Phylogeographic analysis help us interpreting how intraspecific genetic variation originates, becomes subsequently structured, and persist through time, while statistical and model-based species delimitation approaches are then used to determine which phylogeographic lineages merit species status (Huang, 2020). In addition, phylogeographic approaches within a spatially and temporally explicit framework are a powerful way to test hypotheses regarding the causal relationship among geographic phenomena, species distributions, and the mechanisms driving speciation (Avise, 2000; Hickerson et al., 2010). Alternately, species distribution models (SDM) can also be implemented to solve taxonomic issues within an integrative species delimitation approach (e.g., Huang & Knowles, 2016), as well as to generate phylogeographic hypotheses by estimating the potential distributions of independently lineages and elucidating which climatic and/or geographic factors are associated with distinct, bioclimatic niche envelopes (Alvarado-Serrano & Knowles, 2014).

South America harbours the greatest biodiversity on Earth, containing five ecoregions considered global biodiversity ‘hot spots’ (Myers et al. 2000). The mechanisms behind the origin and maintenance of diversity on this continent cannot be restricted to a particular time interval or mechanism (Rull, 2011a; Turchetto-Zolet et al., 2013; Meseguer et al., 2021). In the southern cone of South America, over the past several million years, historic processes such as glaciations (and associated climate change), orogenic, paleo-basins, and inland and/or seashore shifts by marine transgressions have dramatically altered the landscape, creating complex scenarios for species diversification (Ortiz-Jaureguizar & Cladera, 2006; Sersic et al., 2011). The mid-latitude South American geographic region (latitudinal range between 24° N and 36° S), currently comprises a mosaic of Neotropical ecosystems, including formations of the

Atlantic Forest and Pampa biomes. This region is therefore suitable for evaluating the impact of paleogeographic and climate changes on biodiversity (Silva et al., 2018), since there are likely different responses for species associated with forest and open vegetation areas (Turchetto-Zolet et al., 2013).

Amphibians possess several attributes including their nearly global distribution, usually strong genetic structure, and ease of sampling, that make them an excellent model for phylogeographic studies (Zeisset & Beebee, 2008). In addition, their tendency to exhibit conservative morphological evolution (stasis) generates a repository of hidden diversity, and an ideal model for species delimitation studies (Stuart et al., 2006). Moreover, limited dispersal ability as well as ecological and physiological constraints, exposes amphibians to be heavily impacted by climate change and habitat degradation/fragmentation (Blaustein et al., 2010; Brum et al., 2013), consequently, these constraints make that the amphibians generally displaying deep phylogeographic structure (Rodríguez et al., 2015).

The Neotropical treefrog genus *Scinax* Wagler 1830 it is the most specious genus within the family Hylidae and currently houses 128 recognized species (Frost, 2022), comprising two major clades: the *S. catharinae* and *S. ruber* clades (Faivovich, 2002; Faivovich et al., 2005; Frost, 2022). The snouted treefrog *Scinax granulatus* (Peters, 1871) is widely distributed in the Pampa biome and on restricted areas of the southernmost portion of the Atlantic Forest. According to Frost (2022), *S. granulatus* ranges from northeastern Argentina to the south of Buenos Aires Province, the States of Paraná, Santa Catarina, and Rio Grande do Sul in Brazil, throughout most of Uruguay; and it is also expected to occur in southern Paraguay. It is a species that occupies open areas and forest remnants in the Pampa biome, or the edges of forests and adjacent open areas in the Atlantic Forest, but it is also frequently found in anthropized environments (Achaval & Olmos, 2007; Conte et al., 2010). *Scinax granulatus* as a representative of the *ruber* clade has been traditionally related and confused with other species of the group, such as *S. fuscovarius, S. perereca, S. nasicus* and *S. x-signatus* (Langone & Cardoso, 1997; Kwet, 2001b). The high phenotypic similarity of *S. granulatus* with some species of *ruber* clade (i.e., *S. fuscovarius, S. perereca, and S. nasicus*), hinders taxonomic determination of specimens, especially with those that occur partially in sympatry (Kwet, 2001b; Dalmolin et al., 2017). Typically, they have been differentiated in taxonomic keys on the basis of the proportional sizes of the tympanum and the adhesive disc of the third finger (Ziegler &

Maneyro, 2008; Kwet, Lingnau & Di Bernardo†, 2010). As such, were pointed that the *Scinax* genus is a taxonomically confusing group particularly due to the morphological conservatism and similarity among species (e.g., Pombal et al., 1995; Nunes et al., 2012). Until today, the populational studies of *S. granulatus* have focused on distribution, bioacoustics or morphological data, carrying out comparative analyses with other *ruber* group species (Kwet, 2001b; Conte et al., 2010). Nevertheless, none of these previous studies observed lineage divergence suggesting independent evolutionary units within the nominal species. Additionally, there has not been any genetic analysis to address evolutionary history and demographic dynamics, and to evaluate how many actual species exist within the *S. granulatus* complex.

During recent fieldwork in the lowlands of the Atlantic Forest in northeastern Rio Grande do Sul State, we found specimens of *S. granulatus* that we judged to be a new species based on overall morphology, which was also observed in previous studies (Colombo et al., 2008; Wachlevski & Duarte Rocha, 2010). Consequently, we investigated if these peripheral populations constitute either a conspecific lineage of *S. granulatus* or if they belong to a distinct, independently evolving lineage. Herein, we assessed for the first time the genetic diversity and phylogeographic structure of *Scinax granulatus* with the hypothesis that this taxon constitutes a species complex, and validated candidate species with genetic, distribution, and bioclimatic datasets.

2 | MATERIAL AND METHODS

2.1 | *Molecular data*

We used genetic data of 85 individuals of *S. granulatus* from 48 localities sampled across its known distribution range based on fieldwork and museum/collection loan (Fig. 1A). As outgroups for phylogenetic inferences, we included five species of the *ruber* clade (*S. fuscovarius*, *S. perereca*, *S. nasicus, S. ruber*, and *S. x-signatus*), and two species of the *catharinae* clade (*S. berthae* and *S. catharinae*). Voucher numbers, localities, and geographical information are given in Table S1.

Genomic DNA was obtained from liver or muscle samples using a saline extraction protocol (MacManes, 2013). We amplified a fragment of the mitochondrial *Cytochrome b* gene (*cytb*)

(amplicon length 816 bp, length of final alignment 552 bp) in 85 ingroup individuals (Table S1). For the outgroup species of the *ruber* clade, we amplified five sequences of *cytb* (amplicon length 820 bp, length of final alignment 612 bp) from *S. perereca* (N = 1), *S. fuscovarius* (N = 2), and *S. nasicus* (N = 2) (Table S1). The partial coding sequence of the nuclear *Recombination Activation* gene (*RAG1*) (amplicon length 460 bp, length of final alignment 397 bp) was amplified in 41 individuals, including one sample of *S. perereca* (Table S1). DNA fragments were amplified by PCR with the primers of c*ytb*: MVZ15 (5'-GAA CTA ATG GCC CAC ACW WTA CGN AA-3') and MVZ16 (5'-AAA TAG GAA RTA TCA YTC TGG TTT RAT-3') (Moritz et al., 1992); *Rag1*: R1-GFF (5'-GAG AAG TCT ACA AAA AVG GCA AAG-3') and R1-GFR (5'-GAA GCG CCT GAA CAG TTT ATT AC-3') according to Faivovich et al. (2005). PCR master mix for 20 μL reactions included: 10–50 ng of template DNA, 0.2 μL of each primer at 10 μM each, 0.2 mM of each dNTP, 1X Buffer with 1.5 μM MgCl2, and 1 unit of Taq DNA polymerase (Sigma-Aldrich). The cycling profiles of amplification for both fragments were an initial denaturation step of 2 min at 95 °C, 40 cycles (45 cycles for *RAG1*) of 30 s of denaturation at 95 °C + 45 s of annealing at 45 °C (of 30 s at 52 °C for *RAG1*) + 2 min of extension at 72 °C for both fragments, and a final extension step of 7 min at 72 °C for both fragments (following the conditions for *cytb* of Canestrelli & Nascetti, 2008; and for RAG1 of Guayasamin et al., 2008 with modifications). PCR fragments were checked in 1% agarose gel, purified with ExoSap (Exonuclease I and Shrimp Alkaline Phosphatase GE Healthcare), and Sanger sequenced in Macrogen (www.macrogen.com). All new sequences were submitted to GenBank (Table S1). We utilized sequences of *Scinax catharinae, Scinax fuscovarius, S. nasicus, S. perereca, S. ruber* and *S. x-signatus* available in GenBank for *cytb* (accession number AY844001, MT503819, EF364238, and EF364246) and *RAG1* (AY844517, AY844520, MK266640, MT504110, and AY844521).

2.2 | *Genetic diversity and gene tree*

We used the Blast tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to confirm the identity of sequences. The electropherograms were reviewed and edited in Geneious v11.1.5 (http://www.geneious.com, Kearse et al., 2012) and MEGA7 (Kumar et al, 2016), and sequences were aligned by the alignment algorithm of Geneious (Needleman & Wunsch, 1970) using default settings. For solving the haplotype gametic phases of the nuclear fragment *RAG1*, we executed the seqPHASE input/output interconversion tool (Flot, 2010) and the PHASEv.2.1

software (Stephens et al., 2001). The best-fit partition schemes and substitution models were chosen following the Bayesian information criterion (BIC) using PartitionFinder v.2.1.1 (Lanfear et al., 2016). The best model was inferred separately for each locus (see Table S2).

We estimated the haplotype diversity with DnaSP 6 (Rozas et al., 2017), and built a TCS network for each locus using a statistical parsimony approach (Clement et al., 2002) implemented in PopART (Leigh & Bryant, 2015). We computed the average genetic distance within and among the mitochondrial lineages of with MEGA 7 (Kumar et al., 2016) using uncorrected p-distance. In this analysis, we also included sequences of outgroups (i.e., *S. fuscovarius*, *S. perereca*, and *S. nasicus*).

To assess phylogenetic position of *Scinax granulatus*, we used the mitochondrial dataset containing 85 individuals (ingroup) and 10 outgroups terminals. We chose as outgroups those species of the *ruber* clade that have traditionally been related to *S. granulatus* (i.e., *S. fuscovarius, S. perereca, S. nasicus*, *S. ruber* and *S. x-signatus*), we included also two representatives of the *catharinae* clade (*S. berthae* and *S. catharinae*). The mitochondrial tree was built with Beast 2.6.2 (Bouckaert et al., 2019) with the Birth Death speciation model and a Relaxed Log Normal molecular clock. Due to the lack of fossil records for tree calibration, we adopted the evolutionary rates available in the literature for *Cytb* (mean = 1.61% substitution rate per lineage per Mya, with sigma = 0.01) in a normal distribution (Stöck et al., 2012). We executed three independent analyses using different seeds, each one of them consisted of 10 million generations sampled every 2 x 10^3. The runs were combined utilizing LogCombiner, and we obtained the maximum clade credibility (MCC) tree with a 20% burn-in in the TreeAnnotator post-processing tool (Bouckaert et al., 2019).

2.3 | *Species discovery*

The assignment of individuals to putative species was achieved in two steps: discovery and validation (Carstens et al., 2013). We implemented two tree-based and one distance-based method to delimit genetic structure based on the mitochondrial-only dataset of the *Scinax granulatus* complex: (1) the Bayesian implementation of the Generalized Mixed Yule-coalescent model (bGMYC; Reid & Carstens, 2012), (2) the Bayesian implementation of the Poisson Tree Process (bPTP; Zhang et al. 2013), and (3) the Assemble Species by Automatic

Partitioning (ASAP; Puillandre et al., 2021). The bGMYC method identifies the branching events within and between species, distinguishing the transition points between coalescence and speciation, accounting for uncertainty in phylogenetic estimation (Reid & Carstens, 2012). We used the unique haplotypes of *cytb* to build ultrametric trees with Beast 2.6.2 (Bouckaert et al., 2019), implementing a Yule speciation model and a strict molecular clock. We executed two independent analyses using different seeds, each one consisting of 20 million generations sampled every 2 x 10^3. We obtained the maximum clade credibility (MCC) tree with a 20% burn-in in the TreeAnnotator post-processing tool (Bouckaert et al., 2019), which we analyzed with the *bGMYC* package (Reid & Carstens, 2012) in R 4.0.5 (R Core Team, 2021) after prunning off the outgroups to keep only terminals assigned to the *S. granulatus* complex. For the bGMYC analysis, a post-burn-in sample of 1,000 trees was used to calculate the posterior distribution of the model. First, we used the *bgmyc.singlephy* function to set the threshold priors and starting parameters. Next, we performed the bGMYC analysis for 50,000 generations, with a burn-in of 45,000 generations and a thinning interval of 50 samples. Additionally, we applied the *P2C2M.GMYC* package (Fonseca et al., 2021) to assess the statistical fit of the GMYC model to our dataset. The statistical fit was evaluated via implementing the posterior predictive simulation (PPS) strategy used for the Bayesian framework of GMYC according to Fonseca et al. (2021).

The bPTP method uses a heuristic algorithm to distinguish between speciation and coalescent events based on divergence measured as numbers of substitutions rather than absolute time (Zhang et al. 2013). For this analysis, we utilized the same sequences that the bGMYC analysis, we estimated a maximum likelihood (ML) gene tree in RAxML BlackBox (https://raxml-ng.vital-it.ch/#/), and subsequently, we ran bPTP using a web server (http://species.h-its.org/ptp/) (Zhang et al. 2013). Alternatively, ASAP implements a hierarchical clustering algorithm based on genetic gap analysis, using pairwise genetic distances to build a list of partitions ranked by a score. ASAP-scores are computed using the barcode gap widths and the probabilities of groups to be panmictic species, with the best partition being defined as the one with the smallest ASAP-score. For ASAP analysis we used 45 sequences of unique *cytb* haplotypes, and ran the analysis using a web server (https://bioinfo.mnhn.fr/abi/public/asap, Puillandre et al., 2021).

2.4 | *Species validation*

We implemented the Bayesian Phylogenetics and Phylogeography species delimitation software, BPP v4 (Flouri et al., 2018). We used Dataset B and all sequences of *S. fuscovarius, S. perereca, S. nasicus S. ruber and S. catharinae* available for this study. Dataset B comprises a subsample of individuals (i.e., 40 samples) that have information to both *cytb* and *RAG1* (Table S1). BPP uses the Markov chain Monte Carlo (MCMC) approach under the MSC model to jointly estimate both species limits and the species tree (Flouri et al., 2018). We carried out two kinds of inferences: A00 (Rannala & Yang, 2003) for estimation of parameters under the MSC, and A10 for species delimitation using a fixed guide tree. For the fixed guide tree, we considered the topology obtained with StarBEAST (Heled & Drummond, 2009) based in the high support of the estimates (see next section). We calculated observed summary statistics to set priors of the theta (θ) and tau (τ) parameters; we used an inverse gamma prior for $\theta \sim IG(\alpha, \beta)$ and $\tau \sim IG(\alpha, \beta)$. We set $\alpha=3$ and the corresponding β parameter was adjusted using the equation $m=\beta/(\alpha-1)$ (for $\alpha>1$, Flouri et al., 2018) with the median (m) estimates of nucleotide diversity (i.e., π, Nei 1987) for θ, and of node height for τ. We plot the inverse-gamma density and calculated the 95% prior interval using the *invgamma* package (https://github.com/dkahle/invgamma) in R v.4.0.0 (R Development Core Team, 2020).

We executed the A00 analysis for parameter estimation using only samples of *S. granulatus*, utilizing the tree topology obtained with StarBEAST as initial species phylogeny and the inferred priors. We performed this analysis with automatic fine-tune adjustments, using heredity scalars, and two replicates to check for convergence and proper mixing in plots of the MCMC log file. We also performed the A10 analysis to infer the best species delimitation model using the same topology as fixed guide tree (see above). We used the samples of *S. granulatus* that have sequences of both loci (Dataset B) and included samples of *S. fuscovarius, S. perereca, S. nasicus, S. ruber* and *S. catharinae*. We configured three different runs to analyze the autosomal and mitochondrial loci separately, for evaluating possible heterogeneity in species delimitation (Flouri et al., 2018), and a run with both loci combined. For the three analyses we set an inverse gamma prior for $\theta \sim IG(\alpha, \beta)$ and to $\tau \sim IG(\alpha, \beta)$ parameters. We explored the sensitivity and convergence of each analysis using default priors θ ($\alpha=3$, $\beta=0.02$) and τ ($\alpha=3$, $\beta=0.004$) (Flouri et al., 2018), and empirical priors based our data. The runs were executed for 5×10^5 generations with 1×10^4 burn-in generations.

Additionally, we ran the A00 analysis using empirical priors to generate the posterior distribution for the parameters τ and θ, and to estimate of genealogical divergence index (*gdi*). We utilized this empirical criterion with a lineage/species tree (the guide tree) to test if the combination BPP + *gdi* supports the same or fewer species than BPP alone. To ensure convergence and mixing, we first ran four replicates for each analysis, and subsequently, the runs were combined to generate the posterior distributions required to calculate the *gdi* with the equation: $gdi = 1 - e^{-2\tau/\theta}$ (Jackson et al., 2017) using the R script of Leaché et al. (2019). Because $-2\tau/\theta$ is the population divergence time in coalescent units, $2\tau_{AB}/\theta_A$ indicates if the population A is distinct from B, while $2\tau_{AB}/\theta_B$ is used to differentiate population B from A. When the *gdi* < 0.2, the populations belong to the same species, and if *gdi* > 0.7, the candidate species are considered validated. Values of 0.2 < *gdi* < 0.7 suggest ambiguous species status (Jackson et al., 2017; Leaché et al., 2019).

2.5 | *Divergence times and species tree*

A time-calibrated species tree was inferred using StarBEAST in BEAST v.2.6.2 (Bouckaert et al., 2019). We used as outgroups the four species from the *ruber* group, (*S. fuscovarius, S. perereca, S. nasicus* and *S. ruber*), and *S. catharinae* from the *catharinae* group. For this analysis we used Dataset A, which include the complete dataset with all sequences of *cytb* and *RAG1* (see Table S1). Also, we included DNA sequences of GenBank (see Molecular data above). *Scinax berthae* and *S. x-signatus* were not included as terminals due to the lack of *RAG1* data. We calibrated the tree using the same set of priors for the mitochondrial DNA (mtDNA) substitution rate as described above (see mitochondrial tree settings). Meanwhile, for *RAG1* we used a range of substitution rates from the literature (i.e., 0.10–0.15% substitutions per lineage per million years) (Hugall et al., 2007; Gehara et al. 2017). Based on both available rates, we set as prior a normal distribution with mean 0.00125 and 10% standard deviation. We implemented a Birth-Death speciation model with a Strict clock model for nuclear locus and a Relaxed clock Log Normal model for mitochondrial locus. We ran two independent replicates of 3×10^7 generations, with parameters and topologies sampled every 5×10^3 generations. The convergence and stationarity were inspected in Tracer 1.7.1 (Rambaut et al., 2018), and we made sure that all parameters reached effective sample size (ESS) values higher than 200. The replicates of each analysis were combined using LogCombiner, and we obtained the maximum clade credibility (MCC) tree with a 20% burn-in in TreeAnnotator (Bouckaert et al., 2019).

2.6 | *Species distribution models*

We used 19 bioclimatic variables obtained in the WorldClim database (Fick & Hijmans 2017) to model the geographic distributions of four lineages of the *Scinax granulatus* complex. We selected a subset of variables for each lineage with correlations lower than |0.5| using the values from georeferenced occurrence sites. This procedure is recommended to avoid multicollinearity among predictor variables in the modelling process. Next, we chose nine bioclimatic variables for building the ESMs models, using a different subset of them for each lineage. The variables selected in question were: the annual mean temperature (BIO 1), the mean diurnal range (BIO 2), the temperature seasonality (BIO 4), the mean temperature of wettest quarter (BIO 8), the mean temperature of driest quarter (BIO 9), the precipitation of wettest month (BIO 13), the precipitation seasonality (BIO 15), the precipitation of warmest quarter (BIO 18), and the precipitation of coldest quarter (BIO 19).

As the *Scinax granulatus* complex lineages present a low number of occurrences, we chose to model their distributions using the Ensemble of Small Models approach (ESM, Breiner et al. 2015). The ESM technique consists in building bivariate models with all the combinations of predictors pairs, to deal with the overfitting issue when modelling rare species, but without losing the ability to estimate the species niche (Breiner et al. 2015). We used four different algorithms: Bioclim (SRE, Booth et al. 2014), gradient boosting machine (GBM, Friedman 2001), classification and regression trees (CTA, Breiman et al., 1984), and artificial neural networks (ANN, Ripley, 1995), which had a good performance in modeling rare species using ESM (Breiner et al., 2018). The results of different algorithms were later combined into an ensemble model based on performance values (Araújo & New, 2007). As only presence data were available, and the algorithms need absence data, we created a set of 10,000 random points in each lineage's modeling extent. The modeling extent for each lineage was defined as a bounding box that considered the extreme coordinate for each candidate species.

The occurrence data for each lineage were randomly split in 70% for training and 30% for testing the models. We ran five replicate runs with each algorithm. The performance of each bivariate model was evaluated using AUC (Area Under the Curve) and Boyce's index, because they are threshold-independent and complementary to each other, as AUC needs presence and

absence data, while Boyce's index only uses presence data (Hirzel et al., 2006). The different models were combined by using ensemble averaging, based on the Somers' D value to weight them. Only models presenting Somers' D values > 0 were used in the ensemble, because they represent better predictions than random (Breiner et al. 2015). We converted the suitability values into a binary prediction for each lineage using the TSS (True Skilled Statistics) threshold. We performed the SDMs analyses with the software R v.3.2.4 (R Development Core Team, 2016) using the packages, *ecospat* (Di Cola et al., 2017), *biomod2* (Thuiller et al., 2016), *spThin* (Aiello-Lammens et al., 2015) and *usdm* (Naimi, 2015). We used the classification of biomes and ecoregions by Olson et al. (2001) to define the Atlantic Forest and Pampa boundaries (see Section 3.1 and Fig. 1a).

2.7 | *Phylogeographic diffusion*

We reconstructed the spatial spread through continuous space and time using BEAST 1.8.4 (Drummond et al., 2013). The analyses were performed for each evolutionary unit according to the species delimitation results (see species discovery and delimitation section), using the *cytb* data set. We used a Relaxed Random Walk (RRW) model with a Cauchy distribution (Lemey et al., 2010), and a relaxed molecular clock prior with a lognormal distribution. For the tree model we utilized a GMRF Bayesian Skyride coalescent prior with time-aware smoothing (Minin et al., 2008). The analysis was run twice with 3 x 10^7 generations sampled every 1,000 generations. We used the MCC gene tree to reconstruct the spatiotemporal dynamics using the SpreaD3 v.0.9.6 software (Bielejec et al., 2016). We compared the timing of changes in population dynamics with the duration of glacial cycles based on the dating of marine isotope stages (MIS) according to Lisiecki and Raymo (2005). Ocean sediments provide an important record of climatic change because they contain stacks of benthic $\delta^{18}O$, which track temperature and global ice volume (Lisiecki & Raymo, 2005).

3 | RESULTS

3.1 | *Genetic diversity and species discovery*

The alignment of *cytb* sequences was 552 bp long and *RAG1* sequences were 397 bp long. There were no indels and we found an open reading frame in all sequences. The nucleotide (π) and

haplotype diversity (*H*) values for each locus were: $\pi = 0.06994$ and $H = 0.96$) for *cytb*, and $\pi = 0.01394$ and $H = 0.86$ for *RAG1*. We detected 45 haplotypes for *cytb* and 16 for the *RAG1* locus. There were 112 synonymous and 15 non-synonymous mutations in *cytb*, while 16 synonymous and four non-synonymous substitutions were found in *RAG1*.

Four mtDNA lineages (A, B, C, and D) were identified in the *Scinax granulatus* complex based on both the haplotype network (Fig. 1b) and the gene tree of *cytb* (Fig. S1a). The A lineage occurs in the Pampa biome from northern Rio Grande do Sul (RS) State to southern Uruguay and northern Buenos Aires (BA) Province, Argentina. The B lineage spreads further toward the east in RS and northern Uruguay (Cerro Largo Department). The C lineage occurs in the highlands of eastern Santa Catarina (SC) and south Paraná (PR) States, Brazil, and in one locality from San Pedro, Misiones (MN) Province in Argentina, inhabiting areas of the Araucaria Moist Forest (AMF) and Alto Paraná Atlantic Forest (APAF). Finally, the D lineage is found in the lowlands of Serra do Mar Coastal Forest (SMCF) and Atlantic Coast Restinga (ACR) ecoregions in RS and SC, Brazil (Fig. 1a). There is complete lineage sorting between the four lineages in the *cytb* tree and incomplete lineage sorting (ILS) in the *RAG1* tree (Fig. S1a-b). The *cytb* network exhibits a strong genetic divergence of the D lineage from the other lineages of *Scinax granulatus*: there are 86 mutational steps between the C and D lineages, 85 between the B and D, and 98 between A and D lineages (Fig. 1b).

Position FIGURE 1

The genetic p-distance showed different degrees of divergence among the lineages of *S. granulatus* complex. Genetic distances were lower than 5% in all pairwise comparisons between A, B and C lineages, but divergence was always higher than 16% when these lineages were compared with D (Table 1). The comparisons of these lineages of *S. granulatus* with *S. fuscovarius, S. perereca* and *S. nasicus* exhibited mean values of genetic divergence above 16% (Table 1).

The bGMYC analysis indicates that there exist five distinct evolutionary units –i.e., A1, A2, B, C and D (Fig. 2). In this partition scheme, only the D lineage owns a high nodal support (posterior probability, PP > 0.95), while the other lineages were recovered with posterior probabilities lower to 0.95 (Fig. S2a). The P2C2M.GMYC test indicated that the data fit the model without violating it. On the other hand, bPTP methods recovered two candidate species:

one consisting of the *cytb* lineages A, B and C with Bayesian posterior probability of 0.89, and the other constituted by the D lineage with stronger support (PP = 0.97, Fig. S2b). Additionally, the best-scoring ASAP analysis delimited the same two species as bPTP with a distance threshold of 9.8% (Table S3, Fig. S2c). Based on the high divergence and the putative species delineation, hereafter we will refer to the D lineage of *S. granulatus* as *S.* sp. D and to the rest of the lineages as *S. granulatus* sensu stricto (i.e., A, B and C; Fig. 2).

Position TABLE 1

3.2 | *Species delimitation*

We set the following priors in all delimitation analyses with BPP: $\theta \sim IG(3, 0.0200)$ and $\tau \sim IG(3, 0.0784)$. Posterior distributions obtained in the first A00 analysis were plot together with the prior distributions for each parameter to show how the information in our data produce precise estimates within the wider prior bounds (Fig. S3). The A10 analysis of BPP was executed using the topology ((((*S. granulatus* ABC, *S.* sp. D), *S. perereca*), ((*S. ruber*, *S. nasicus*), *S. fuscovarius*)), *S. catharinae*) as the fixed guide tree based on the StarBEAST results (Fig. 2). The A10 analyses (i.e., with c*ytb* alone, *RAG1* alone, and both loci) validated the hypothesis of seven species with maximum support, recovering in all runs the same results, using both the default and the empirical priors (Table S4).

Position FIGURE 2

We executed the A00 analysis with both loci because we did not observe any incongruency among the three A10 analyses (see above). We used a lineage/species tree (((A, B), C), D) as fixed guide tree. We estimated the *gdi* comparing all three lineages of *S. granulatus* sensu stricto (i.e., A, B and C) with the *S.* sp. D lineage. We carried out a BPP + *gdi* analysis to further validate the lineage *S.* sp. D as a good candidate species and the other lineages of *S. granulatus* sensu stricto as conspecific. We performed three different A00 analyses to calculate the *gdi* for pairs of lineages by inferring the most recent lineage/species divergences between A and B, AB vs C, and ABC vs D following the approach in Leaché et al. (2019). The *gdi* analysis indicated that the specific status of the A, B and C lineages are uncertain due their low (mean *gdi* A = 0.16; AB = 0.16; C = 0.22; Fig S4a-b) and intermediate values (mean *gdi* B = 0.37; ABC = 0.57;

Fig S4a, c). On the contrary, the results suggested that the *S.* sp. D would be a distinct species with high support (mean *gdi* = 0.9, Fig. S4c). We conservatively conclude that A, B, and C are conspecific lineages of *S. granulatus* sensu stricto, and that *S.* sp. D is a candidate new species (Fig. 2).

3.3 | *Divergence times and species trees*

We used *Scinax granulatus* sensu stricto (i.e., A, B and C) and *S.* sp. D as species or minimum units to infer the species tree. The species tree (Fig. 2) exhibited the same topology as the mitochondrial tree (Fig. S1). The *S.* sp. lineage D was recovered as monophyletic with strong support for the mtDNA tree and in the species tree (PP > 0.97), but with weak support in the *RAG1* tree (PP = 0.81). The relationships between the remaining *S. granulatus* lineages were not resolved, and they appear in the *RAG1* tree as paraphyletic (Fig. S1). In our phylogenetic hypothesis of the mitochondrial tree, *Scinax perereca* is recovered as the sister taxa of the *S. granulatus* complex (also supported in the *RAG1,* Fig. S1 and species trees, Fig. 2). Further back in time, among *ruber* group species, *S. ruber* and *S. x-signatus* appear as sister taxa, the next node branches off to *S. nasicus*, and the subsequent older node to *S. fuscovarius*, in a pectinate branching pattern (Fig. S1). The *S. ruber* group was recovered as monophyletic in relation with the *catharinae* group species, *Scinax berthae* and *S. catharinae*, which were recovered as monophyletic with maximum support (PP = 1, Fig. S1).

The species tree estimate was performed without *S. berthae* and *S. x-signatus* (see section 2.2 Divergence times, gene and species trees). Nevertheless, the node support and the topology recovered were similar to that of the mitochondrial phylogenetic hypothesis (Fig. 2, S1). The calibrated divergence times of the species tree obtained by StarBEAST showed an early diversification between the *S.* sp. D lineage and the ancestor of *S. granulatus* sensu stricto in the late Pliocene around 4.43 Mya (Fig. 2).

Position FIFURE 3.

3.5 | *Species distribution models*

The SDM predictions for the four lineages of *S. granulatus* complex showed good performance based on the high AUC and low to moderate Boyce index values (Table S5). The selection of variables for each lineage were based in the correlation threshold (i.e., 0.5). The most important bioclimatic variables for each lineage were: the precipitation seasonality (BIO 15) for the A lineage, precipitation of wettest month (BIO 13) for the B lineage, the mean temperature of wettest quarter (BIO 8) for the C lineage, and temperature seasonality (BIO 4) for the D lineage (Fig. S5). The predictive distribution maps for the four lineages displayed areas with high climatic suitability, with most of the sampling localities falling within the area with the highest predicted suitability (Fig. 3a-d). In addition, the models projected some areas of high suitability for lineage C for which we do not have any sample, including areas in Uruguay, the coastal line of Rio Grande do Sul in Brazil, and portions of Buenos Aires in Argentina (Fig. 3c). Moreover, the models predicted that the lineages A and C would overlap in regions of the Uruguayan Savanna, AMF, and APAF ecoregions (Fig. 3e).

3.6 | *Phylogeographic diffusion*

Phylogeographic diffusion reconstruction analysis placed the diversification center for *Scinax granulatus* sensu stricto in the Depressão Central of Rio Grande do Sul according to Rambo (1950). The first expansion event started at 430 ka in two main directions: to the south in Uruguay and north to the Santa Catarina Plateau (Fig. 4a), They constituted the initial long-distance colonizations that reached the northern and southern limits around 230 ka (Fig. 4bi). The subsequent long-distance colonization was completed about 120 ka (Fig 4ci). From here, the dispersal was predominantly of short distance (Fig 5ci, di). On the other hand, *Scinax* sp. D expanded from an area near to Jaguaruna in Santa Catarina State around 230 ka, and from here, it dispersed north towards Imbituba and south to Arroio do Silva in Santa Catarina (Fig. 4bii). Up to the present, the dispersion events have been of short distance, without further changes in the diffusion rates (Fig. 4cii-dii and e).

Position FIGURE 4.

4 | DISCUSSION

4.1 | *Diversity and species delimitation*

In this study, we elucidated for the first time the genetic diversity and phylogeographic structure of the *Scinax granulatus* complex across its distribution range. We recovered four mitochondrial lineages with moderate genetic differentiation between the A, B, and C lineages, but a high divergence of the D lineage, which we named as *S.* sp. D (Fig. 1b; Fig. 2). The gene trees (i.e., *cytb* and *RAG1*) recovered *S.* sp. D as monophyletic and divergent of *S. granulatus* sensu stricto (Fig. S1a-b). However, the relationships among the A, B and C lineages were unresolved with shared haplotypes in the network and paraphyly in the nuclear gene tree (Fig. 1B; Fig. S1b). Moreover, when we compared the genetic distances between the lineages the *S. granulatus* complex, the genetic p-distance ranged between 2.7 and 4.5% for the A, B, and, C lineages, but increased to 16% for *S.* sp. D (Table 1). This divergence magnitude was similar to that one found when comparing any of the lineages of S. *granulatus* complex with *S. fuscovarius, S. perereca* and *S. nasicus* (Table 1). The *cytb* genetic divergence for different vertebrate species within a genus averages 10.69% (± 1.34), but increases to 14% or higher among amphibians (see Kartavtsev & Lee, 2006). In frogs, this degree of differentiation suggests species-level distinctness (e.g., Rojas et al., 2018; Sabbag et al., 2018), which is consistent with the divergence of *Scinax* sp. D from other lineages of *S. granulatus* sensu stricto and closely related species of the *ruber* group (Table 1). Additionally, we observed six non-synonymous mutations exclusive of *S.* sp. D, five that belong to *cytb* (e.g., on position 529) and one to *RAG1* (i.e., on position 104). These sites are molecular diagnostic characters that distinguish *S. granulatus* sensu stricto from *S.* sp. D, pending further molecular and geographic sampling to confirm fixation of these mutations in each lineage.

The discovery analyses with bPTP and ASAP detected two candidate species: *S. granulatus* sensu stricto and *S.* sp. D. Alternately, bGMYC analysis suggested five distinct species, splitting the terminals of mitochondrial A lineage in two putative species. Nevertheless, it seems like bGMYC cannot distinguish the transition point between coalescence and speciation, showing over-splitting of the *cytb* data, resulting potentially in artifactual detection of non-existent species boundaries. For instance, it is known that bGMYC is prone to over-splitting species in simulated as well as empirical data (Papakostas et al., 2016; Dellicour & Flot, 2018). Consequently, we took the conservative decision that *S. granulatus* complex is constituted at most by two species, based on the cross-evidence among several species discovery methods (Fig. 2) following the approach of Edwards & Knowles (2014).

Herein, we followed a model-based coalescent approach to validate a candidate species belonging to *S. granulatus* complex using genetic data. The genetic validation method BPP indicated with high support that *S.* sp. D represents a divergent evolutionary lineage within *S. granulatus* complex (Fig. 2). Recently, coalescent-based methods for species delimitation, particularly BPP, have been strongly criticized for ignoring the impact of gene flow and population structure that leads to over-splitting divergent lineages as species (Sukumaran & Knowles, 2017; Barley et al., 2018; Leaché et al., 2019). Consequently, we also implemented a BPP + *gdi* strategy to evaluate if the divergence of the lineage *S.* sp. D corresponds to that one expected at the species-level. Indeed, the *gdi* index confirmed the hypothesis proposed by bGMYC, bPTP, ASAP, and BPP that supported *S.* sp. D is a distinct species (Fig. 2). However, the status of the other lineages of *S. granulatus* sensu stricto remain uncertain, suggesting conservatively that they are conspecific (Fig. 2, S4).

There has been no phylogenetic analysis of the *S. granulatus* complex and closely related species of the *ruber* group until this study. The mitochondrial tree recovered *Scinax perereca* as the sister taxon of the *S. granulatus* complex (i.e., *S. granuatus* sensu stricto and *S.* sp. D). These three species were recovered as a sister clade of another one formed by the remaining species of the *ruber* group included in our study. In this clade, *S. ruber* and *S. x-signatus* were recovered as sister to each other in the clade along with *S. nasicus* and *S. fuscovarius*. The relationships among these four species are in accordance with the topology proposed in the amphibian tree of life (see Jetz and Pyron, 2018). Our findings indicate that *S. granulatus* sensu stricto, *S.* sp. D e *S. perereca* are closely related to *S. fuscovarius, S. nasicus, S. ruber* and *S. x-signatus*. In any case, to confirm this hypothesis, it would be necessary a comprehensive phylogenetic analysis including all nominal species of *Scinax* (i.e., 128 species) plus the innumerable species that need yet to be described (e.g., *S.* sp. D).

4.2 | *Biogeography and diversification patterns*

We observed that *S. granulatus* sensu stricto and the candidate species *S.* sp. D occupy different patterns of distribution. While *S. granulatus* sensu stricto occurs in open areas of the Pampa biome, and of the AMF and APAF ecoregions, *S.* sp. D has a more restricted range in forest and open areas in lowlands of the Atlantic Forest (i.e., SMCF and ACR). These patterns of diversity,

genetic divergence and distribution would be a consequence of the historic processes that have affected the populational dynamics and evolutionary history of these linages. Our results agree with the idea that the current Neotropical biodiversity is the result of a complex interplay of ecological and evolutionary processes across spatial and temporal scales that began with orogenic events in the Neogene and climatic changes in the Pleistocene (Rull, 2011a; Turchetto-Zolet et al., 2013; Rull et al., 2020), altering the landscape and paleogeography of different biomes and ecoregions.

First, the divergence between *S. granulatus* sensu stricto and *S.* sp. D coincides with the late uplift phase of the Serra do Mar and concomitants changes in the landscape. In southeastern Brazil, the Serra do Mar and associated ranges emerged from the arching and fracturing of the ancient Brazilian Shield, which experimented a significant increase in the rate of uplift in three periods of epeirogenic movements. The first of them probably happened at the end of the Cretaceous and the last two in the Tertiary, with the third one probably extending into the Quaternary (Freitas, 1951). These tectonic processes of Serra do Mar would have affected the climate along the coast and nearby inland regions, modifying dramatically the temperature and rainfall regime, and therefore, altering the distribution of humid and dry habitats (Oliveira-Filho & Fontes, 2000; Mata et al., 2009; Dantas et al., 2011). This period of environmental changes would have occurred during the early Pliocene about 5.6 Ma (Vasconcelos et al., 1992; Dantas et al., 2011), being able to affect the diversification patterns of the biota these regions (e.g., Mata et al., 2009; this work). The diversification of the *S. granulatus* complex began with the split of the lineage *S. sp.* D around 4.43 Mya in the Zanclean age of the Pliocene epoch in the Neogene period (Fig. 2). These divergence times are also congruent with the diversification patterns for frogs (Brunes et al., 2010; Thomé et al., 2010), birds (Mata et al., 2009) and plants (Silva et al., 2018) that co-occur in the Atlantic Forest and Pampa biomes.

Second, the phylogeographic reconstruction indicated that the diversification within *S. granulatus* sensu stricto and *S.* sp. D occurred in different periods, but in both species the dispersal and diversification events would have been driven by paleogeographic reorganizations and paleoclimatic fluctuations. The initial diversification of *S. granulatus* sensu stricto most likely occurred in the central portion of the Peripherical Depression of Rio Grande do Sul. This geomorphological unit consists of a vast depression supported by rocks from the Paraná basin sequence, showing characteristics of an interplanaltic depression surrounded to the south and

east by the Sul-Rio-Grandense Plateau and, to the north and west, by the Meridional Plateau (Rambo, 1950; Verdum et al., 2019). The initial colonization event occurred about 420 ka in the mid Pleistocene, during a cold-warm transition phase (Fig. 4a). Subsequent dispersal events gave rise to the three mitochondrial lineages (i.e., A, B and C), which occurred predominantly during warmer phases (Fig. 4b1-c1). This pattern is similar to that one observed in another Pampean frog species, *Pseudopaludicola falcipes*, that colonized southern portions of its current distribution during interglacial periods (Langone et al., 2016).

In the case of *S.* sp. D, the origin of dispersion would be in the southern tip of Santa Catarina, from where it dispersed towards the north (i.e., Imbituba) around 230 ka, and to the south near Arroio do Silva in Santa Catarina (Fig. 4bii, showing the populational dynamics of about 200 ka). The subsequent dispersions occurred from the Late Interglacial (~120 ka) until today (Fig. 4cii-dii). *Scinax* sp. D is distributed in the recently formed South Atlantic Coastal Plain (SACP), a geological formation that originated by successive marine transgression/regression events: three in the Pleistocene and one in the Holocene, dating to around 400, 325, 125, and 7 ka, respectively (Tomazelli & Villwock, 2005; Dillenburg & Barboza, 2014). However, a recent study dated the second event to a more recent age range: 238–235 ka (Lopes et al. 2014), and it could be the actual mechanism that facilitated the initial dispersal of *S.* sp. D, which took place in the interglacial phase of the MIS 7 Stage. The next dispersal event of *S.* sp. D can also be associated with the SACP formation, in this case with the third event, which occurred at ~125 ka during the LIG of the MIS 5 Stage. These same geomorphological events could be related with the evolutionary history and diversification of fish species (Beheregaray et al., 2002), mammal (Peçanha et al., 2017), plants (Fregonezi et al.,2015; Silva et al., 2018) and frogs (this work) that co-occur in the SACP.

We found differentiation between the climatic suitability envelopes of *S. granulatus* sensu stricto (i.e., the A, B and C lineages) and the lineage *S.* sp. D. Because amphibians have strong environmental constraints due to their ectothermic physiology, distribution patterns within regions are strongly dependent on humidity and temperature (Buckley & Jetz, 2007). The probability of occurrence of the lineages of *S. granulatus* sensu stricto seems to be associated with bioclimatic variables related to precipitation (i.e., BIO 15 and BIO 13), while the climatic niche suitability of *S.* sp. D was better explained by thermal variables (i.e., BIO 4 and BIO 1). In this vein, the temperature seasonality (BIO 4) has been suggested as a good biogeographic

predictor because cold winter temperatures limit the dispersal of tropical hylid taxa (e.g., *Scinax staufferi*) in more temperate regions (Wiens et al., 2006b). Our results support the idea that species complexes distributed across biomes with divergent climates –e.g., grassland and rainforest– often harbour hidden cryptic diversity (see also Fouquet et al., 2007). Consequently, ecological conditions can play an important role in speciation when adaptation to different ecological settings drives evolutionary divergence in incipient species (Wiens, 2004). Herein, we observed differentiation in the climatic suitability between *S. granulatus* sensu stricto and *S.* sp. D (see Fig. 3, S5), which was congruent with strong genetic divergence. Therefore, the ecological differentiation (i.e., habitat and climate), and the genetic isolation among lineages could have jointly led to the divergence of these species within the speciation continuum (sensu de Queiroz, 2007; Arnegard et al., 2014).

5 | FINAL REMARKS

We found compelling evidence in this study to recognize *S.* sp. D as distinct lineage within the *S. granulatus* complex, based on genetic and ecological niche differentiation. We do not know the actual degree of range overlap between *S. granulatus* sensu stricto and *S.* sp. D. However, our SDMs predict that *S. granulatus* sensu stricto and *S.* sp. D would have limited range overlap, for instance, in areas close to the Osorio municipality from Rio Grande do Sul State where typical ecotonal areas of grassland (i.e., Pampa biome) and rainforest (i.e., Atlantic Forest) might facilitate contact and genetic exchange between these lineages (see Fig. 3e). Although we tested for gene flow among these lineages using an isolation with migration model in IMa3, model parameters could not be estimated confidently, possibly due to the small sample sizes (results not shown). Therefore, it would be necessary to collect sequences from more loci or genome-level data to detect possible signatures of gene flow and/or hybridization in this species complex. Further sampling of multiple data types from these potential contact areas are necessary to address evolutionary processes underlying speciation in this complex, and to assess the relative importance of hybridization vs. reproductive isolation between these lineages. Additionally, a re-determination of specimens deposited in museums and collections of Argentina, Brazil, and Uruguay will be necessary, including genetic, morphological and bioacoustic data, if available, to ascertain the degree of misidentification, range overlap, and trait variation in sympatric vs. allopatric areas between these lineages.

There are a few published studies recording the presence of *S. granulatus* in the lowlands of the Atlantic Forest from Rio Grande do Sul and Santa Catarina States (Colombo et al., 2008; Wachlevski & Duarte Rocha, 2010; Campos & Lourenço-de-Moraes 2017). However, misidentification may have been made in some of these works by confusing these specimens with *S. fuscovarius* (see Fig. 2.9 in Colombo et al., 2008) or referring to them as *S. granulatus* (see Fig. 2B in Wachlevski & Duarte Rocha, 2010). We believe that these records actually correspond to *S.* sp. D (i.e., with a putative diagnosis related to a supralabial white stripe line, see Fig. 2). Kwet (2001b) pointed out the difficult diagnosis of *S. granulatus, S. perereca*, and *S. fuscovarius* due to their similarity in call and morphological traits. Because these species, together with *S. nasicus*, exhibit high intraspecific variation in coloration patterns, and co-occur in several areas, they have been confused with each other and misidentified in the field and in collections (e.g., see Achaval & Olmos, 2007; Kwet, Lingnau & Di Bernardo†, 2010; Dalmolin et al., 2017). Finally, we emphasize the need to increase the geographic sampling of *S.* sp. D, including adult and larval data and call recordings to carry out the formal description of this divergent lineage, and we encourage to apply an integrative taxonomy approach before making any taxonomic decisions. Moreover, we highlight the need to carry out integrative studies of species delimitation with multiple types of data within lineages of the *Scinax ruber* group, which are currently classified as a single species (e.g., *S. nasicus*, *S. fuscovarius*, and *S. perereca*), but that might also foster hidden diversity and constitute additional species complexes.

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CONFLIC T OF INTEREST

There are no conflicts of interest.

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FIGURE 1. (A) Geographic distribution of samples of *Scinax granulatus* used in this study (B) Haplotype networks *cytb* and *RAG1.* The white triangle denotes the type locality (i.e., Porto Alegre, RS - Brazil) of *Scinax granulatus.* Brazilian states: PR (Paraná); SC (Santa Catarina); RS (Rio Grande do Sul), Argentinian states: BA (Buenos Aires); MN (Misiones), Uruguay (URY) and Paraguay (PRY).

FIGURE 2. Calibrated species-tree annotated from StarBEAST 's analysis (left), and summary of different delimitation methods (right). Numbers above of nodes indicate posterior probabilities (PP), below indicate mean estimates of node ages. The vertical bars correspond to the candidate lineages detected by discovery methods (i.e., bGMYC, bPTP and ASAP) and the validated lineages by genetic (i.e., BPP, BPP + *gdi*). BPP analysis: i) only *Cytb*, ii) only *RAG1* and iii) multi-locus. BPP + *gdi* based in both loci.

FIGURE 3. Species distribution models and niche comparison of lineages of the *Scinax granulatus* complex. Projection of climatic suitability areas: (a) Stability areas of the A lineage; (b) Predicted areas to the B lineage; (c) Potential areas of the C lineage; (d) Potential areas to the lineage D; (e) Binary map using TSS thresholding, showing the smoothed predictions indicating the percentage of suitable habitat (i.e., presences) for each lineage.

FIGURE 4. Spatio-temporal dynamics of species of *Scinax granulatus* complex. (a, bi, ci and di) Spatial projection of the diffusion of *S. granulatus* sensu stricto. The blue lines represent the branches of the MCC tree and the light blue areas represent the 80%-HPD uncertainty in the location of ancestral branches with a gradient between clear and dark representing older vs. younger diffusion events. (bii, cii and dii) Dynamics of the diffusion of *S.* sp. D. The red lines represent the branches of the MCC tree and the orange areas represent the 80%-HPD uncertainty in the location of ancestral branches with a gradient between clear and dark representing older vs. younger diffusion events. Arrows denote the time slices of each snapshot of diffusion events. (e) Variation of diffusion rates through time: Color lines indicate the mean diffusion rates for each species, while the transparent ribbons represent 95%-HPD (lower and upper values of mean values). The $\delta^{18}O$ curve corresponds to the composite benthic stable oxygen isotope ratios (Lisiecki and Raymo, 2005) and the script plot according to Ruddy (2017).

TABLE 1 Genetic distances (uncorrected p) of *Cytb* averaged within (bold) and among lineages of *Scinax granulatus* complex, compared with outgroups.

	A	B	C	D	*S. perereca*	*S. fuscovarius*	*S. nasicus*
S. granulatus							
A	**0.005**						
B	0.027	**0.003**					
C	0.044	0.035	**0.004**				
D	0.170	0.164	0.160	**0.004**			
Outgroups							
S. perereca	0.196	0.193	0.199	0.174	**n/c**		
S. fuscovarius	0.182	0.184	0.174	0.186	0.208	**0.016**	
S. nasicus	0.166	0.165	0.165	0.176	0.175	0.192	**0.020**

n/c – distance not calculated due to the low number of individuals

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

Table S1. Locality specifications, geographical records, voucher specimens of *Scinax granulatus*, *S. sp.* D, *S. fuscovarius*, *S. perereca* and *S. nasicus*. The GenBank accession numbers of the amplified sequences for *cytb* and *RAG1* genes and samples utilized in each dataset.

Table S2. Analyses performed in BEAST program. For each analysis is presented the locus used, number of sequences (N), fragment size in base pairs (pb), and substitution model estimated by Partition Finder program.

Table S3. Analysis of Assemble species by automatic partitioning (ASAP) showing nine partitions for cytb dataset. We highlight in bold the best partition based in the lower ASAP-score. p-value = probability of panmixia (p-val), W = relative gap width metrics. See the Fig. S2c to observe the ranged and treshold distances of the best partition.

Table S4. Species delimitation hypothesis with *posterior* probabilities for each replicate of the A00 analyses of BPP.

Table S5. Score of the performance of ESMs inferred for each lineage of *S. granulatus* complex assessed by AUC and Boyce index. Mean (SD = Standard deviation).

Figure S1. Gene trees of (a) *cytb* and (b) *RAG1*. Numbers in the nodes indicate Bayesian *posterior* probabilities.

Figure S2. Summary of the lineage delimitation analysis of the *Scinax granulatus* complex. (a) Bayesian implementation of the generalized mixed Yule-coalescent (bGMYC) model. Numbers in each node indicate the posterior probability (PP) sampled from a posterior distribution of 1000 trees. (b) Bayesian implementation of the Poisson Tree Process (bPTP). Here the numbers denote the support (PP) for each delineated candidate species. (c) Results of Assemble Species by Automatic Partitioning (ASAP) method, showing the barcode gap and the distance threshold (denoted with line red) that indicate best partition for the hypothesis of the species boundaries.

Figure S3. Distribution density results of MCMC sampling for theta and tau parameters from A00 analysis: (a) Theta parameters; (b) Tau estimates.

Figure S4 Candidate lineages delimitation of *S. granulatus* complex. a-c Density plots of gdi values (a) *gdi* of Sp.A and Sp.B (b) *gdi* of Sp.AB and Sp.C (c) *gdi* of Sp.ABC and Sp.D. According to Jackson et al.(2017), *gdi* <0.2 indicates a single species, *gdi* >0.7 indicates distinct species, and *gdi* values between 0.2 and 0.7 represent ambiguous species status.

Figure S5. Boxplot of the contribution of each environmental variable for the four *S. granulatus* ‘s lineages. The percentage represents the proportion of the ensemble explained by the variable of interest. The variables used were: the annual mean temperature (BIO 1), the mean diurnal range (BIO 2), the temperature seasonality (BIO 4), the mean temperature of wettest quarter (BIO 8), the mean temperature of driest quarter (BIO 9), the precipitation of wettest month

(BIO 13), the precipitation seasonality (BIO 15), the precipitation of warmest quarter (BIO 18), and the precipitation of coldest quarter (BIO 19).

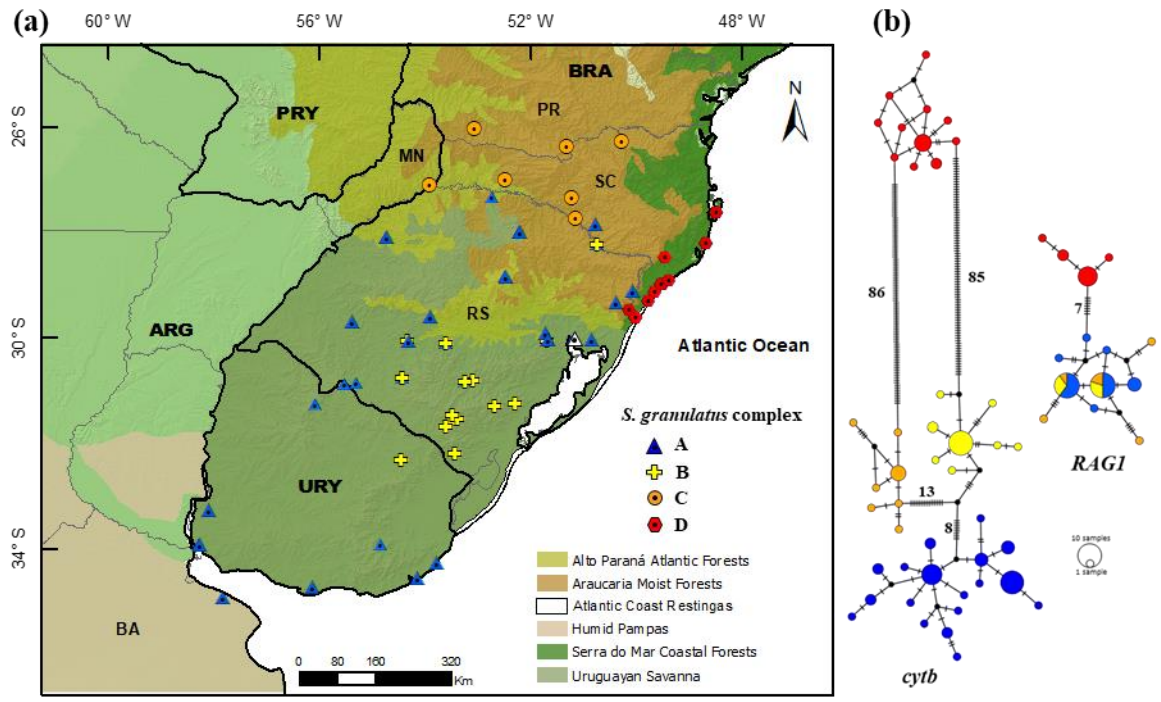
(a)
60° W
56° W
52° W
48° W
26° S
30° S
34° S
BRA
PRY
PR
MN
SC
RS
ARG
URY
BA
N
Atlantic Ocean
S. granulatus complex
A
B
C
D
Alto Paraná Atlantic Forests
Araucaria Moist Forests
Atlantic Coast Restingas
Humid Pampas
Serra do Mar Coastal Forests
Uruguayan Savanna
0 80 160 320
Km
(b)
86
85
7
13
8
RAG1
cytb

FIGURE 1

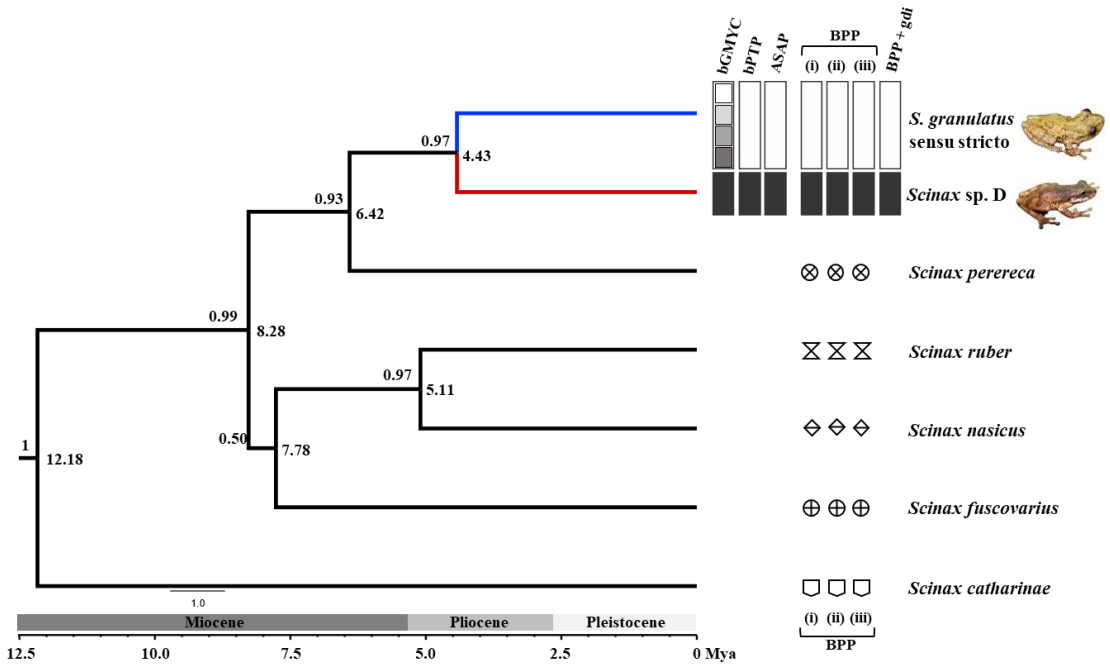
bGMYC
bPTP
ASAP
BPP
(i) (ii) (iii)
BPP+gdi
S. granulatus sensu stricto
Scinax sp. D
Scinax perereca
Scinax ruber
Scinax nasicus
Scinax fuscovarius
Scinax catharinae
0.97
4.43
0.93
6.42
0.99
8.28
0.97
5.11
0.50
7.78
1
12.18
1.0
Miocene
Pliocene
Pleistocene
12.5
10.0
7.5
5.0
2.5
0 Mya

FIGURE 2

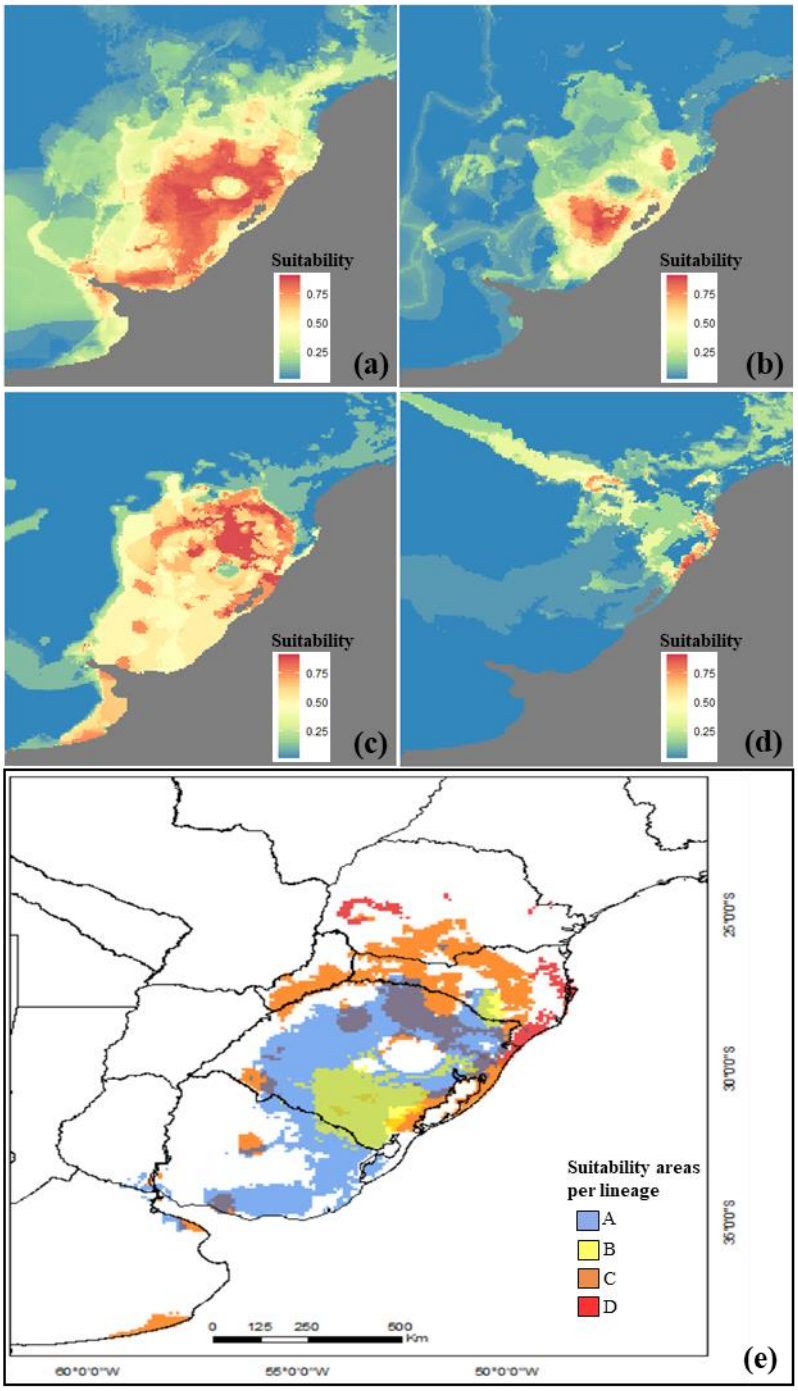
Suitability
0.75
0.50
0.25
(a)
Suitability
0.75
0.50
0.25
(b)
Suitability
0.75
0.50
0.25
(c)
Suitability
0.75
0.50
0.25
(d)
Suitability areas per lineage
A
B
C
D
0 125 250 500 Km
60°0'0"W
55°0'0"W
50°0'0"W
25°0'0"S
30°0'0"S
35°0'0"S
(e)

FIGURE 3

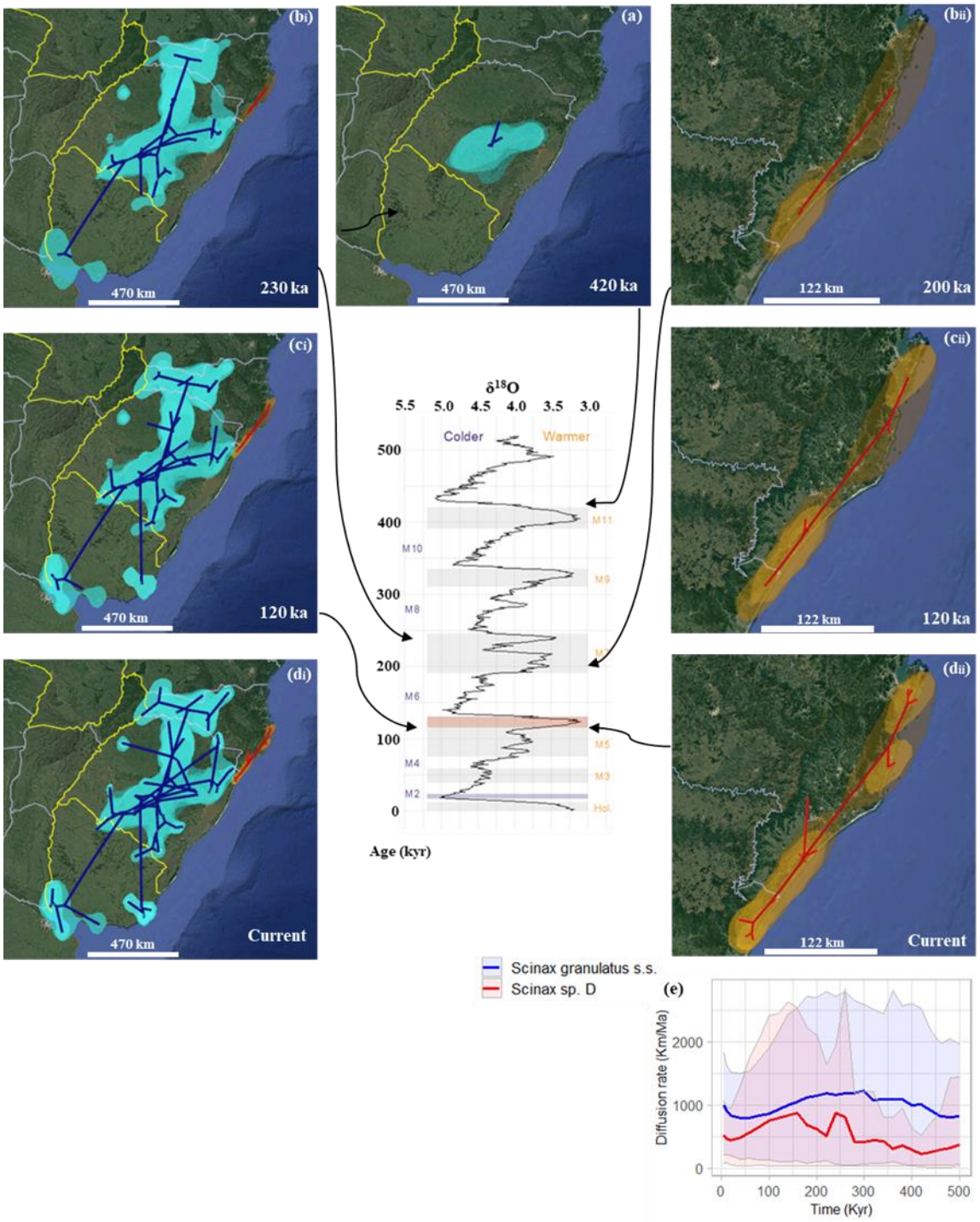
(bi)
470 km
230 ka
(a)
470 km
420 ka
(bii)
122 km
200 ka
(ci)
470 km
120 ka
δ18O
5.5 5.0 4.5 4.0 3.5 3.0
Colder
Warmer
500
400
300
200
100
0
M11
M10
M9
M8
M6
M5
M4
M3
M2
Hol.
Age (kyr)
(cii)
122 km
120 ka
(di)
470 km
Current
(dii)
122 km
Current
Scinax granulatus s.s.
Scinax sp. D
(e)
Diffusion rate (Km/Ma)
2000
1000
0
0 100 200 300 400 500
Time (Kyr)

FIGURE 4

CAPÍTULO II

**Formato nas normas da revista Journal o Biogeography*

Long-time persistence during the Pleistocene and recurrent secondary contact among lineages of *Leptodactylus latinasus* a widely distributed Neotropical frog from the Chaco-Pampean plains

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Long-time persistence during the Pleistocene and recurrent secondary contact among lineages of *Leptodactylus latinasus* a widely distributed Neotropical frog from the Chaco-Pampean plains

Matías M. Malleret | Diego Baldo | Francisco Brusquetti | Priscila Lemes | Laura Verrastro | Arley Camargo

Abstract

Aim: Neotropical diversification would be the result of diverse historical processes; both the geological events of the Neogene and the climatic cycles of the Quaternary seem to have been equally important drivers of the origin of Neotropical biodiversity. We investigated how landscape changes and climatic oscillations shaped the distribution, diversification history, and historical demography of an endemic frog from Pampa and Chaco biomes.

Location: The mid-latitude South American plains including Chaco and Pampa biomes.

Taxon: Frog *Leptodactylus latinasus*

Methods: We analyzed Cytochrome b (*cytb*) sequences of 139 individuals across most of the species' distribution. We generated genome-wide SNP of a subset of 40 samples. We conducted analyses of genetic structure, demography, phylogeographic diffusion, and estimates divergence times. We projected species distribution models on the lineages' distribution for different epochs of Quaternary, to generate hypotheses over the diversification of *L. latinasus*.

Results: The Gran Chaco would be the ancestral area of *Leptodactylus latinasus*, from herein started its diversification around early Pleistocene, which gave place to five mitochondrial lineages. Population genomic analysis identified two spatially structured populations, whose contact zone coincides with the current course of the Paraná River. Mitochondrial lineages remained isolates by long-time, until that recent range expansion events possibilities the secondary contact and the gene flow between populations, leaving signatures in the genome of *L. latinasus*. Diffusion events across of the Last Interglacial and Last Glacial Maximum are according with the probable areas of climatic suitability during these epochs of Quaternary.

Main conclusion: Geomorphological events and climatic changes modified the landscape of Chaco and Pampa biomes, affecting the distribution, population dynamics and diversification the *L. latinasus*. Paraná and Uruguay Rivers could have acted as important biogeographic corridors or barriers during certain periods, allowing or did not the dispersion and gene flow between intermittently geographically isolated lineages.

K E Y W O R D S

Anura, Biogeography, Phylogeography, species-tree diffusion, Quaternary climatic fluctuation, Pampa, ddRAD, genome wide SNPs, ecological niche modelling, historical demography

1 | INTRODUCTION

The mid-latitude South American plains –including Uruguay, northern, central and eastern Argentina, southern Brazil, the southern half of Paraguay and southern Bolivia – is dominated by open areas formations of several different types of vegetation (Turchetto et al., 2014). To the west, it is limited by a belt formed, from north to south, by the Andean Piedmont and subAndean Sierras, and the Pampean Sierras. To the south, the Tandilia and Ventania Sierras delimit this area at 39°S, and to the north/northeast is delimits by the southern Brazilian plateau. In geomorphological and botanical terms, this is a complex region known as Pampas in Brazil, Uruguay and Southern Argentina and as Chaco in its central and northern distribution in Argentina, Paraguay and southern Bolivia (Speranza et al., 2007; Turchetto et al., 2014).

The South American Gran Chaco extends from central Argentina to southeastern Bolivia, occupying the entire western region of Paraguay and entering into Brazil as a narrow strip parallel to the Paraguay River in the State of Mato Grosso do Sul. The Chaco climate is distinguished by its strong seasonality, with summer maxima of up to 49 °C, the highest temperatures recorded in South America, severe winter frosts and the rainfall declining from over 1000 mm/year in the east to less than 500 mm/year in the west, with a dry season in the winter and spring, and a rainy season in the summer (Toby Pennington et al., 2000). It is a semi-arid biome, being covered by xerophytic vegetation that forms a mosaic of grassland, savanna, open woodland, and xeric thorn forest (Prado, 1993a). The substratum of the Chaco is characterized by the absence of stones, with generally alkaline heavy clayish or silt-sandy soils, resulting in the development of compact soils with impeded drainage, that suffer seasonal yearly droughts and frequent floods towards the east (Toby Pennington et al., 2000; Morales et al., 2019).

On the other hand, Pampa biome one of the most important grassland regions in the world, extend between 28° and 38° S latitude, covering about 700,000 km^2 of eastern Argentina, Uruguay, and southern Brazil (Di Giacomo & Krapovickas, 2005; Paruelo et al., 2007). It is a biome of temperate subtropical climate, with NE-SW precipitation gradient that ranges from 1,500 mm/year in southern Brazil to 600 mm/year in central Argentina. Prairies and steppes (grass and low shrubs) are the dominant physiognomic types, and the woody vegetation within the

region is restricted to small areas near water bodies, such as the gallery forests along the large Paraná and Uruguay Rivers and their tributaries (Paruelo et al., 2007). The Pampa plain on the western side is mostly of Quaternary sedimentary origin, while to the east of the Uruguay River in the Uruguayan and Brazilian portions of the region, a diverse array of rocks such as Precambrian granite, Carboniferous sandstone, and Jurassic basalt is exposed to surface and soil-forming processes. The dominant loessic sediments of the Pampas are characterized by their unconsolidated nature, silt to fine-sand texture typical of wind-transported material, and large contributions of volcanic material (Paruelo et al., 2007). Most primary loess has been subsequently reworked by winds and fluvial streams, and by pedogenesis (Zárate, 2003).

Neotropical diversification would be the result of diverse historical processes, both Neogene tectonic events and paleogeographical reorganisations, and Quaternary climatic cycles seem to have been equally important drivers of the origin of neotropical biodiversity (Rull, 2011). The Neogene-Quaternary events have been proposed as important in the population dynamic and/or diversification of the herpetofauna different open areas biomes from South American (e.g., Camargo et al., 2013; Morando et al., 2004; Prado et al., 2012; Werneck et al., 2012), but studies remain scarce for species from the Pampa and Chaco biomes (Brusquetti et al., 2018, 2019; Langone et al., 2016). In the Chaco region, climates and habitats were similar to those of the Pampa region from Argentina during all the Pleistocene, the climates were arid or semiarid and cold with an increase of the mean annual temperature from south to north (Carlini et al., 2004; and references therein), while that in the Mesopotamia, Uruguay and southern Brazil during the entire Pleistocene, climates were wetter and warmer (Carlini et al., 2004).

Leptodactylus latinasus frog Jiménez de la Espada, 1875, represents an ideal model to for studying the influence of historical events on Chaco and Pampean fauna, being widely distributed in Gran Chaco of Argentina, Bolivia, and Paraguay, south and east throughout Uruguay and southern Brazil (Frost, 2021). This frog belongs to the *fuscus* group within the genus *Leptodactylus* (Fitzinger, 1826). This group has been characterized by a reproductive mode linked to terrestrial habitat, because they deposit their eggs in foam nests on constructed subterranean chambers by the males (Heyer, 1969; Pereira et al., 2015). This reproductive mode was suggested to be important to avoidance the desiccation of eggs and tadpoles (Heyer, 1969) and as a defence mechanism against predators (Kokubum & Giaretta, 2005). Although the tadpoles

hatch inside the chamber, they need to reach a water body (usually a temporary pond) to complete their development, which they achieve when rain floods the chamber and breaks the foam nest (Ponssa et al., 2019). *Leptodactylus latinasus* tadpoles have the ability to generate their own foam, producing bubbles with their mouths in the early stages of development (Ponssa & Barrionuevo, 2008), this behaviour can be important to avoid desiccation in case that the dry season is prolonged (Downie & Smith, 2003).

We investigated the recent evolutionary history of Pampa and Chaco through a phylogeographical study of *Leptodactylus latinasus*. We used mitochondrial sequence data with samples from across most of the *L. latinasus'* range and a subset of samples with genome-wide SNP data to investigate the role of historical landscape processes driving the diversification, the population structure, and the demographic history of *L. latinasus*. Based on the reproductive biology of this frog adapted to seasonally-dry conditions, we predicted that the glacial cycles should have caused fragmentation, and at least, partial isolation among deep lineages and subsequent recontact during phases of demographic expansion. In addition, given the trans-biome distribution of this frog in the Chaco-Pampean plains, we also expected the deep lineages should be restricted to these ecoregions if evolutionary divergence has been driven by adaptation to the distinct climatic regimes of the Chaco and Pampas during the Pleistocene.

2 | MATERIALS AND METHODS

2.1 | Sample collection and DNA sequence data

We analysed Cytochrome b (*cytb*) sequences of 139 individuals of *L. latinasus* collected from 90 localities across the species distribution. All specimens are vouchered in Table S1. We extracted total genomic DNA from samples preserved in 96-100% ethanol (liver or muscle), using Salt extraction protocol (McManes, 2013). DNA fragments were amplified via PCR with the primers CytbF (5'-TTT CTA GCA ATA CAY TAC ACA GCY GAT-3') and CytbC-R (5'-CTA CTG GTT GTC CTC CGA TTC ATG T-3') (Santos et al., 2020). We utilized the Blast

tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to confirm the identity of sequences. The electropherograms were examined and edited in Geneious v11.1.5 (http://www.geneious.com, Kearse et al., 2012) and MEGA7 (Kumar et al., 2016), and sequences were aligned by the alignment algorithm of Geneious (Needleman & Wunsch, 1970) using default settings. For each analysis we selected the best-fit partition schemes and substitution models following the Bayesian information criterion (BIC) using PartitionFinder v.2.1.1 (Lanfear et al., 2016) (see Table S2).

2.2 | Mitochondrial DNA: haplotype network

We implement the DNAPARS function of Phylip v.3.695 package (Felsenstein, 2005) to estimate a maximum parsimony tree of *cytb*. We built a haplotype genealogy from maximum parsimony tree using Haploviewer (Salzburger et al., 2011). We also inferred the molecular diversity indices: number of haplotypes (h), nucleotide diversity (π), and haplotype diversity (Hd) using DnaSP v6.11.01 (Rozas et al., 2017). We defined assigned samples to populations based on the mitochondrial lineages (see Results, mitochondrial tree and haplotype network).

2.3 | Gene tree and estimation of divergence times

We estimated the genic tree of *cytb* using a Bayesian approach with BEAST v2.6.2 (Bouckaert et al., 2019). For calibration of the molecular clock, we used a normally distributed evolutionary rate available in the literature (mean = 1.61% substitution rate per lineage per Mya, standard deviation = 0.01) (Stöck et al., 2012) and utilized two species for the rooting: *Leptodactylus gracilis* and *L. albilabris*. Analysis was performed under a strict clock model and the Yule process was implemented as prior for the speciation process. We executed two independent replications of 2×10^7 generations each, sampling every 1000 generations. We verified the convergence and stationarity of all parameters reached effective sample size (ESS) values higher than 200 using Tracer v1.7.1 (Rambaut et al., 2018). The replicates were combined discarding the first 20% of each run as burn-in using LogCombiner 2.6.6 (BEAST package),

and we obtained the maximum clade credibility (MCC) tree with a 10% burn-in in TreeAnnotator (Bouckaert et al., 2019).

2.4 | Historical demography

We performed the Tajima's D (Tajima, 1989) and Fu's Fs (Fu, 1997) tests in order to investigate whether there are significant deviations from the null hypothesis of neutral evolution and constant population size. Significance levels of Fs and D statistics were estimated with 10,000 coalescent simulations. All the statistics and coalescent simulations were inferred with DnaSP v6.11.01 (Rozas et al., 2017).

We estimated the population size dynamics over time using the Gaussian Markov random field (GMRF) Bayesian Skyride plot (Minin et al., 2008), implemented in Beast 1.8.4 (Drummond et al., 2012). We executed the Bayesian Skyride method for each mitochondrial lineage recovered with the mitochondrial network and tree. The GMRF Skyride plot was reconstructed using the cytb data (with the same substitution rate used for the gene tree), implementing a time-aware smoothing and strict clock priors. For each population we executed two independent runs of 20 million generations, sampling every 2000 generations. The convergence of each run was assessed in Tracer v1.7.1 by examination (>200) of the effective sample size (ESS).

We reconstructed the spatial dispersion of *L. latinasus* lineages through continuous space and time using BEAST 1.8.4 (Drummond et al., 2012). The analyses were performed for each lineage independently with the cytb data set. We used a Relaxed Random Walk (RRW) model with a Cauchy distribution (Lemey et al., 2010), and a relaxed molecular clock prior with a lognormal distribution. For the tree model we utilized a GMRF Bayesian Skyride coalescent prior with time-aware smoothing (Minin et al., 2008). The analysis was run twice with 2×10^7 generations sampled every 1000 generations. We used the MCC gene tree to reconstruct the spatiotemporal dynamics using the SpreaD3 v.0.9.6 software (Bielejec et al., 2016) and inferred the variation in diffusion rate over time using the TimeSlicer tool (Lemey et al., 2010).

2.5 | Ecological niche modelling

We used 85 non-duplicated occurrence data points for *L. latinasus* species to map the distribution of four lineages: L1 (n = 29), L2 (n = 14), L3 (n = 31), and L5 (n = 18). First, we spatially thinned the occurrence localities by 10 km to minimize the clustering of records due to biased sampling using thin() in the R package spThin (Aiello-Lammens et al., 2015). We used 19 bioclimatic variables as current candidate predictors with a spatial resolution of 0.08333° (~ 10 km) from the global dataset of PaleoClim (Brown et al., 2018). To avoid problems associated with multicollinearity, the candidate predictors were selected by the variance inflation factor (VIF, Zuur et al., 2010), and we retained only the variables with VIF values < 10 using the function vifstep() from R package sdm (Naimi & Araújo, 2016). Next, we selected six climatic variables for building all models: temperature seasonality (BIO 4), mean temperature of wettest quarter (BIO 8), mean temperature of driest quarter (BIO 9), precipitation of wettest month (BIO 13), precipitation seasonality (BIO 15), and precipitation of warmest quarter (BIO 18). In order to characterize past climate conditions for *L. latinasus* and each lineage separately, we used the same current environmental dataset projected to the Last Interglacial past (LIG, ~130ka), the Last Glacial Maximum (LGM, ~21ka), and the mid Holocene (MH, 8.32–4.20 ka). All global climate slices are available on PaleoClim (Brown et al., 2018).

We used the SDM package (Naimi & Araújo, 2016) in order to develop the ensemble forecasting of ecological niche modelling (Peterson & Soberón, 2012), and the output models were combined to generate a single prediction. For each lineage, we calibrated models using presence-only and presence-absence methods, as follows: domain, bioclim, support vector machine (SVM), boosted regression trees (BRT), and multivariate adaptive regression splines (MARS) models all available in the sdm-package (Naimi & Araújo, 2016). We used the area under the curve (AUC) of the receiver operating characteristic (ROC) to assess the accuracy of ecological niche models (ENMs; Fielding & Bell, 1997). AUC values of 0.5–0.7 correspond to low accuracy, 0.7–0.9 indicate good accuracy and values above 0.9 indicate high accuracy (Sweets, 1988). We also calculated the true skill statistic (TSS), which is equal to the sensitivity + specificity – 1 (Allouche et al., 2006) and ranges from –1 to +1. For each species, we generated ensemble forecasting using the output models for each time slice. We classified continuous predictions into presence/absence maps based on maximizing the true skill statistics (TSS, Allouche et al., 2006).

2.6 | ddRADseq and genome-wide SNP data

We built ddRADseq genomic libraries based on the protocol, barcodes, adapters and indices of (Peterson et al., 2012). We analyzed 40 individuals of *L. latinasus* (Table S1), after checking the DNA quality in agarose gels and measuring DNA concentrations with Qubit. Genomic DNA was double-digested with the restriction enzymes SbfI and MspI (Thermo Fisher Scientific), and the obtained fragments were purified with Serapure beads. After fragments were ligated with barcoded Illumina adapters, samples were pooled and size-selected between of 415–515 bp using a Blue Pippin Prep size fractionator (Sage Science). The final library was PCR amplified and ligated with Illumina indices. Libraries were sequenced in a single Illumina NovaSeq 6000 lane in the QB3 lab of the University of California at Berkeley (USA).

We processed raw Illumina reads with the program Stacks 2.5 (Rochette & Catchen, 2017; Rochette et al., 2019). We demultiplexed the samples using their unique barcode and index (i.e., --inline-index) with process_radtags component. Because there is no reference genome available, we implemented the denovo_map.pl program. For the minimum stack depth (-m) parameters and distance between catalog loci (-n), we used the default values, and allowed a maximum of four mismatches (M = 4) between groups of reads. We obtained 3047 SNPs (Matrix A) from 40 individuals, retaining only loci that appeared in 60 percent of them (r60) and allowing a bound of 5% for the minimum allele frequency for each locus (--min-maf = 0.05). We generated a second matrix with 1335 SNPs (Matrix B), after an additional filter to specify a single SNP called from each putatively unlinked locus, in order to exclude strongly linked SNPs within loci from subsequent analyses. Subsequently, we applied for each SNP matrix, alternative filtering steps to remove monomorphic loci, and loci or individuals based on percentage of missing data using the dartR package (Gruber et al., 2018) We used the following criteria: (1) no monomorphic loci, (2) <30% (matrix A) and <38% (matrix B) of missing data per locus, (3) <40% (matrix a) and <45% (matrix b) of missing data per individual (Gruber et al., 2018). Finally, the locus metrics were recalculated for the filtered data using the function gl.recalc.metrics (Gruber et al., 2018).

2.7 | Genetic structure and phylogeny of ddRAD loci

We executed STRUCTURE method (Pritchard et al., 2000) to investigate genetic structure among individuals and localities without a priori assignment to populations using 1,335 RAD loci (matrix B). We configured the run used a constant lambda, an admixture model, and tested a variety of k values (k = 1–5, ten runs for each k) with a burn-in period of 10,000 MCMC generations followed by another 200,000 iterations. The most likely K was determined based on the method of Evanno et al. (2005) via the on-line program Structure Harvester v.0.6.94 (Earl et al., 2012). We assembled the multiple runs for each K in CLUMPP v.1.1.2 (Jakobsson & Rosenberg, 2007) and visualized the clustering and individual assignment results with the bar graph tool in an Excel spreadsheet.

For estimating the phylogenetic relationships among individuals based on genome-wide SNP data, we used the concatenated matrix of 3047 loci (matrix A) utilizing a maximum likelihood (ML) approach in IQ-TREE 1.4.3 (Nguyen et al., 2015) implemented in IQ-TREE web server (Trifinopoulos et al., 2016). We configured the Auto option for selecting best-fit substitution model and assessed support of each node by means of SH-aLRT test (Shimodaira–Hasegawa approximate Likelihood Ratio Test, 1,000 replicates; Guindon et al., 2010). In addition, we inferred a phylogenomic tree implementing a Bayesian approach using BEAST v2.6.2 software (Bouckaert et al., 2019). We used the partition scheme GTR+G+I as substitution model, a Log Normal relaxed clock model and the Birth Death speciation model.

3 | RESULTS

3.1 | Mitochondrial genetic diversity

We amplified a fragment of *cytb* (amplicon length of 900 pb, length of final alignment of 713 pb) for all 139 individuals. Overall, we identified 146 variable sites, of which 26 were singletons, which corresponded to 74 haplotypes. The haplotype network showed a strong genetic structure consisting of five mitochondrial lineages with little geographic overlap (Fig. 1a, 2a). The L1 and L2 lineages contain haplotypes mainly for populations of Rio Grande do Sul in Brazil and Uruguayan populations, with exception of three samples of L1 lineage that occur in

Argentina, two in Entre Ríos, and one in Corrientes Province. The L3 lineage is the most widely distributed, occurring from the south of Argentina in Buenos Aires to its northern limit in Salta Province. The L3 lineage also appears in two localities from Brazil (Quaraí and São Lorenço do Sul in Rio Grande do Sul) and one from Cerro Largo in Uruguay. L4 lineage is restrict to three localities: Doctor Manuel Belgrano, El Carmen, and Ledesma from Jujuy Province in northern Argentina. The L5 lineage is also distributed across northern Argentina (Salta, Santiago del Estero, Chaco and Formosa) and in the Presidente Hayes Department in Paraguay. Genetic diversity levels were low for L1 and L2, and high for L3 and L5. L4 lineage exhibited a high haplotype diversity, showing a haplotype for each sample (Table 1).

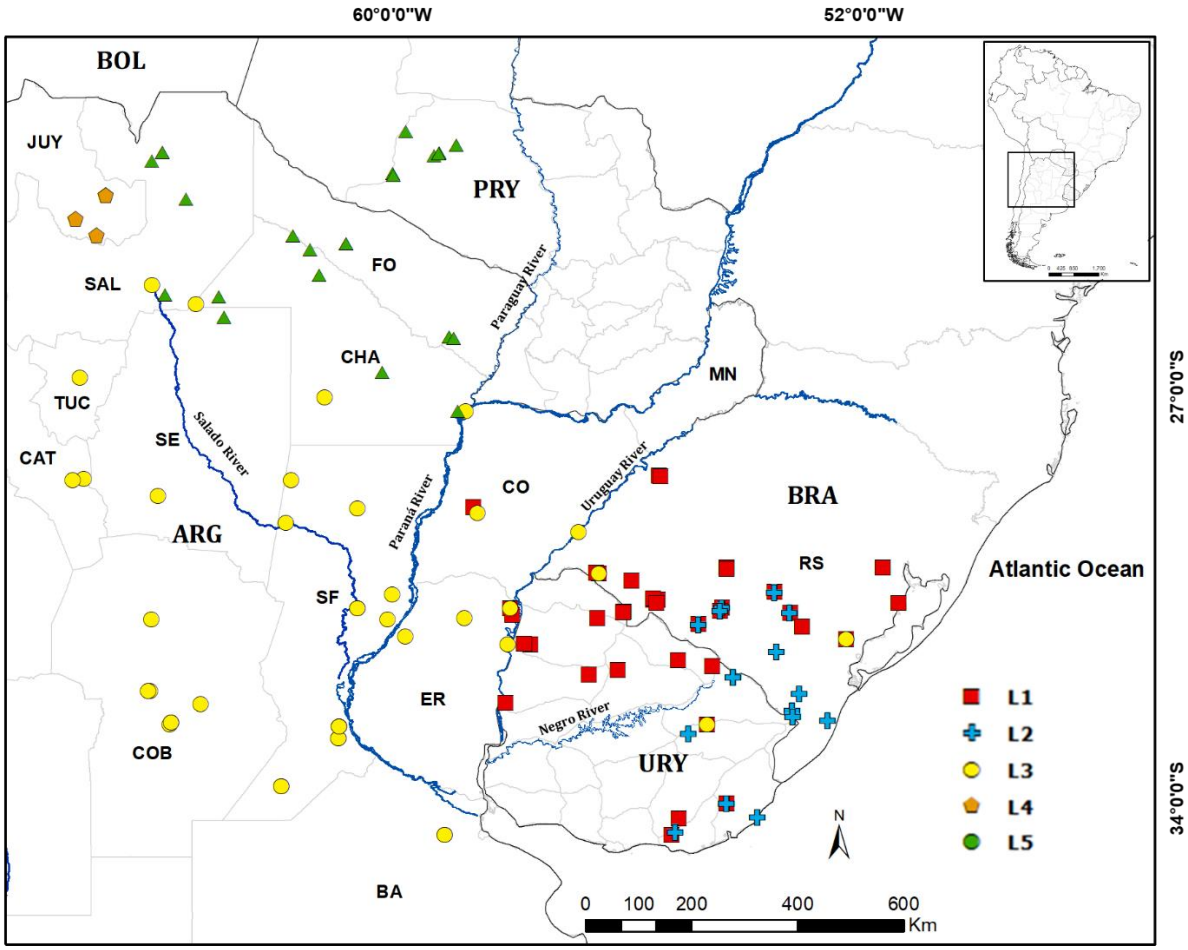

FIGURE 1 Geographic distribution of samples of *Leptodactylus latinasus* used in this study. Distribution of sampling localities with *cytb* data, the colors represent the five mitochondrial lineages. The codes of identification, geographic coordinates and localities are given in Table S1. Localities: BA = Buenos Aires, ER = Entre Ríos, CO = Corrientes, COB = Córdoba, SF = Santa Fe, SE = Santiago del Estero, CAT = Catamarca, TUC = Tucumán, CHA = Chaco, FO = Formosa, SAL = Salta and JUY = Jujuy in Argentina; RS = Rio Grande do Sul in Brazil; Presidente Hayes in Paraguay; URY = Uruguay; BOL = Bolivia.

3.2 | Phylogenetic inferences and divergence times

The Bayesian gene tree estimated with BEAST analysis indicated the same five lineages recovered with haplotype genealogy (Fig. 2a-b). All the nodes showed maximum support (posterior probability, PP = 1.0) sustaining the divergence of the five lineages. The relationships of the outgroup species *L. gracilis* and *L. albilabris* had moderate support (PP = 0.89). The calibrated divergence times show an early diversification around 1.93 Mya [95% of the highest posterior density (HPD) = 0.80–4.84 Mya] between the L5 lineage and the rest of the lineages of *L. latinasus* (Fig. 2b). The remaining cladogenetic events among the *L. latinasus* lineages are more recent, with L1-L4 ancestor diversifying around 1.34 Mya [95% HPD = 0.52–3.32 Mya], and the subsequent events occurred about 0.90 Mya for the ancestors of the L1+L2 [95% HPD = 0.34–2.24 Mya] and of L3+L4 lineages [95% HPD = 0.34–2.23 Mya] (Fig. 2b).

3.3 | Demographical analyses

Neutrality tests presented significant values for L3 and L5 lineages. Recent demographical expansion of L3 lineage was supported by both neutrality statistics, however for L5 lineage only Fu's FS test was significant (Table 1). GMRF Skyride analysis revealed that L3 lineage had an increase in effective population size at around 250 ka (Fig. S1c). On the other hand, Skyride analysis suggested that the L5 lineage could have experimented an increase of its effective population size, but this shift is less evident when compared to the L3 lineage (Fig. S1c-d).

Phylogeographic diffusion reconstruction analysis suggest that the first expansion events started at 350 ka, but the first long-distance colonisations begun around of 250 ka (i.e., for L3 and L5 lineages) (Fig 3d). The majority the long-distance diffusion events for all lineages took place from the LIG until the LGM. In this period, the colonisations were toward northwestern Argentina for the L3 and L5 lineages, and there were dispersal events of northwest at southeast from Uruguay for L1, L2 and L3 lineages (Fig 3a-b). The diffusion analysis indicates that L1 lineage experimented an increase in the diffusion rates around 150 ka, and such a diffusion would have happened recently. Nonetheless, the L2, L3 and L5 lineages raised their diffusion

rates more recently about 50 ka (Fig. 3d). Dynamic visualizations of the phylogeographic diffusion for each lineage can be explored at Appendix S1.

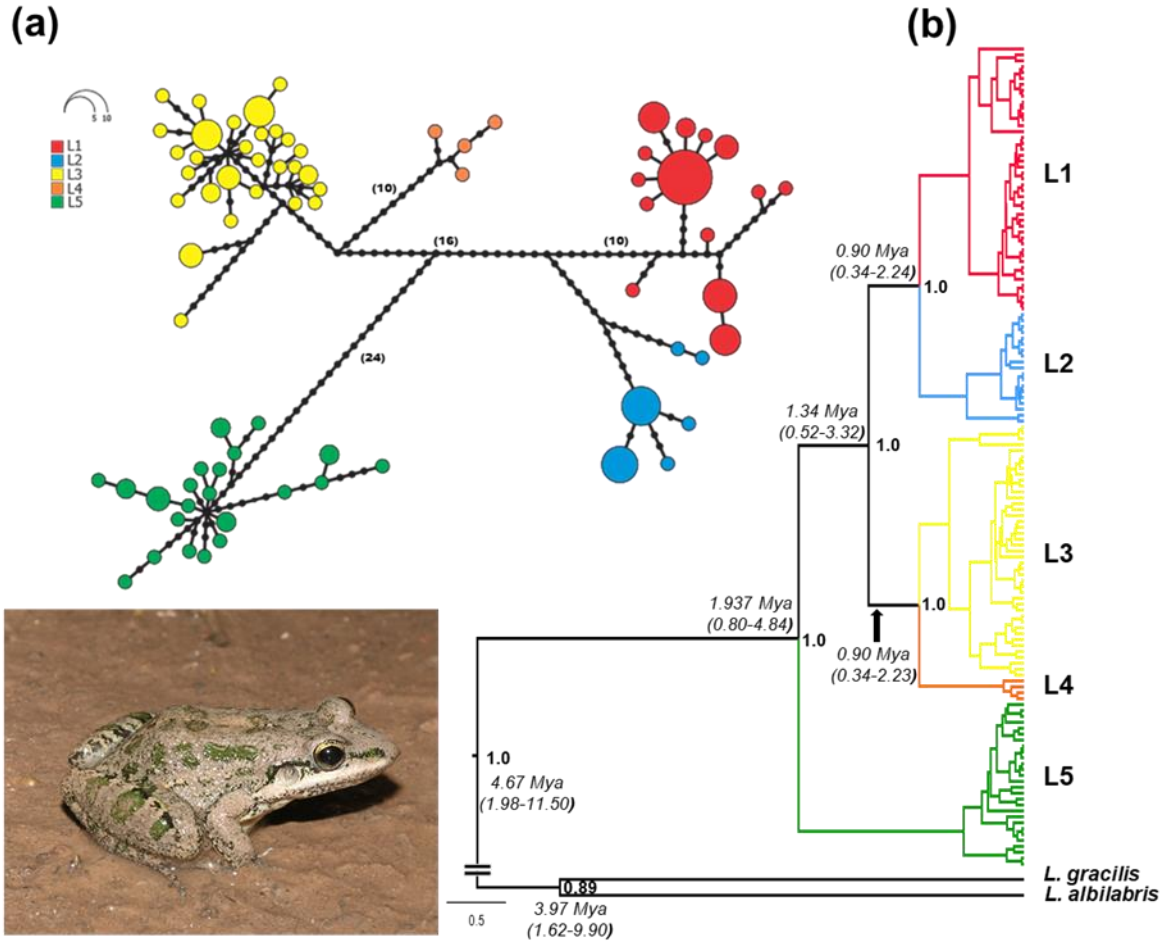

FIGURE 2 Reconstruction of genealogical relationships of *Leptodactylus latinasus* lineages from the *cytb* data set. (a) Haplotype network of *cytb* sequences using a maximum parsimony approach (b) Gene tree of *cytb* using a Bayesian approach. Bold numbers in nodes indicate the posterior probability (PP). Italic numbers indicate the MRCA times in millions of years (Mya) with 95% High Posterior Density (HPD) inside of parenthesis. Outgroup species: *Leptodactylus gracilis* and *L. albilabris.* Foto: *L. latinasus* specimen from Chaco region (Diego Baldo).

3.4 | Ecological niche modelling

Ensemble projections of ENMs under current and past climatic conditions generally fit well with the observed distributions (mean AUC = 0.918 ± 0.052; mean TSS = 0.826 ± 0.096 (Table S3). The L4 lineage was not included due to the scarcity of occurrence data to construct reliable models.

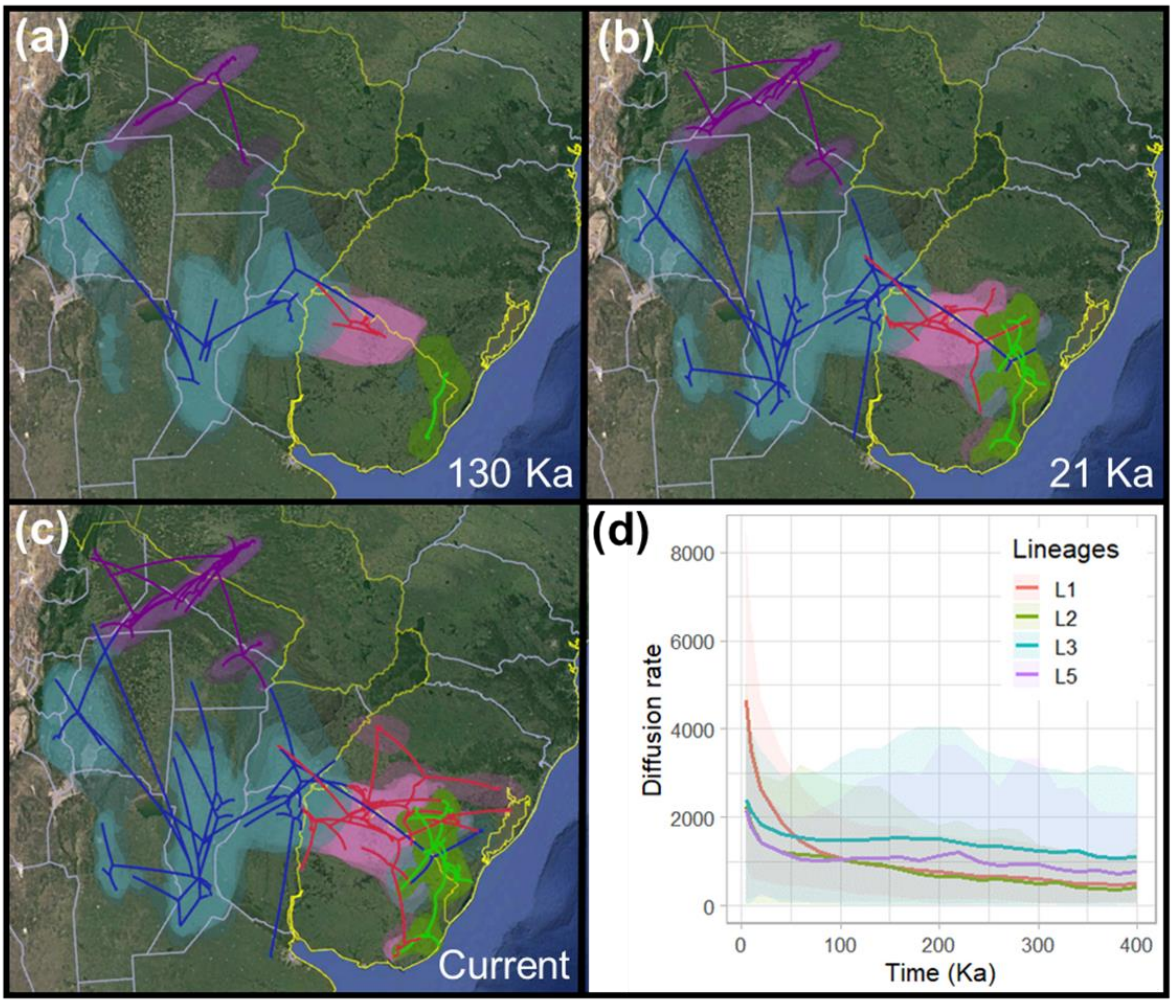

FIGURE 3 Phylogeographic diffusion of *Leptodactylus latinasus* lineages. (a-d) Spatial reconstruction of the diffusion pattern of the L1, L2, L3 and L5 lineages through time: (a) 130 Ka (b) 21 Ka (c) Present time (d) Variation through time in the diffusion rates.

For both L1 and L2 lineages, the precipitation seasonality (BIO 15) was the most important variable based on both correlation and AUC metric (Table S4), while the temperature seasonality (BIO 4) was the most important for the L3 and L5 lineages (Table S4). According to consensus models, the climate suitability of the L1 lineage decreased as the climate became warmer, but remained climatically suitable in the regions such as the southwest of Rio Grande do Sul, Brazil (Fig. 4). Few areas have maintained climatic suitability for the L2 lineage, mainly in Uruguay and the central-southern portion of Rio Grande do Sul, Brazil. Since the middle-Holocene, the L3 lineage has remained in a suitable climate in the north-eastern part of Argentina and the western part of the Paraná River basin where there are predominantly subtropical and tropical climates (Fig. 4). As long as the climate became drier and warmer, the L5 lineage tracked its climate suitability from Corrientes to Formosa Provinces primarily in a subtropical climate (Fig. 4).

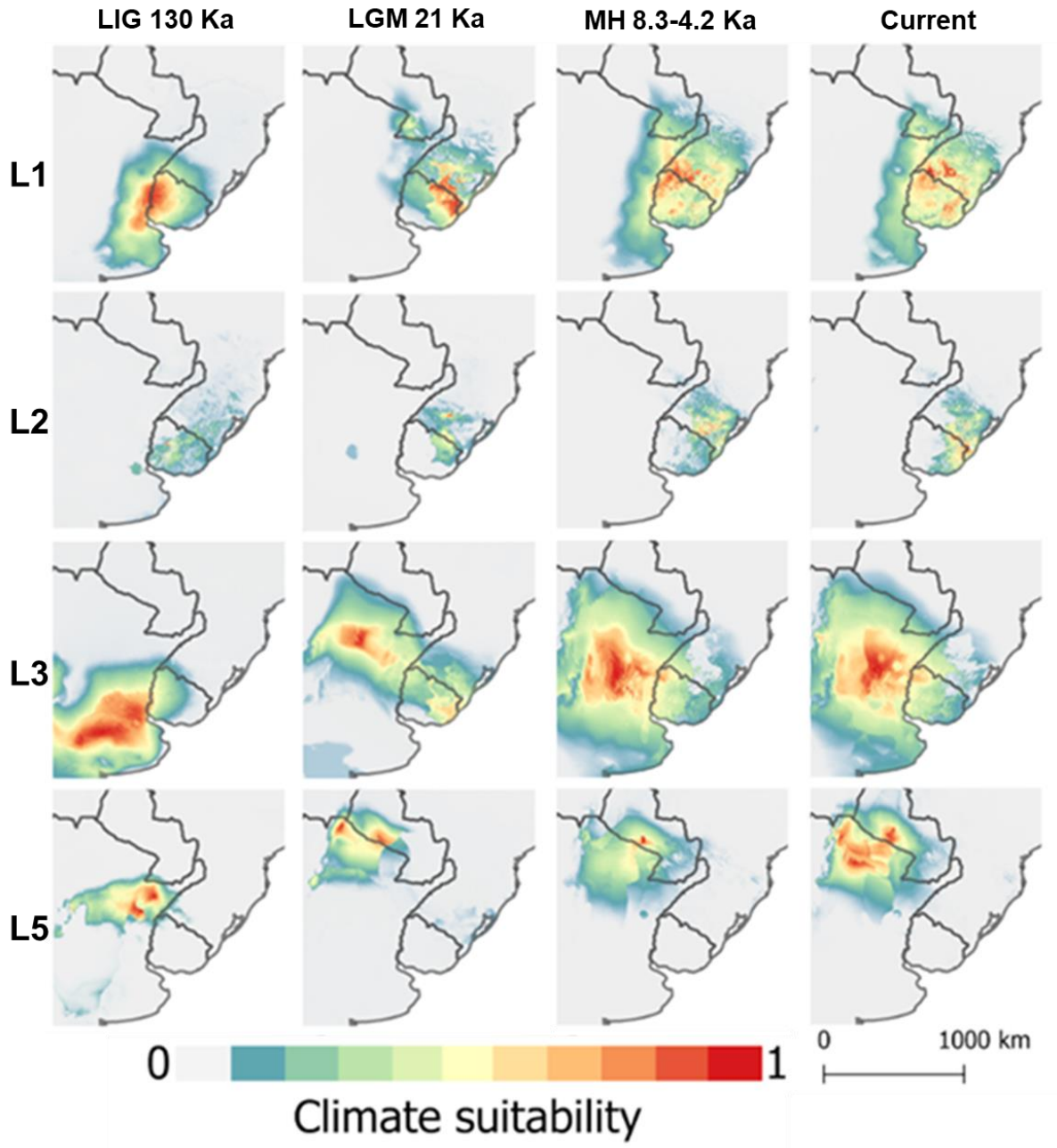

FIGURE 4 Ecological niche models for *Leptodactylus latinasus* lineages (i.e., L1, L2, L3 and L5), representing the relative climatic suitability areas over time. Projections for four epochs, three of Quaternary: Late Inter Glacial (LIG), Late Glacial Maximum (LGM) and Middle Holocene (MH); and the current climate scenario.

3.5 | Genetic structure and phylogenomic trees

According to Evanno's ΔK, the most likely number of clusters obtained in STRUCTURE was K = 2 (Table S5; Fig. S2a-b). There are six samples (i.e., 26-31 samples) from central Argentina that have an assignment probability of about 50% (see Bayesian clustering and maps Fig. 5a-

b). The RAD-based Bayesian tree recovered the same two clusters defined by the STRUCTURE analysis; this topology was sustained by the high nodal support (Fig. S3). Maximum Likelihood analysis also suggested two clusters, showing moderato to high nodal support through the phylogeny (Fig. S3).

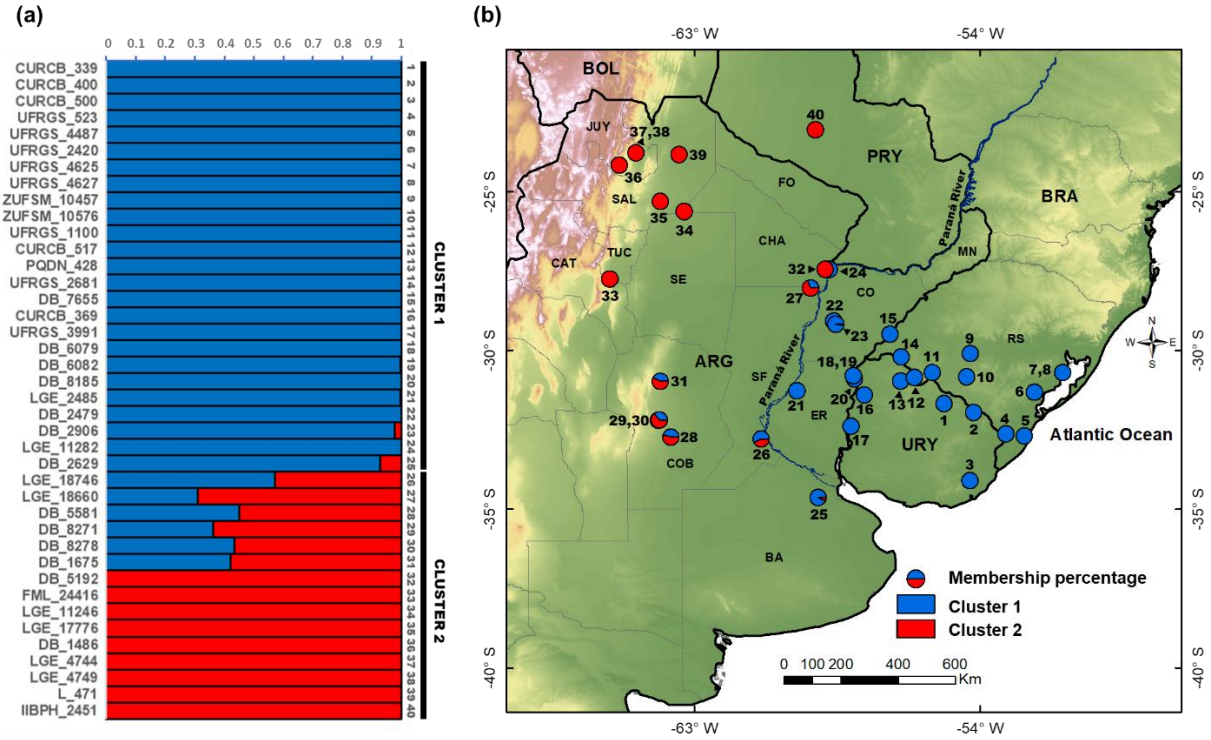

FIGURE 5 Bayesian clustering analysis and distribution map of *Leptodactylus latinasus*, using the genomic dataset with 1335 SNPs (unlinked unique SNPs) for 40 individuals. (a) Bayesian population clustering with STRUCTURE, showing the most likely number of the clusters K = 2. Horizontal bars represent the individuals with the membership probability for each cluster (axis x of 0 to 1.0). (b) Sampling with RADseq data, showing the membership percentage of the individuals for each cluster. The codes of identification, geographic coordinates and localities are given in Table S1. Colours represent to the two identified nuclear clusters, cluster 1 (blue) and cluster 2 (red). Localities: BA = Buenos Aires, ER = Entre Ríos, CO = Corrientes, COB = Córdoba, SF = Santa Fe, SE = Santiago del Estero, CAT = Catamarca, TUC = Tucumán, CHA = Chaco, FO = Formosa, SAL = Salta and JUY = Jujuy in Argentina; RS = Rio Grande do Sul in Brazil; Presidente Hayes in Paraguay; URY = Uruguay; BOL = Bolivia.

4 | DISCUSSION

4.1 | Mitochondrial diversity, diffusion patterns biogeographic and historical demographic

Leptodactylus latinasus is spatially structured in five mitochondrial lineages: the L1 and L2 lineages that occur in the Pampa biome of Brazil and Uruguay, L3 in Argentinian localities of

the Pampa and southern Chaco, L4 occupies habitats in the ecoregion of the Yungas from Jujuy in Argentina, and L5 at north from Chaco in Argentina and Paraguay. The differentiation among lineages is deep and they do not share haplotypes (Fig. 2a-b). The first cladogenetic event happened around 1.93 Mya (in the early Pleistocene), giving origin to the ancestral of L5 lineage and the clade L1-L4, thus, this diversification event likely had place in the Chaco region, as suggested previously by others authors (Cáffaro et al., 2022). The biogeographic region that comprises the Argentinian and Paraguayan Chaco and Argentinian Mesopotamia has experimented several geomorphological and environmental changes that could have driven the *L. latinasus* diversification. In the late Pliocene about 3 Mya during Diaguita phase of Andean orogenic, the neotectonic movements formed the Paraná River paleochannel (Amsler et al., 2020). Posteriorly, around 3-1.7 Mya (Plio-Pleistocene transition) still during Diaguita phase, occurred the elevation of the eastern orographic systems of Argentina, *Pampean Sierras*, and the uplift of Mesopotamian region, which caused changes paleogeographic and environmental in this region (Chernicoff et al., 2002; Ortiz-Jaureguizar & Cladera, 2006). These historical processes would have facilitated the fragmentation of the ancestral population of *L. latinasus* and the subsequent colonization to other areas.

The next cladogenetic event, the diversification of the L1-L2 and L3-L4 matrilineal clades, dating for around 1.34 Mya (Fig. 2b), could to be associated to successive glacial cycles. First, between about 2.18 and 1.43 Ma (early and middle Pleistocene), happened as minimum seven glaciations, which would have modified the landscapes in the Southern South America (Rabassa, 2008; Rutter et al., 2012). Subsequently, the Greatest Patagonian Glaciation (GPG) that dating between 1 and 1.2 Mya (Rabassa et al., 2005), also would have influenced over the environmental and climate of the Patagonia and neighbouring ecoregions (Cosacov et al. 2010). Additionally, the next branching events that gave origin to the remaining *L. latinasus* lineages (i.e., L1, L2, L3 e L4) and dating for about 0.90 Mya (Fig. 2b), are agree with the warm phase comprised between the GPG and the coldest Pleistocene glaciation (CPG, ~0.7 Mya). According to some authors, these glacial periods (GPG and CPG) have had a strong influence on the distribution of the Chaco biome, with stronger effects on the current southern distribution due to the proximity of glaciers and the expansions of Patagonian Steppe to the north (Cosacov et al., 2010; Brusquetti et al., 2019). Brusquetti et al. (2019) in turn proposed that the demographic expansion observed in *Leptodactylus bufonius* would be attributed to the occasioned bioclimatic effects by these glacial periods. Alternately, Rabassa et al. (2005) indicate that the glacial cycles

provoked climatic and environmental changes, which had a great influence on the landscape and Patagonian/Pampean ecosystems development during the last 5 Myr. These evidences indicate that the geomorphologic processes as well as the glacial cycles had a strong impact in the dynamic of Chaco and Pampa regions, therefore both phenomena would have been important in the diversification and populational dynamic of the flora and fauna of region (Moreno et al., 2018; Brusquetti et al., 2019).

Genetic diversity, the great divergence observed between mitochondrial lineages of *L. latinasus* and the discrepancies among different areas –i.e., Uruguayan Savanna vs. Argentinian Pampa and Chaco, would be a hint that they experimented different selective pressures. The haplotype diversity exhibited into genealogical network of L1 and L2 lineages, give insight that this region (i.e., Uruguayan Savanna) was more stable through of LIG and LGM (see haplotype network Fig. 2a; paleomodels of species distribution Fig. 3e). Indeed, populations from this area harbour less genetic variation (i.e., more common haplotypes) than that found in the Argentinian Pampa and Chaco. On the other hand, the L3, L4 and L5 lineages show a major genetic variation (i.e., many unique haplotypes and few common haplotypes), these signatures would indicate that they were subject to an environment more dynamic with frequent climate oscillations. In the Chaco region of north-central Argentina, Paraguay, and south-eastern Bolivia, climates and habitats were similar to those of the Pampean region during all the Pleistocene, the climates were arid or semiarid and cold with an increase of the mean annual temperature from south to north (Carlini et al., 2004; and references therein), while that in the Mesopotamia, Uruguay and southern Brasil during the entire Pleistocene, climates were wetter and warmer (Carlini et al., 2004). According to Ortiz-Jaureguizar & Cladera (2006) during Early Paleocene to Late Pleistocene in southern South America (latitudes below 15°S), the overall trend of environmental change was climatic conditions became colder, dryer and more seasonal; nevertheless, this cold and dry climate was intermittent during the Quaternary as a consequence of glacial cycles. This cyclic pattern of changes produced the provincialism that characterized the South American biota from Early Pleistocene to the present day.

On the other hand, the relatively high haplotype diversity of L3, L4 and L5 lineages could result of long-term persistence, which would have allowed it maintain diversity over time. Long-term persistence was saw in different scenarios: allopatric divergence followed by secondary contact and subsequent populational expansion (e.g., Senczuk et al., 2017); isolation in a peripheral

area of the core population (Cosacov et al., 2010). The diversity of L3 and L5 lineages can have been keep in long persistence by means of stepping stones populations, that underwent with range shifts in reply to climate changes previous to LGM. Otherwise, the L4 lineage that only occur in three localities from northwest of Argentina, could have persisted in a refugium in this peripheral area (Fig. 1a). Regions that retain stable environmental conditions can facilitate the long-term persistence of populations and act as refugium (Kiedrzyński et al., 2017), some authors also observed divergent samples for frogs of the same region (Camargo et al., 2006).

Recent population expansion was inferred for the L3 lineage, starting around 250 Ka in the middle Pleistocene (Fig. S1c). The L5 lineage could also have experimented populational expansion because of the pattern of low nucleotide diversity and high haplotype diversity (as with L3 lineage) which suggest recent population expansion despite the not significant Tajima's D test (Brusquetti et al., 2019; Miranda et al., 2019). However, this pattern of numerous unique haplotypes could be associated with deleterious mutants being maintained at low frequencies (e.g., selective sweeps and background selection), both them can produce patterns of haplotype diversity similar to those produced by population expansion (Wlasiuk et al., 2003).

The diffusion reconstructions indicate that had an increase in the diffusion rates at begin from of LIG period, and until the LGM occurred the majority of the long-distance colonisations (Fig. 3a-b). These dispersive events produced of south to north in Argentina (i.e., L3 and L5 lineages) and west to east in the Brazil and Uruguay frontier (L1, L2 and L3, Fig. 3 a-b). There are two samples (i.e., from Buenos Aires) that do not follow the general pattern and would have experimented long-distance dispersal toward southern (Fig. 3a-c). This colonization event may have taken place by passive dispersion via Hydrochory, a common transportation mechanism in larval and adult of frogs (Langone et al., 2016; Pabijan et al., 2015; and references there), which could have allowed such long-distance colonization event (Fig. 3a-c). Diffusion results are in according with the predictions of paleomodels, the suitability areas of L3 and L5 lineages during the LIG and LGM (Fig. 3e), coincide with the dispersion observed in the phylogeographic diffusion (Fig. 3a-b). Further, the displacements of L1 and L2 lineages also are correspond with the changes of suitability areas across the LIG and LGM (Fig. 3a-b).

4.2 | Mito-nuclear discordance

In the last years, the use of large genomic datasets has become much more commonplace and in consequence, the incongruent evolutionary histories were most frequently identified in diverse taxa, bird (Zhang et al., 2019), spiders (Graham et al., 2020), snakes (Marshall et al., 2021) and frogs (Cairns et al., 2021; Firneno et al., 2021). Discordance between mitochondrial and nuclear data is a pattern frequently found in phylogeographic studies, and such a signal conflict remains as the result of the underlying processes of lineage divergence between (e.g., Zhang et al., 2019) or within species (e.g., Cairns et al., 2021). We observed discordance between mtDNA and nuclear dataset of *L. latinasus*: while we recovered five deep mitochondrial lineages (i.e., see gene tree and haplotype network), the genomic data suggest two clusters as the best partition for the data (i.e., see STRUCTURE and concatenate nuclear tree). The L3 mtDNA lineage was split in the nuclear dataset, as on the clustering analysis as the phylogeny, with their individuals being assigned to both nuclear clusters, while all the samples of the L1 (i.e., 1, 3, 6-9, 11-13, 16-18, 20 and 22 samples) and L2 (i.e., 2, 4-5 and 10) lineages were classified for the C1 cluster, and the individuals of L4 (i.e., 36-38) and L5 (i.e., 32, 39-40 samples) for C2 (Fig. 5a-b, S3). The L3 individuals of C1 cluster (i.e., 14-15, 19, 21, 23-25 samples) showed assignment probability greater than 0.9, whilst the remaining L3 samples (i.e., 26-31 samples) designated to C2, exhibited different degrees of admixture with the C1 cluster (Fig. 5a-b, S3).

Mito-nuclear discordance can arise by diverse factors including introgression, gene flow, incomplete lineage sorting (ILS), and sex-biased dispersal (Toews & Brelsford, 2012; Zhang et al., 2019), or by local adaptation of distinct mitochondrial DNA (mtDNA) lineages (e.g., Pavlova et al., 2013). The distinction between ILS and introgression/gene flow can be very difficult; nonetheless, it has been noted that discordance arising from ILS is not expected to leave any predictable biogeographic pattern between mitochondrial and nuclear markers (Funk & Omland, 2003; Toews & Brelsford, 2012). Herein, we observed that there is an evident biogeographic pattern that could explain the observed discordance scenario in *L. latinasus*. The Paraná River could have functioned as biogeographic barrier or corridor, allowing the isolation and secondary contact in different periods, however, the environmental possibly would have had a pivotal role in the maintenance of observed genetic diversity. Alternately, the gradient of habitats in buffers areas (i.e., Espinal, Humid Chaco and Humid Pampa ecoregions) between drier (i.e., Dry Chaco and Yungas ecoregions) and moister climate regions (i.e., Uruguayan Savanna), could to ease the gene flow between nuclear clusters, otherwise, the more extreme

regions would allow for the groups to progress beyond in the speciation continuum (de Queiroz, 2007; Edwards et al., 2016). A similar pattern with deep mitochondrial differentiation and signatures of apparent nDNA gene flow, was observed in the Spring Peeper frog (Cairns et al., 2021). Cairns et al. (2021), in turn, pointed out that the origin and maintenance of the genetic diversity of Spring Peeper frog, would be associated to riverine barriers (i.e., Mississippi River) and environmental gradients (i.e., aridity), from which the isolation and secondary contact scenarios were generated.

The Paraná River was suggested as a barrier to gene flow that could have driven divergence and speciation in birds (Kopuchian et al., 2020; and references here), mammals (Carlini et al., 2004), and frogs and reptiles (Gallardo, 1979). On the other hand, during the Pleistocene the paleochannels of the Paraná River would have experimented a migratory trend from south to north, successively capturing different segments of the Paraguay River (Orfeo, 2005). Alternately, some authors suggested that in the middle Pleistocene (~780 to 130 Ka) the Paraná River migrated its channel toward the Uruguay River by means of the paleochannel of the Agapey and Miriñay Rivers in the Mesopotamian region (Popolizio, 2006; Amsler et al., 2020). Alternately, it was suggested that the Paraná and Uruguay Rivers may have acted as important biogeographical corridors animals and plants (Arzamendia & Giraudo, 2009; Moreno et al., 2018; Nores et al., 2005). Because passive dispersion via hydrochory is a common transportation mechanism in larval and adult frogs (Langone et al., 2016; Pabijan et al., 2015; and references there), this process could account for the distribution and genetic composition of the sample from Buenos Aires, that have a 95% of probability of belonging to the nuclear C1 cluster (i.e., 25 sample, Fig. 5a-b). These events could explain the mito-nuclear discordance, while the mitochondrial lineages show a deep divergence without haplotype sharing, the nuclear clusters exhibit a convoluted history of gene flow, which could have been possible by the migration of channels of rivers or by the passive transportation across them.

4.3 | Conclusions and future directions

Our results suggest that early Pleistocene orogenic events might have played a prominent role in the early diversification of *L. latinasus*. Posteriorly, the successive periods of climatic shifts (i.e., GPG, CPG, LIG and LGM) would have been important drivers in the colonization, frag-

mentation, isolation and secondary contact of lineages. In this way, for explain the diversification and current biogeographic pattern of *L. latinasus*, we propose a divergency model with long-time isolation and subsequent secondary contact resultant of recent range expansion (Zhang et al., 2008; Boria et al., 2020). Sensitivity to environmental changes makes vicariance a common explanation for phylogeographic patterns in anurans as even small shifts in climate, vegetation or more strong barriers as rivers can present impediments to gene flow and promote genetic isolations (Vences & Wake 2007).

Additionally, the long-term persistence could have been favoured by the tendency toward a terrestrial mode of life of *L. latinasus*, characterized by deposition eggs in foam nests in self-constructed subterranean chambers on land (Heyer, 1969). This reproductive mode was proposed as crucial for the persistence and dispersion of *L. bufonius*, endemic frog from Gran Chaco (Brusquetti et al., 2019). After long-term isolation (see Fig. 4a-e), more recently around of the LIG, the events of range expansion of L1 and L3 lineages (see Fig. 3a-b) would have put them in secondary contact, allowing their gene exchange (see Fig. 5a). The migration and gene flow across of Paraná and/or Uruguay Rivers could have been able by the migration the paleo channels (Orfeo, 2005; Asler et al., 2020) or by passive dispersion via Hydrochory (Arzamendia & Giraudo, 2009; Langone et al., 2016). Future studies that use coalescent simulation-based approaches as IMa3 could provide insight on whether there is the genetic exchange among the *L. latinasus* clusters. The modelling of different scenarios with and without gene flow might shed light for understand the biogeographic pattern observed in this frog and maybe will allows to test hypothesis on the role of the Paraná and Uruguay Rivers as important biogeographic barriers.

Table 1 Mitochondrial genetic diversity indices and neutrality tests for *Leptodactylus latinasus* lineages. N = number of sequences; h = number of haplotypes; Hd = haplotype diversity; π = nucleotide diversity; Fu's FS and Tajima's D neutrality tests. SD = standard deviation. *P < 0.05, **P < 0.01.

Lineages	N	h	Hd ± SD	π ± SD	Fu's FS	Tajima's D
L1	45	14	0.839 ± 0.042	0.008 ± 0.0009	−0.3807	−0.7499
L2	19	6	0.713 ± 0.073	0.005 ± 0.0019	1.7095	−1.1579
L3	43	29	0.969 ± 0.014	0.011 ± 0.0011	−12.784 **	−1.464 *
L4	4	4	1.000 ± 0.177	0.005 ± 0.0013	−	−
L5	28	21	0.976 ± 0.016	0.009 ± 0.0011	−8.888 **	−1.273
All	139	74	0.974 ± 0.006	0.042 ± 0.0013	−	−

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BIOSKETCH

Matías Maximiliano Malleret is broadly interested in patterns and processes of diversification of Neotropical anuran species. This study is part of his PhD work at Universidade Federal de Rio Grande do Sul, in Brazil, on the biogeography, phylogeography, species boundaries and evolutionary history of Chaco and Pampa anurans.

Author Contributions: MMM and AC conceived and designed the study. MMM, DB, FB and AC carried out fieldwork. MMM and AC performed the experiments. LV and AC contributed with reagents and material tools. MMM, PL and AC analysed the data. MMM wrote the paper and all authors contributed on the final draft.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Appendix S1 KML file viewable in Google Earth (http://google.com/earth/) showing a dynamic phylogeographic visualization of the diffusion for each lineage

CONCLUSÕES

Nesta tese se investiga que processos históricos influenciaram na diversidade, dinâmica populacional e divergência de linhagens de duas espécies de anuros, amplamente distribuidos em biomas do sul de América do Sul. Utilizamos os táxons *Scinax granulatus* e *Leptodactylus latinasus* para compreender os padrões de diversidade e história evolutiva idiossincráticos de cada espécie e como os mudanças climáticas e paleogeográficos teriam afetado cada taxón.

No primeiro capítulo, com base em métodos de delimitação de espécies e uma abordagem de taxonomia integrativa identifico a linhagem divergente *Scinax* sp. D dentro do complexo *S. granulatus*, e sugiro que esta espécie candidata deveria ser descrita. Discuto o papel que teriam jogado os eventos neotectônicos, mudanças climáticas e ambientais na divergência de linhagens, e sobre a diferenciação de nicho ecológico exibida entre *S. granulatus stricto sensu* e *S.* sp. D. Discuto sobre a importância do uso de dados genéticos, quando analisamos espécies que exibem elevado conservadorismo morfológico, e da utilização da taxonomia integrativa para compreender a diversidade destes táxons taxonomicamente confusos. Ressalto a necessidade de aumentar a amostragem de indivíduos, incluir dados mofológicos de adultos e girinos e de cantos de anúncios para tomar decisões taxonômicas e descrever esta espécie. Também, indico que são necessarios amostragens de especimens provénientes de áreas de ecotono de ambientes de Pampa e Mata Atlântica, priorizando potenciais áreas de sobreposição de linhagens, considerando que poderiam representar zonas hibridas. Além, sugiro a utilização de dados de SNP de genoma amplo para modelar e detectar fluxo gênico ou hibridização entre especies do complexo *Scinax granulatus.*

No segundo capítulo, analiso a diversidade, estrutura genética, demografia histórica, e deduzo que as prováveis vias de colonização de linhagens de *Leptodactylus latinasus*. Além disso, faço projeções das distribuições potenciais de populações ancestrais durante diferentes períodos do Quaternário. de *L. latinasus*, teria se originado no Gran Chaco. O ancestral comum mais recente diversificou em cinco linhagens mitocondriais (L1-L5), os quais teriam permanecido isolados por um longos períodos. Recentemente, aproximadamnete durante o último interglacial, as linhagens L1 e L3 como resultado de pulsos de difusão teriam ficado em contacto secundário, o qual teria possibilitado o intercâmbio gênico entre estas linhagens. Remarco o papel das mudanças climática paleoambientais na fragmentação e diversificação de linhagens e os efeitos

destes fenômenos na persisténcia e isolamento de linhagens durante longos períodos. Além disso, destaco o rol que poderiam ter desempenhado os rios Paraná e Uruguai como barreiras ou corredores biogeográficos influenciando na dinâmica populacional e fluxo gênico. Por fim, ressalto a necessidade de incrementar a amostragem de indivíduos, particularmente de amostras do noroeste de Argentina para conseguir compreender a dinâmica populacional e inferir modelos de ditribuição de populacões ancestrais e atuais da linhagem L4 de *L. latinasus.*

Conhecer a diversidade, estrutura populacional e identificar os limites entre espécies é fundamental para compreender a verdadeira diversidade biológica. A identificação e distinção entre variação intraespecífica e interespecífica também é essencial para entender a real diversidade, devido a que espécies amplamente distribuídas podem ter elevada variação intraespecífica com populações estruturadas (Barraso, 2014; Felappi et al., 2015; Fernández, 2016; Langone et al., 2016; Elgue, 2018; Alves da Silva, 2019; este trabalho) ou não exibir estrutura aparente (Alves da Silva, 2019; Brusquetti et al., 2019; Miranda et al., 2019). Não entanto, distinguir a divergência do nível populacional e específico é essencial para não confundir divergência do nível de populações e espécies, e distinguir entre táxons amplamente distribuídas (p. ex., Brusquetti et al., 2014; Miranda et al., 2019) de complexos de espécies (Baldo et al., 2019; Schneider et al., 2019; Villamil et al., 2019; este trabalho). Por isso, é fundamental que ao investigar a diversidade biológica se utilizem abordagens multidisciplinares, usando múltiplas linhas de evidencia para poder testar e propor hipóteses filogenéticas ou taxonômicas. A diversidade biológica é multimodal e a evolução de distintos caracteres acontece a diferentes taxas, além que a evolução de muitos deles pode estar restringida por seleção ou adaptação. Alternativamente, o uso de modelos de distribuição de espécies pode indicar quais fatores climáticos e/ou ecológicos podem estar associados à divergência de linhagens ou com processos históricos que definiram os padrões atuais de diversidade e distribuição de populações ou espécies (Alvarado-Serrano & Knowles, 2014; Cabanne et al., 2019; Camurugi et al., 2021).

Os eventos históricos geomorfológicos e incursões marinhas do Neogeno teria modificado o ambiente e o clima, dando forma ao provincialismo caracteristico da biota de América do Sul, estes processos também têm sido indicados como promotores da diversificaçao de alguns grupos de vertebrados (Morando et al., 2014; Brusquetti et al., 2018). Adicionalmente, os sucessivos ciclos de climas frios e quentes durante os ciclos glaciais (GPG e LGM) e interglaciais (LIG) também teriam jogado um papel importante na reorganização

paleogeográfica de diferentes biomas (Villagrán & Hinojosa, 1997; Rabassa et al., 2005), com oscilações climáticas promovindo a diversificação e afetando a dinâmica populacional de diferentes espécies de plantas (Cosacov et al., 2010; Turchetto et al., 2014; Moreno et al., 2018) e animais (Camargo et al., 2013; Langone et al., 2016; Brusquetti et al., 2019; este trabalho). Esta tese confirma o já sugerido por vários autores, sustentando que a diversificação e história evolutiva da biota neotropical é o resultado de eventos históricos do Neogeno-Quaternário, e que cada espécie teria respondido diferencialmente baseado em seus requerimentos ecológicos e restrições espécie específicas. Finalmente, também cabe destacar a importância do uso de anfíbios como modelos para estudos de filogeografia e delimitação de espécies, e a relevância deles para estudar os efeitos dos processos históricos na fauna de diferentes biomas e ecorregiões de América do Sul.

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APÊNDICES

CAPÍTULO I

Supporting Information

Phylogeography and species delimitation of the Neotropical frog complex (Hylidae: Scinax granulatus)

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Table S1. Locality specifications, geographical records, voucher specimens of *Scinax granulatus*, *S. sp.* D, *S. fuscovarius*, *S. perereca* and *S. nasicus*. The GenBank accession numbers of the amplified sequences for *cytb* and *RAG1* genes and samples utilized in each dataset.

Specie	Locality/State or Province/Country	Latitude	Longitude	DNA Voucher	GenBank accession numbers		Dataset A		Daset B
					cytb	*RAG1*	*cytb*	*RAG1*	cytb/RAG1
S. granulatus	Bañados Medina/CL/URY	-32.356293	-54.442166	CURCB 423	MZ579601	MZ645853	x	x	x
S. granulatus	Cañas/TA/URY	-31.304409	-56.058552	PQDN 0078	MZ579584		x		
S. granulatus	Toledo Chico/MO/URY	-34.778375	-56.141388	CURCB 510	MZ579567	MZ645841	x	x	x
S. granulatus	Valizas/RO/URY	-34.333846	-53.795532	CURCB 534	MZ579563	MZ645837	x	x	x
S. granulatus	Villa Soriano/SO/URY	-33.32873	-58.11302	CURCB 588	MZ579565	MZ645839	x	x	x
S. granulatus	Carmelo/CO/URY	-33.98110	-58.28853	CURCB 593	MZ579566	MZ645840	x	x	x
S. granulatus	Carmelo/CO/URY	-33.98110	-58.28853	CURCB 594	MZ579582		x		
S. granulatus	Arachania/RO/URY	-34.612837	-54.145249	CURCB 549	MZ579564	MZ645838	x	x	x
S. granulatus	Mariscala/LA/URY	-33.98160	-54.83601	CURCB 615	MZ579596		x		
S. granulatus	Rivera/RV/URY	-30.927880	-55.509550	CURCB 617	MZ579597		x		
S. granulatus	Rivera/RV/URY	-30.927880	-55.509550	CURCB 618	MZ579598		x		
S. granulatus	Arvoredo/SC/BRA	-27.051812	-52.467974	UFRGS 4274	MZ579627		x		
S. granulatus	Arvoredo/SC/BRA	-27.051812	-52.467974	UFRGS 4275	MZ579621	MZ645861	x	x	x
S. granulatus	San Pedro/MN/ARG	-27.145278	-53.912500	LGE 01455	MZ579620	MZ645860	x	x	x
S. granulatus	La Plata/BA/ARG	-34.983333	-57.85	DB 5279	MZ579568	MZ645842	x	x	x
S. granulatus	Sertão/RS/BRA	-28.042628	-52.216267	CAUPF 2304	MZ579581		x		
S. granulatus	Sertão/RS/BRA	-28.042628	-52.216267	CAUPF 2261	MZ579562	MZ645836	x	x	x
S. granulatus	Sertão/RS/BRA	-28.042628	-52.216267	CAUPF 2259	MZ579580		x		
S. granulatus	Cerro Largo/RS/BRA	†-28.147841	-54.739997	UFRGS 3381	MZ579589		x		
S. granulatus	Cerro Largo/RS/BRA	†-28.147841	-54.739997	UFRGS 4157	MZ579572	MZ645846	x	x	x
S. granulatus	Cerro Largo/RS/BRA	†-28.147841	-54.739997	UFRGS 4158	MZ579592		x		
S. granulatus	Pedras Altas/RS/BRA	-31.647729	-53.527802	UFRGS 4837	MZ579614		x		
S. granulatus	Pinheiro Machado/RS/BRA	-31.510538	-53.458675	UFRGS 5194	MZ579604	MZ645856	x	x	x
S. granulatus	Pinheiro Machado/RS/BRA	-31.510538	-53.458675	UFRGS 5195	MZ579615		x		

S. granulatus	São Lourenço do Sul/RS/BRA	-31.305083	-52.289972	UFRGS 5348	MZ579605	MZ645857	x	x	x
S. granulatus	Canguçu/RS/BRA	-31.350000	-52.663694	UFRGS 5385	MZ579606	MZ645858	x	x	x
S. granulatus	Santana de Boa Vista/RS/BRA	-30.854669	-53.089809	UFRGS 5417	MZ579616		x		
S. granulatus	São Jerônimo/RS/BRA	†-29.959439	-51.728178	UFRGS 5668	MZ579573	MZ645847	x	x	x
S. granulatus	Eldorado do Sul/RS/BRA	-30.091603	-51.672062	UFRGS 6691	MZ579594		x		
S. granulatus	Eldorado do Sul/RS/BRA	-30.091603	-51.672062	UFRGS 6692	MZ579617		x		
S. granulatus	Eldorado do Sul/RS/BRA	-30.091603	-51.672062	UFRGST 5058	MZ579576	MZ645850	x	x	x
S. granulatus	Pedras Altas/RS/BRA	†-31.733637	-53.587170	UFRGS 6784	MZ579613		x		
S. granulatus	Pinheiro Machado/RS/BRA	†-31.580593	-53.384620	UFRGS 6796	MZ579618		x		
S. granulatus	Viamão/RS/BRA	-30.091946	-50.842821	UFRGS 6935	MZ579574	MZ645848	x	x	x
S. granulatus	Santana de Livramento/RS/BRA	-30.902314	-55.300343	UFRGST 4672	MZ579595		x		
S. granulatus	Santana Maria/RS/BRA	-29.648583	-53.916883	UFRGS 7448	MZ579575	MZ645849	x	x	x
S. granulatus	São Francisco de Paula/RS/BRA	-29.377527	-50.389096	UFRGS 7463	MZ579577	MZ645851	x	x	x
S. granulatus	Bom Jesus/RS/BRA	-28.276519	-50.728165	UFRGS 4124	MZ579603	MZ645855	x	x	x
S. granulatus	Bom Jesus/RS/BRA	-28.276393	-50.727920	UFRGS 4125	MZ579590		x		
S. granulatus	Bom Jesus/RS/BRA	-28.276625	-50.726951	UFRGS 4126	MZ579591		x		
S. granulatus	Campo Belo do Sul/SC/BRA	†-27.896657	-50.761994	UFRGS 4063	MZ579571	MZ645845	x	x	x
S. granulatus	Anita Garibaldi/SC/BRA	-27.771999	-51.140294	UFRGS 4021	MZ579622	MZ645862	x	x	x
S. sp. D	Imbituba/SC/BRA	†-28.239057	-48.655293	UFRGS 4130	MZ579633	MZ645869	x	x	x
S. sp. D	Imbituba/SC/BRA	†-28.239057	-48.655293	UFRGS 4131	MZ579634	MZ645870	x	x	x
S. granulatus	Nonoai/RS/BRA	-27.363875	-52.724523	UFRGS 4346	MZ579593		x		
S. sp. D	Araranguá/SC/BRA	-28.943569	-49.369222	UFRGS 7438	MZ579636	MZ645872	x	x	x
S. sp. D	Arroio do Silva/SC/BRA	-29.021772	-49.484261	UFMG 21051	MZ579629	MZ645865	x	x	x
S. sp. D	Arroio do Silva/SC/BRA	-29.021772	-49.484261	UFMG 21052	MZ579639		x		
S. granulatus	Três Barras/SC/BRA	-26.311801	-50.267636	UFMG 21075	MZ579619	MZ645859	x	x	x
S. granulatus	Três Barras/SC/BRA	-26.311801	-50.267636	UFMG 21076	MZ579625		x		
S. granulatus	Três Barras/SC/BRA	-26.311801	-50.267636	UFMG 21077	MZ579626		x		
S. granulatus	Porto Alegre/RS/BRA	-30.052667	-51.176119	MMM 026	MZ579599		x		

S. granulatus	Porto Alegre/RS/BRA	-30.052667	-51.176119	MMM 027	MZ579600		x		
S. sp. D	Capão da Canoa/RS/BRA	-29.652177	-50.000047	MMM 029	MZ579644		x		
S. sp. D	Capão da Canoa/RS/BRA	-29.652177	-50.000047	MMM 031	MZ579645		x		
S. sp. D	Arroio do Silva/SC/BRA	-29.018035	-49.507438	MMM 076	MZ579631	MZ645867	x	x	x
S. sp. D	Arroio do Silva/SC/BRA	-29.018035	-49.507438	MMM 077	MZ579632	MZ645868	x	x	x
S. sp. D	Balneário Gaivota/SC/BRA	-29.163038	-49.62189	BG 026	MZ579643		x		
S. sp. D	Itati/RS/BRA	-29.511675	-50.109397	DP 140	MZ579635	MZ645871	x	x	x
S. sp. D	Torres/RS/BR	-29.346905	-49.76544	DP 057	MZ579630	MZ645866	x	x	x
S. granulatus	Lavras do Sul/RS/BRA	-30.80739	-54.413202	ZUFSM 10578	MZ579602	MZ645854	x	x	x
S. granulatus	Lavras do Sul/RS/BRA	-30.80739	-54.413202	ZUFSM 10579	MZ579609		x		
S. granulatus	Lavras do Sul/RS/BRA	-30.80739	-54.413202	ZUFSM 10580	MZ579583		x		
S. granulatus	Jaguarão/RS/BRA	-32.247485	-53.410489	ZUFSM 10592	MZ579607		x		
S. granulatus	Jaguarão/RS/BRA	-32.247485	-53.410489	ZUFSM 10593	MZ579608		x		
S. granulatus	Soledade/RS/BRA	-28.883418	-52.470743	ZUFSM 10646	MZ579586		x		
S. granulatus	Soledade/RS/BRA	-28.883418	-52.470743	ZUFSM 10647	MZ579570	MZ645844	x	x	x
S. granulatus	Santana de Boa Vista/RS/BRA	-30.862414	-53.234347	ZUFSM 10494	MZ579610		x		
S. granulatus	Santana de Boa Vista/RS/BRA	-30.862414	-53.234347	ZUFSM 10502	MZ579585		x		
S. granulatus	São Gabriel/RS/BRA	-30.096886	-54.330487	ZUFSM 10442	MZ579611		x		
S. granulatus	São Gabriel/RS/BRA	-30.102814	-54.312478	ZUFSM 10480	MZ579569	MZ645843	x	x	x
S. granulatus	São Sepé/RS/BRA	-30.144151	-53.584747	[#] ZUFSM-TSP002	MZ579587		x		
S. granulatus	São Sepé/RS/BRA	-30.144151	-53.584747	[#] ZUFSM-TSP003	MZ579588		x		
S. granulatus	São Sepé/RS/BRA	-30.144151	-53.584747	[#] ZUFSM-TSP004	MZ579612		x		
S. granulatus	Alegrete/RS/BRA	-29.750164	-55.379213	ZUFSM 10325	MZ579561	MZ645835	x	x	x
S. granulatus	Alegrete/RS/BRA	-29.750769	-55.360658	ZUFSM 11646	MZ579579		x		
S. sp. D	Treviso/SC/BRA	†-28.518485	-49.455933	CFBH 9849	MZ579642		x		
S. granulatus	Campos Novos/SC/BRA	†-27.399053	-51.223673	CFBH 13630	MZ579623	MZ645863	x	x	x
S. granulatus	General Carneiro/PR/BRA	†-26.425803	-51.316859	CFBHT 11177	MZ579624	MZ645864	x	x	x
S. granulatus	Cambará do Sul/RS/BRA	-29.158800	-50.080336	CFBH 30367	MZ579578	MZ645852	x	x	x

S. granulatus	Francisco Beltrão/PR/BRA	†-26.078450	-53.056136	CFBH 40193	MZ579628		x		
S. sp. D	Florianopolis/SC/BRA	†-27.653681	-48.477128	CFBH 40194	MZ579640		x		
S. sp. D	Florianopolis/SC/BRA	†-27.653681	-48.477128	CFBH 40195	MZ579638	MZ645874	x	x	x
S. sp. D	Florianopolis/SC/BRA	†-27.653681	-48.477128	CFBH 40196	MZ579637	MZ645873	x	x	x
S. sp. D	Florianopolis/SC/BRA	†-27.653681	-48.477128	CFBH 40197	MZ579641		x		
S. fuscovarius	Valle del Lunarejo/RV/UR	-31.177083	-55.906111	CURCB 321	MZ603884		x		
S. fuscovarius	San Javier/MN/ARG	-27.909722	-55.270000	LGE 01336	MZ603885		x		
S. nasicus	Torres/RS/BRA	-29.346905	-49.76544	DP 060	MZ603888		x		
S. nasicus	Osorio/RS/BRA	-29.856511	-50.234169	UFRGS 5087	MZ603887		x		
S. perereca	Três Barras/SC/BRA	-26.308928	-50.270397	UFMG 21080	MZ603886	MZ645875	x	x	

Abreviations: CL = Cerro Largo, CO = Colonia, LA = Lavalleja, MO = Montevideo, RV = Rivera, RO = Rocha, SO = Soriano, TA = Tacuarembó, Uruguay; BA = Buenos Aires, MN = Misiones, Argentina; PR = Paraná, RS = Rio Grande do Sul, SC = Santa Catarina, SP = São Paulo, Brazil.
x = samples used in each dataset
# Tadpole's samples
†Geographic records obtained from https://censo2010.ibge.gov.br/sinopse/index.php?dados=8

Table S2. Analyses performed in BEAST program. For each analysis is presented the locus used, number of sequences (N), fragment size in base pairs (pb), and substitution model estimated by Partition Finder program.

Analysis	Locus	N	Fragment	Substitution model
Gene tree	*Cytb*	95	612	HKY+G+I
	Rag1	46	397	TRNEF+G
Species tree	*Cytb*	95	612	HKY+G+I
	Rag1	46	397	TRNEF+G
bGMYC	*Cytb*	50	552	HKY+I
Diffusion *S. granulatus* ABC	*Cytb*	68	552	TRN
Diffusion *S.* sp. D	*Cytb*	17	552	TRN

Table S3. Analysis of Assemble species by automatic partitioning (ASAP) showing nine partitions for cytb dataset. We highlight in bold the best partition based in the lower ASAP-score. p-value = probability of panmixia (p-val), W = relative gap width metrics. See the Fig. S2c to observe the ranged and treshold distances of the best partition.

Number of species	ASAP-score	P-value (rank)	W (rank)	Treshold distance
2	**1.00**	**6.36e-02 (1)**	**7.79e-04 (1)**	**0.098008**
4	2.00	5.37e-01 (2)	5.27e-04 (2)	0.016517
5	3.00	7.52e-01 (3)	8.94e-05 (3)	0.007287
3	4.00	8.52e-01 (4)	8.28e-05 (4)	0.027670
22	6.00	9.58e-01 (6)	6.95e-05 (6)	0.001814
7	6.50	9.56e-01 (5)	6.68e-05 (8)	0.003637
45	7.50	1.00e+00 (10)	6.95e-05 (5)	0.000907
10	8.00	9.94e-01 (9)	6.68e-05 (7)	0.003632
6	8.50	9.68e-01 (7)	4.36e-05 (10)	0.004545

Table S4. Species delimitation hypothesis with *posterior* probabilities for each replicate of the A00 analyses of BPP.

Model	Θ ~ IG	τ ~ IG	Trees with node probabilities	PP*
Cytb run1	3, 0.02	3, 0.004	((((*S. granulatus* ABC, *S. sp*. D):1.00, *S. per*):1.00, ((*S. rub, S. nas*):0.98, *S. fus*):0.99)1.00, *S. cat*)1.00	0.98
Cytb run2	3, 0.02	3, 0.004	((((*S. granulatus* ABC, *S. sp*. D):1.00, *S. per*):1.00, ((*S. rub, S. nas*):0.96, *S. fus*):0.99)1.00, *S. cat*)1.00	0.96
Rag1 run1	3, 0.02	3, 0.004	((((*S. granulatus* ABC, *S. sp*. D):1.00, *S. per*):1.00, ((*S. rub, S. nas*):0.85, *S. fus*):0.99)1.00, *S. cat*)1.00	0.85
Rag1 run2	3, 0.02	3, 0.004	((((*S. granulatus* ABC, *S. sp*. D):1.00, *S. per*):1.00, ((*S. rub, S. nas*):0.85, *S. fus*):0.99)1.00, *S. cat*)1.00	0.85
Cytb + *Rag1* run1	3, 0.02	3, 0.004	((((*S. granulatus* ABC, *S. sp*. D):1.00, *S. per*):1.00, ((*S. rub, S. nas*):0.90, *S. fus*):0.99)1.00, *S. cat*)1.00	0.90
Cytb + *Rag1* run2	3, 0.02	3, 0.004	((((*S. granulatus* ABC, *S. sp*. D):1.00, *S. per*):1.00, ((*S. rub, S. nas*):0.88, *S. fus*):0.97)1.00, *S. cat*)1.00	0.88
	Empirical	Empirical		
Cytb run1	3, 0.02	3, 0.0784	((((*S. granulatus* ABC, *S.* sp. D):1.00, *S.* per):1.00, ((*S. rub*, *S. nas*):0.97, *S. fus*):0.99)1.00, *S. cat*)1.00	0.97
Cytb run2	3, 0.02	3, 0.0784	((((*S. granulatus* ABC, *S.* sp. D):1.00, *S.* per):1.00, ((*S. rub*, *S. nas*):0.97, *S. fus*):0.99)1.00, *S. cat*)1.00	0.97
Rag1 run1	3, 0.02	3, 0.0784	((((*S. granulatus* ABC, *S.* sp. D):1.00, *S.* per):1.00, ((S. *rub*, *S. nas*):0.85, *S. fus*):0.99)1.00, *S. cat*)1.00	0.85
Rag1 run2	3, 0.02	3, 0.0784	((((*S. granulatus* ABC, *S.* sp. D):1.00, *S.* per):1.00, ((*S. rub*, *S. nas*):0.85, *S. fus*):0.99)1.00, *S. cat*)1.00	0.85
Cytb + *Rag1* run1	3, 0.02	3, 0.0784	((((S. granulatus ABC, S. sp. D):1.00, S. per):1.00, ((S. rub, S. nas):0.87, S. fus):0.97)1.00, S. cat)1.00	0.87
Cytb + *Rag1* run2	3, 0.02	3, 0.0784	((((S. granulatus ABC, S. sp. D):1.00, S. per):1.00, ((S. rub, S. nas):0.87, S. fus):0.97)1.00, S. cat)1.00	0.87

* Probability posterior of the number of delimitated species

S. per = Scinax perereca, S. fus = Scinax fuscovarius, S. rub = Scinax ruber, S. nas = Scinax nasicus, S. cat = Scinax catharinae

Table S5. Score of the performance of ESMs inferred for each lineage of *S. granulatus* complex assessed by AUC and Boyce index. Mean (SD = Standard deviation).

	S. granulatus A	*S. granulatus* B	*S. granulatus* C	*S. sp.* D
AUC	0.96 (0.0044)	0.98 (0.012)	0.94 (0.022)	0.97 (0.05)
Boyce	0.21 (0.44)	0.61 (0.33)	0.45 (0.51)	0.81 (0.14)

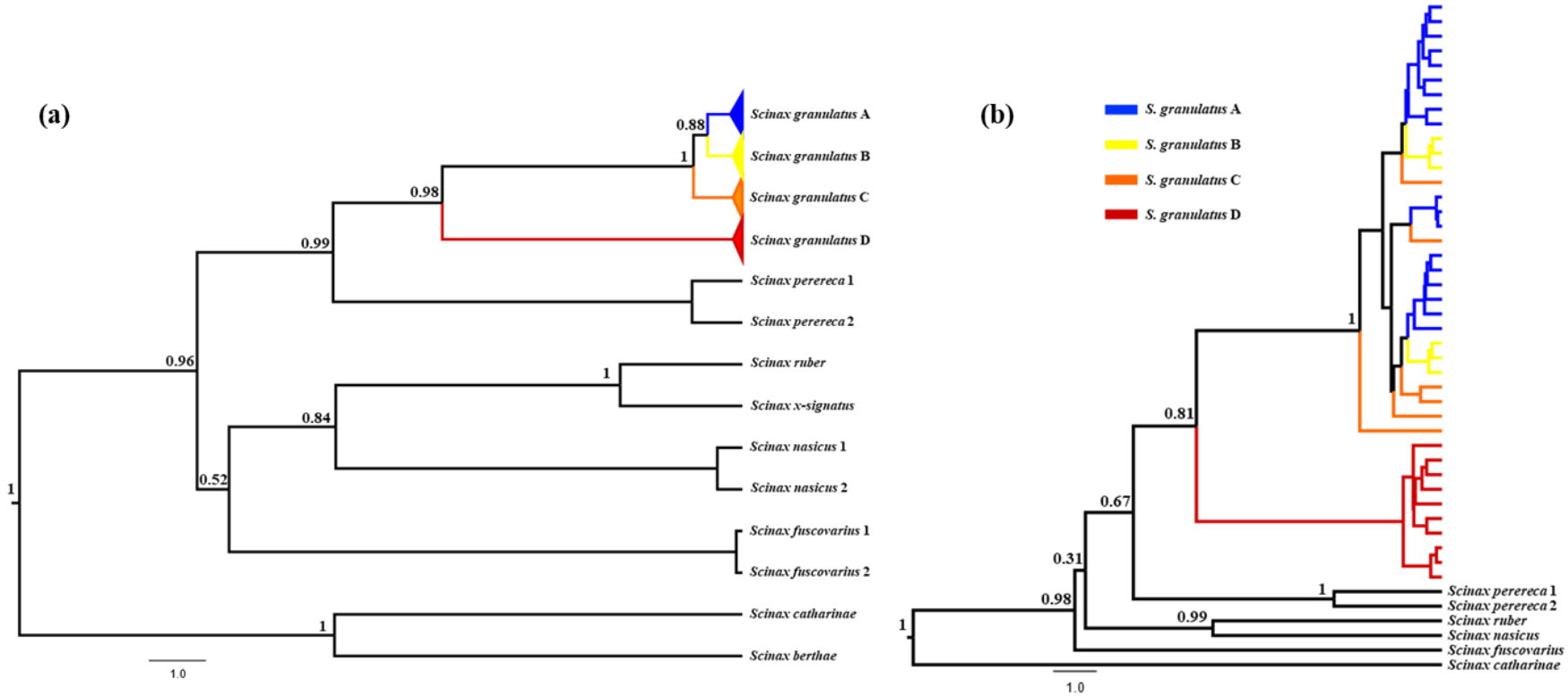

Figure S1. Gene trees of (a) *cytb* and (b) *RAG1*. Numbers in the nodes indicate Bayesian *posterior* probabilities.

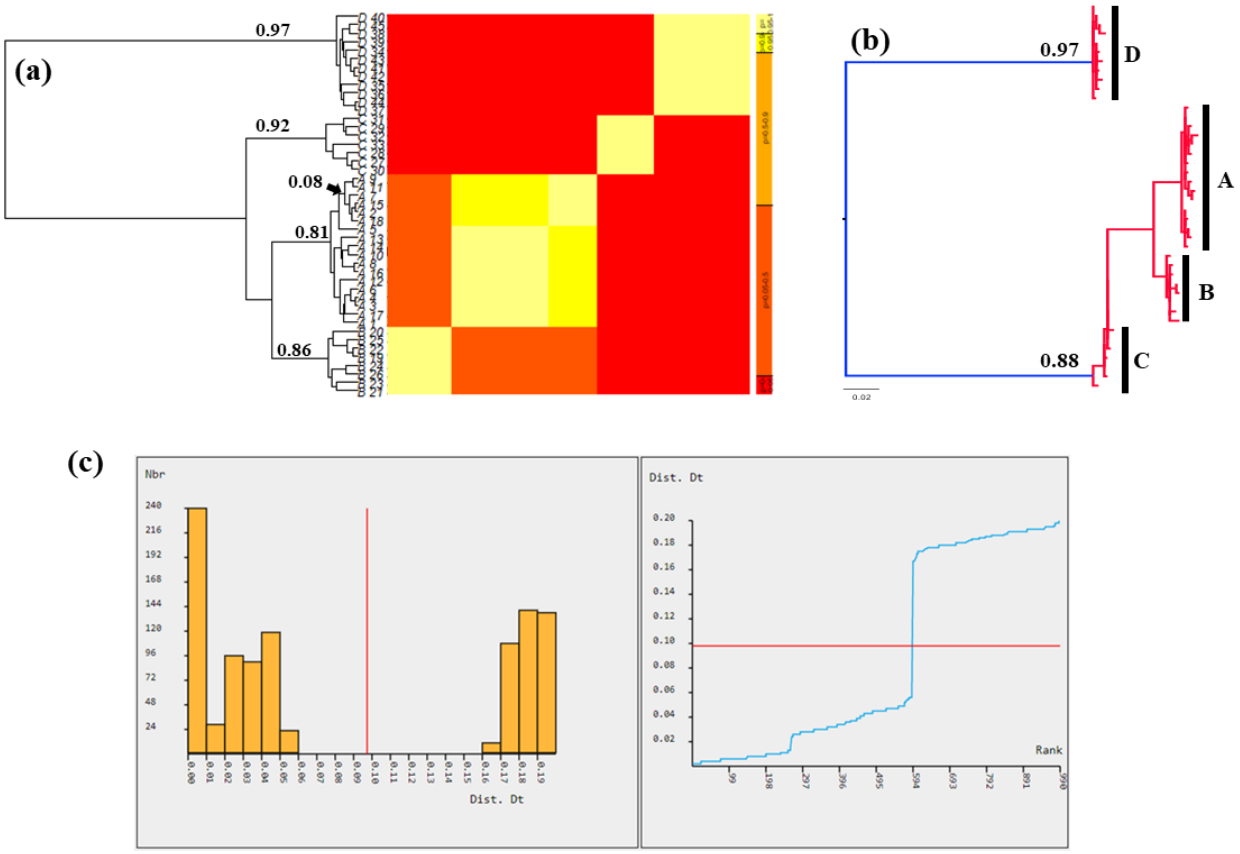

Figure S2. Summary of the lineage delimitation analysis of the *Scinax granulatus* complex. (a) Bayesian implementation of the generalized mixed Yule-coalescent (bGMYC) model. Numbers in each node indicate the posterior probability (PP) sampled from a posterior distribution of 1000 trees. (b) Bayesian implementation of the Poisson Tree Process (bPTP). Here the numbers denote the support (PP) for each delineated candidate species. (c) Results of Assemble Species by Automatic Partitioning (ASAP) method, showing the barcode gap and the distance threshold (denoted with line red) that indicate best partition for the hypothesis of the species boundaries.

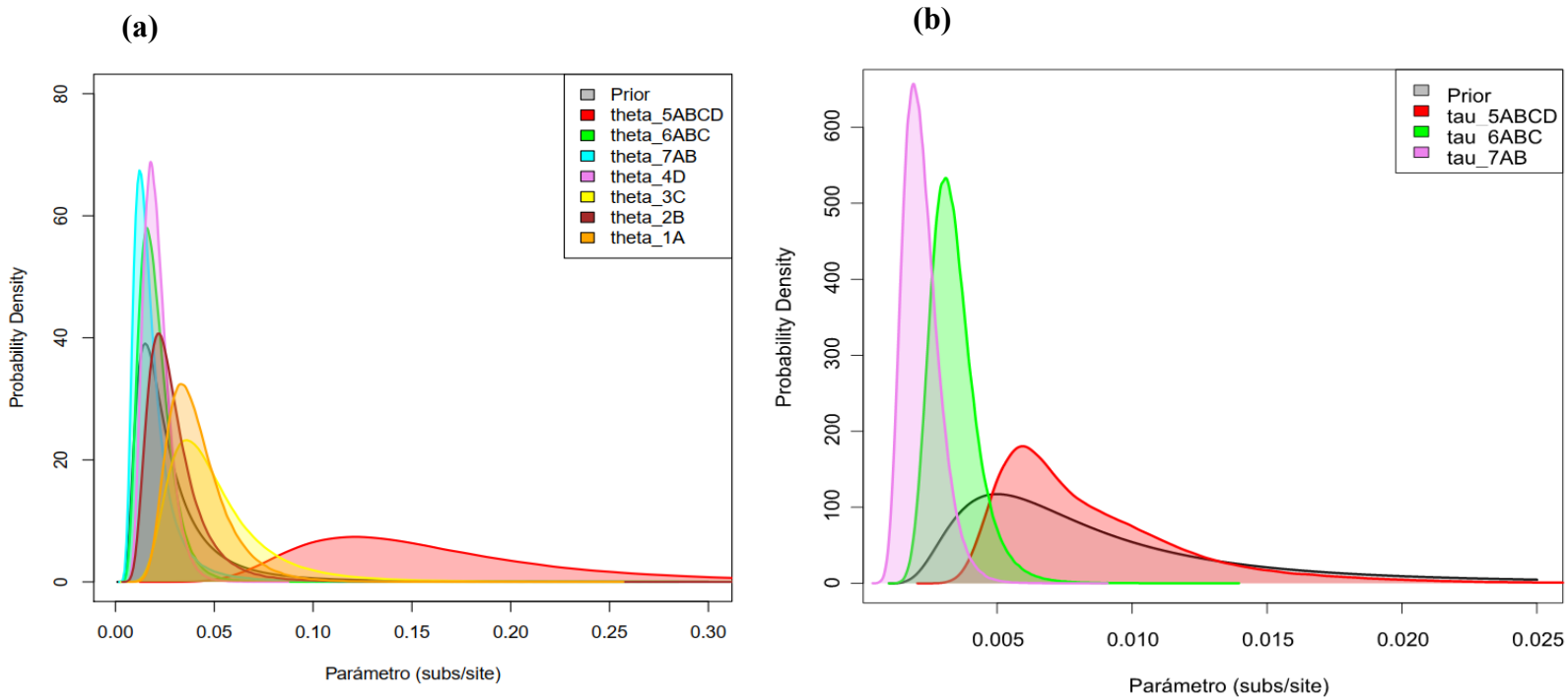

Figure S3. Distribution density results of MCMC sampling for theta and tau parameters from A00 analysis: (a) Theta parameters; (b) Tau estimates.

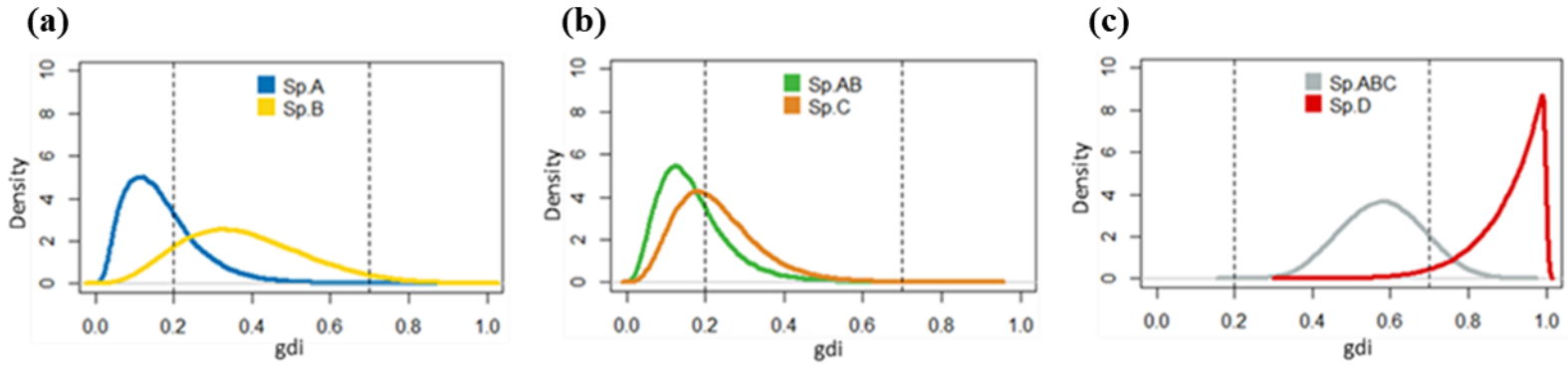

Figure S4 Candidate lineages delimitation of *S. granulatus* complex. a-c Density plots of gdi values (a) *gdi* of Sp.A and Sp.B (b) *gdi* of Sp.AB and Sp.C (c) *gdi* of Sp.ABC and Sp.D. According to Jackson et al.(2017), *gdi* <0.2 indicates a single species, *gdi* >0.7 indicates distinct species, and *gdi* values between 0.2 and 0.7 represent ambiguous species status.

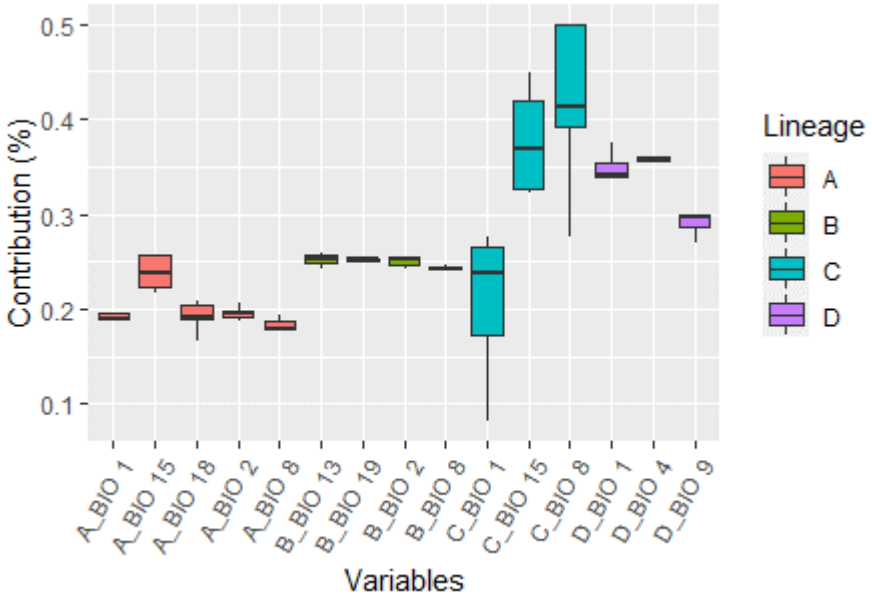

Figure S5. Boxplot of the contribution of each environmental variable for the four *S. granulatus* 's lineages. The percentage represents the proportion of the ensemble explained by the variable of interest. The variables used were: the annual mean temperature (BIO 1), the mean diurnal range (BIO 2), the temperature seasonality (BIO 4), the mean temperature of wettest quarter (BIO 8), the mean temperature of driest quarter (BIO 9), the precipitation of wettest month (BIO 13), the precipitation seasonality (BIO 15), the precipitation of warmest quarter (BIO 18), and the precipitation of coldest quarter (BIO 19).

CAPÍTULO II

Supporting Information

Long-time persistence during the Pleistocene and recurrent secondary contact among lineages of *Leptodactylus latinasus* a widely distributed Neotropical frog from the Chaco-Pampean plains

Matías M. Malleret | Diego Baldo | Francisco Brusquetti | Priscila Lemes | Laura Verrastro | Arley Camargo

TABLE S1 Information regarding the samples analysed in the current study.

Species	Locality/State or Province/Country	Geographical coordinates (Latitude, Longitude)	DNA Voucher	Dataset		GeneBank accession number
				cytb	ddRAD	
L. latinasus	Rivera/Rivera/URY	-31.67164, -55.14055	CURCB_339	x	x	-
L. latinasus	Rivera/Rivera/URY	-31.76106, -54.56699	CURCB_354	x		-
L. latinasus	Salto/Salto/URY	-31.39421, -57.65255	CURCB_369	x		-
L. latinasus	Salto/Salto/URY	-31.3832, -57.7577	CURCB_380	x		-
L. latinasus	Salto/Salto/URY	-31.3832, -57.7577	CURCB_381	x		-
L. latinasus	Tacuarembo/Tac/URY	-31.83768, -56.16375	CURCB_393	x		-
L. latinasus	Cerro Largo/CL/URY	-32.7588, -54.65574	CURCB_437	x		-
L. latinasus	Rocha/Rocha/URY	-34.09833, -54.3176	CURCB_500	x	x	-
L. latinasus	Artigas/Artigas/URY	-30.83928, -56.06938	CURCB_516	x		-
L. latinasus	Artigas/Artigas/URY	-30.83928, -56.06938	CURCB_517	x		-
L. latinasus	Artigas/Artigas/URY	-30.83928, -56.06938	CURCB_518	x		-
L. latinasus	Artigas/Artigas/URY	-30.845598, -56.075036	CURCB_521	x		-
L. latinasus	Dom Pedrito/RS/BRA	-31.05524, -54.78912	ZUFSM_12003	x		-
L. latinasus	Lavras do Sul/RS/BRA	-30.77394, -54.39483	ZUFSM_10559	x		-
L. latinasus	Artigas/Artigas/URY	-30.9568, -56.51189	PQDN_428	x		-
L. latinasus	Quaraí/RS/BRA	-30.31152, -55.93598	ZUFSM_11595	x		-
L. latinasus	Santo Antônio das Missões/RS/BRA	-28.52348, -55.46773	ZUFSM_10351	x		-
L. latinasus	Santo Antônio das Missões/RS/BRA	-28.53667, -55.44053	ZUFSM_10365	x		-
L. latinasus	São Gabriel/RS/BRA	-30.09177, -54.31637	ZUFSM_10453	x	x	-

L. latinasus	Quaraí/RS/BRA	-30.1925, -56.4927	UFRGS_2684	x		-
L. latinasus	Santana do Livramento/RS/BRA	-30.64337, -55.48903	UFRGS_1088	x		-
L. latinasus	Santana do Livramento/RS/BRA	-30.693371, -55.509157	UFRGS_1100	x		-
L. latinasus	São Lourenço do Sul/RS/BRA	-31.30611, -52.28914	UFRGS_2420	x	x	-
L. latinasus	Eldorado do Sul/RS/BRA	-30.0916, -51.672062	UFRGS_3685	x		-
L. latinasus	Eldorado do Sul/RS/BRA	-30.091603, -51.672062	UFRGS_4518	x		-
L. latinasus	Paysandú/Pay/URY	-32.38163, -58.07209	UFRGS_3991	x	x	-
L. latinasus	Sanatan de Boa Vista/RS/BRA	-30.8552, -53.24198	ZUFSM_10533	x		-
L. latinasus	Minas/Lavalleja/URY	-34.35551, -55.1359	CURCB_602	x		-
L. latinasus	Nueva Carrara/Maldonado/URY	-34.63887, -55.24855	CURCB_607	x		-
L. latinasus	Santo Antônio das Missões/RS/BRA	-28.52348, -55.46773	ZUFSM_10352	x		-
L. latinasus	Santana do Livramento/RS/BRA	-30.63846, -55.55771	UFRGS_1083	x		-
L. latinasus	Quaraí/RS/BRA	-30.176, -56.538333	UFRGS_5085	x		-
L. latinasus	Curuzú Cuatiá/Corrientes/ARG	-29.06861, -58.61666	DB_2479	x		-
L. latinasus	Chajarí/Entre Río/ARG	-30.78833, -57.9975	DB_6079	x	x	-
L. latinasus	Chajarí/Entre Río/ARG	-30.89277, -57.96111	DB_8185	x		-
L. latinasus	Lavras do Sul/RS/BRA	-30.82635, -54.42457	ZUFSM_10577	x		-
L. latinasus	Piratini/RS/BRA	-31.08768, -53.036767	ZUFSM_11943	x		--
L. latinasus	Piratini/RS/BRA	-31.08768, -53.036767	ZUFSM_11944	x		
L. latinasus	Tapes/RS/BRA	-30.68985, -51.39585	UFRGS_4625	x	x	-
L. latinasus	Tapes/RS/BRA	-30.68985, -51.39585	UFRGS_4627	x	x	-
L. latinasus	São Gabriel/RS/BRA	-30.08997, -54.31015	ZUFSM_10457	x		-
L. latinasus	São Gabriel/RS/BRA	-30.11757, -54.31358	ZUFSM_10465	x		-

L. latinasus	Santana do Livramento/RS/BRA	-30.61773, -55.56238	UFRGS_1094	x		-
L. latinasus	Caçapava do Sul/RS/BRA	-30.5075, -53.503833	UFRGS_5080	x		-
L. latinasus	Paysandú/Paysandú/URY	-31.90668, -56.65531	CURCB_577	x		-
L. latinasus	Cerro Largo/Cerro Largo/URY	-31.94989, -54.2028	CURCB_400	x	x	-
L. latinasus	Cerro Largo/Cerro Largo/URY	-31.94989, -54.2028	CURCB_402	x		-
L. latinasus	Cerro Largo/Cerro Largo/URY	-32.90921, -54.95848	CURCB_425	x		-
L. latinasus	Jaguarão/RS/BRA	-32.51994, -53.202359	ZUFSM_11922	x		-
L. latinasus	Lavras do Sul/RS/BRA	-30.77394, -54.39483	ZUFSM_10561	x		-
L. latinasus	Lavras do Sul/RS/BRA	-30.82635, -54.42457	ZUFSM_10576	x	x	-
L. latinasus	Jaguarão/RS/BRA	-32.61994, -53.19061	UFRGS_523	x	x	-
L. latinasus	Jaguarão/RS/BRA	-32.62339, -53.19152	UFRGS_707	x		-
L. latinasus	Jaguarão/RS/BRA	-32.62339, -53.19152	UFRGS_708	x		-
L. latinasus	Pinheiro Machado/RS/BRA	-31.51564, -53.47727	UFRGS_2343	x		-
L. latinasus	Rio Grande/RS/BRA	-32.68552, -52.60887	UFRGS_4487	x	x	-
L. latinasus	Caçapava do Sul/RS/BRA	-30.5075, -53.503833	UFRGS_5079	x		-
L. latinasus	Dom Pedrito/RS/BRA	-31.05524, -54.78912	ZUFSM_12000	x		-
L. latinasus	Arroio/RS/BRA	-32.231213, -53.086802	MCP_12308	x		-
L. latinasus	Santana de Boa Vista/RS/BRA	-30.850772, -53.244941	ZUFSM_10534	x		-
L. latinasus	Rocha/Rocha/URY	-34.09833, -54.3176	CURCB_498	x		-
L. latinasus	Rocha/Rocha/URY	-34.09833, -54.3176	CURCB_499	x		-
L. latinasus	El Eden/Maldonado/URY	-34.58118, -55.18827	CURCB_610	x		-
L. latinasus	Valizas/Rocha/URY	-34.330031, -53.79229	CURCB_528	x		-
L. latinasus	Cerro Largo/Cerro Largo/URY	-32.7588, -54.65574	CURCB_438	x		-

L. latinasus	São Lourenço do Sul/RS/BRA	-31.30611, -52.28914	UFRGS_2421	x		-
L. latinasus	São Lourenço do Sul/RS/BRA	-31.30611, -52.28914	UFRGS_2422	x		-
L. latinasus	Colón/Córdoba/ARG	-30.96666, -64.08333	DB_1675	x	x	-
L. latinasus	Luján/Buenos Aires/ARG	-34.62706, -59.10822	DB_2628	x		-
L. latinasus	Luján/Buenos Aires/ARG	-34.62706, -59.10822	DB_2629	x	x	-
L. latinasus	Curuzú Cuatiá/Corrientes/ARG	-29.16666, -58.55	DB_2906	x	x	-
L. latinasus	San Martín/Córdoba/ARG	-32.41027, -63.25416	DB_3655	x		-
L. latinasus	San Justo/Santa Fe/ARG	-30.9765, -60.07675	DB_4618	x		-
L. latinasus	General Lopéz/Santa Fe/ARG	-33.80372, -61.88591	LGE_1658	x		-
L. latinasus	Juárez Celman/Córdoba/ARG	-32.75638, -63.77222	DB_5223	x		-
L. latinasus	Juárez Celman/Córdoba/ARG	-32.72388, -63.74777	DB_5580	x		-
L. latinasus	Juárez Celman/Córdoba/ARG	-32.72388, -63.74777	DB_5581	x		-
L. latinasus	Chajarí/Entre Río/ARG	-30.78833, -57.9975	DB_6081	x		-
L. latinasus	Chajarí/Entre Río/ARG	-30.78833, -57.9975	DB_6082	x	x	-
L. latinasus	San Martín/Corrientes/ARG	-29.48277, -56.8425	DB_7655	x	x	-
L. latinasus	Tercero Arriba/Córdoba/ARG	-32.1834, -64.1076	DB_8271	x	x	-
L. latinasus	Tercero Arriba/Córdoba/ARG	-32.1906, -64.14193	DB_8278	x	x	-
L. latinasus	Rosario/Santa Fe/ARG	-32.78344, -60.90083	LGE_18744	x		-
L. latinasus	Rosario/Santa Fe/ARG	-32.78344, -60.90083	LGE_18746	x	x	-
L. latinasus	9 de Julio/Santa Fe/ARG	-28.606722, -61.708583	LGE_20736	x		-
L. latinasus	General Lopéz/Santa Fe/ARG	-33.80372, -61.88591	LGE_1657	x		-
L. latinasus	Rosario/Santa Fe/ARG	-32.99025, -60.90675	LGE_18725	x		-
L. latinasus	La Paz/Entre Ríos/ARG	-31.26572, -59.776	LGE_2485	x	x	-

L. latinasus	San Justo/Santa Fe/ARG	-30.77777, -60.59055	LGE_16970	x		-
L. latinasus	San Justo/Santa Fe/ARG	-30.77777, -60.59055	LGE_16971	x		-
L. latinasus	San Javier/Santa Fe/ARG	-30.54891, -59.99847	LGE_18695	x		-
L. latinasus	Chacabuco/Chaco/ARG	-27.2, -61.15	DB_2774	x		-
L. latinasus	Rosario/Santa Fe/ARG	-32.99025, -60.90675	LGE_18723	x		-
L. latinasus	Rosario/Santa Fe/ARG	-32.78344, -60.90083	LGE_18745	x		-
L. latinasus	Federal/Entre Ríos/ARG	-30.954, -58.771389	LGE_18883	x		-
L. latinasus	Federal/Entre Ríos/ARG	-30945583, -58.789861	LGE_18891	x		-
L. latinasus	Quaraí/RS/BRA	-30.1925, -56.4927	UFRGS_2681	x	x	-
L. latinasus	Concordia/Entre Rios/ARG	-31.393425, -58.03194	UFRGS_5096	x		-
L. latinasus	El Alto/Catamarca/ARG	-28.585, -65.229722	DB_2656	x		-
L. latinasus	Corrientes/Corrientes/ARG	-27.43333, -58.75	LGE_11282	x	x	-
L. latinasus	Lules/Tucumán/ARG	-26.868888, -65.295833	LGE_12825	x		-
L. latinasus	Anta/Salta/ARG	-25.292222, -64.079444	LGE_17776		x	-
L. latinasus	Anta/Salta/ARG	-25.292222, -64.079444	LGE_17777	x		-
L. latinasus	Ojo de Agua/SE/ARG	-28.877222, -63.980833	LGE_2328	x		-
L. latinasus	Vera/Santa Fe/ARG	-29.079805, -60.586361	LGE_20658	x		-
L. latinasus	9 de Julio/Santa Fe/ARG	-29.324305, -61.803472	LGE_20690	x		-
L. latinasus	El Alto/Catamarca/ARG	-28.6111111, -65.415277	DB_2672	x		-
L. latinasus	Almirante Brown/Chaco/ARG	-25.614611, -63.327694	LGE_11242	x		-
L. latinasus	Doc. Manuel Belgrano/Jujuy/ARG	-24.154722, -65.372499	DB_1486	x	x	-
L. latinasus	Ledesma/Jujuy/ARG	-23.7625, -64.849499	LGE_4749	x	x	-
L. latinasus	El Carmen/Jujuy/ARG	-24.441305, -65.001944	GS_3518	x		-

L. latinasus	Ledesma/Jujuy/ARG	-23.7625, -64.849499	LGE_4744	x	x	-
L. latinasus	San Fernando/Chaco/ARG	-27.433333, -58.883333	DB_5192	x	x	-
L. latinasus	Patiño/Formosa/ARG	-24.5855, -60.772361	DB_6326	x		-
L. latinasus	G. José de San Martín/Salta/ARG	-23.034444, -63.890527	L_425	x		-
L. latinasus	G. José de San Martín/Salta/ARG	-23.034444, -63.890527	L_419	x		-
L. latinasus	El Quebrachal/Salta/ARG	-25.4438889, -63.848333	LGE_9281	x		-
L. latinasus	Cmte. Fernández/Chaco/ARG	-26.76522, -60.161367	LGE_10073	x		-
L. latinasus	General Güemes/Chaco/ARG	-24.679966, -61.391033	LGE_12157	x		-
L. latinasus	G. José de San Martín/Salta/ARG	-23.174222, -64.069472	LGE_4993	x		-
L. latinasus	General Güemes/Chaco/ARG	-25.021805, -61.521361	LGE_17017	x		-
L. latinasus	Pirané/Formosa/ARG	-26.158, -59.029638	LGE_9415	x		-
L. latinasus	Formosa/Formosa/ARG	-26.180982, -58.944312	GS_3617	x		-
L. latinasus	Presidente Hayes/PRY	-23.41426, -59.97325	IIBPH_1628	x		-
L. latinasus	Presidente Hayes/PRY	-23.38412, -59.98315	IIBPH_1640	x		-
L. latinasus	Presidente Hayes/PRY	-23.08201, -59.29131	IIBPH_1683	x		-
L. latinasus	Presidente Hayes/PRY	-23.03645, -59.18913	IIBPH_2427	x		-
L. latinasus	Presidente Hayes/PRY	-23.05367, -59.20287	IIBPH_2463	x		-
L. latinasus	Presidente Hayes/PRY	-23.05367, -59.20287	IIBPH_2466	x		-
L. latinasus	Presidente Hayes/PRY	-22.6771, -59.77132	IIBPH_1450	x		-
L. latinasus	Patiño/Formosa/ARG	-24.5855, -60.772361	LGE_12671	x		-
L. latinasus	Presidente Hayes/PRY	-22.89909, -58.90724	IIBPH_5397	x		-
L. latinasus	La Paloma/Chaco/ARG	-25.47975, -62.932444	L_535	x		-
L. latinasus	Taco Pozo/Chaco/ARG	-25.614611, -63.327694	LGE_11291	x		-

L. latinasus	General Güemes/Chaco/ARG	-25.111766, -61.228766	LGE_12231	x		-
L. latinasus	Copo/SE/ARG	-25.822916, -62.852694	LGE_13235	x		-
L. latinasus	Presidente Hayes/PRY	-23.41426, -59.97325	IIBPH_1629	x		-
L. latinasus	Pozo Hondo/Salta/ARG	-23.823555, -63.49	L_471	x	x	-
L. latinasus	Presidente Hayes/PRY	-23.0415, -59.19502	IIBPH_2451	x	x	-
L. latinasus	General Güemes/Chaco/ARG	-24.438611, -61.682805	LGE_12791	x		-
L. latinasus	General Obligado/Santa Fe/ARG	-28.027777, 59.348138	LGE_18660		x	-
L. latinasus	Taco Pozo/Chaco/ARG	-25.61461, -63.327694	LGE_11246		x	-
L. latinasus	La Cocha/Tucumán/ARG	-27.742777, -65.676111	FML_24416		x	-
L. gracilis			CURCB_348	x		-

Localities: SE = Santiago del Estero in Argentina; RS = Rio Grande do Sul in Brazil; ARG = Argentina; BRA = Brazil; PRY = Paraguay; URY = Uruguay.

TABLE S2 The best-fitting partition schemes and substitution models based on the Bayesian information criterion (BIC) in PartitionFinder for cytb locus are shown for each analysis.

Analysis	Codon partition	Number partition	Substitution model
Gene tree	Position 1 Position 2 Position 3	3	K80+I+G F81 TIM+G
Bayesian Skyride plot - L1	Position 1 Position 2 Position 3	3	K80 F81 TRN
Bayesian Skyride plot - L2	Position 1 Position 2 Position 3	3	JC F81 HKY
Bayesian Skyride plot - L3	Position 1 Position 2 Position 3	3	K80 F81 TRN
Bayesian Skyride plot - L5	Position 1 Position 2 Position 3	3	K80 F81 TIM
Phylogeographic diffusion - L1	Position 123	1	HKY
Phylogeographic diffusion - L2	Position 123	1	HKY
Phylogeographic diffusion - L3	Position 123	1	HKY+G
Phylogeographic diffusion - L5	Position 123	1	K81UF
Species-tree diffusion	Position 1 Position 2 Position 3	3	K80+G F81 TIM+I

TABLE S3 Modelling algorithms used to identify the paleo-niches of *Leptodactylus latinasus* lineages from the current distribution. Models are calibrated under current conditions and projected to current and paleoclimate scenarios. Model performance is evaluated through TSS and AUC.

Model	AUC				TSS			
	L1	**L2**	**L3**	**L5**	**L1**	**L2**	**L3**	**L5**
bioclim	0.83	0.82	0.85	0.8	0.67	0.64	0.71	0.6
domain	0.95	0.9	0.92	0.94	0.9	0.81	0.84	0.88
brt	0.97	0.95	0.95	0.92	0.89	0.89	0.84	0.83
svm	0.95	0.98	0.94	0.95	0.86	0.96	0.84	0.88
mars	0.95	0.95	0.94	0.91	0.9	0.89	0.96	0.84

TABLE S4 Aspects of variables used for the consensus model generated for the four *L. latinasus* lineages. The % permutation importance corresponds to the process of model building and it was based on two metrics, correlation and AUC.

Variable	Correlation (%)				AUC (%)			
	L1	L2	L3	L5	L1	L2	L3	L5
Temperature Seasonality (BIO 4)	59.6	47.4	73.2	59.4	45.6	32.6	63.2	50.4
Mean Temperature of Wettest Quarter (BIO 8)	24.1	37.9	19.8	55.4	15.6	15.3	12	35.7
Mean Temperature of Driest Quarter (BIO 9)	18	51.9	25.6	38.5	12	32.3	23.5	26.5
Precipitation of Wettest Month (BIO 13)	26.6	30.9	23.5	25.8	21	21.5	21	23.6
Precipitation Seasonality (BIO 15)	63.9	66.8	13.9	24.7	47.4	54.1	7.5	12.3
Precipitation of Warmest Quarter (BIO 18)	36	35	44.4	28.4	26	21.2	22.5	21.4

TABLE S5 Table output of the Evanno's method results. Bolded value of Delta K indicates the most likely number of clusters (i.e., K = 2). Data as in Fig. 1

K	Reps	Mean LnP(K)	Stdev LnP(K)	Ln'(K)	|Ln''(K)|	Delta K
1	10	-36821.27	0.8883	NA	NA	NA
2	10	-29765.43	10.7971	7055.84	6177.25	**572.119**
3	10	-28886.84	40.2513	878.59	888.31	22.069
4	10	-28896.56	30.8771	-9.72	169.50	5.489
5	10	-29075.78	380.147	-179.22	NA	NA

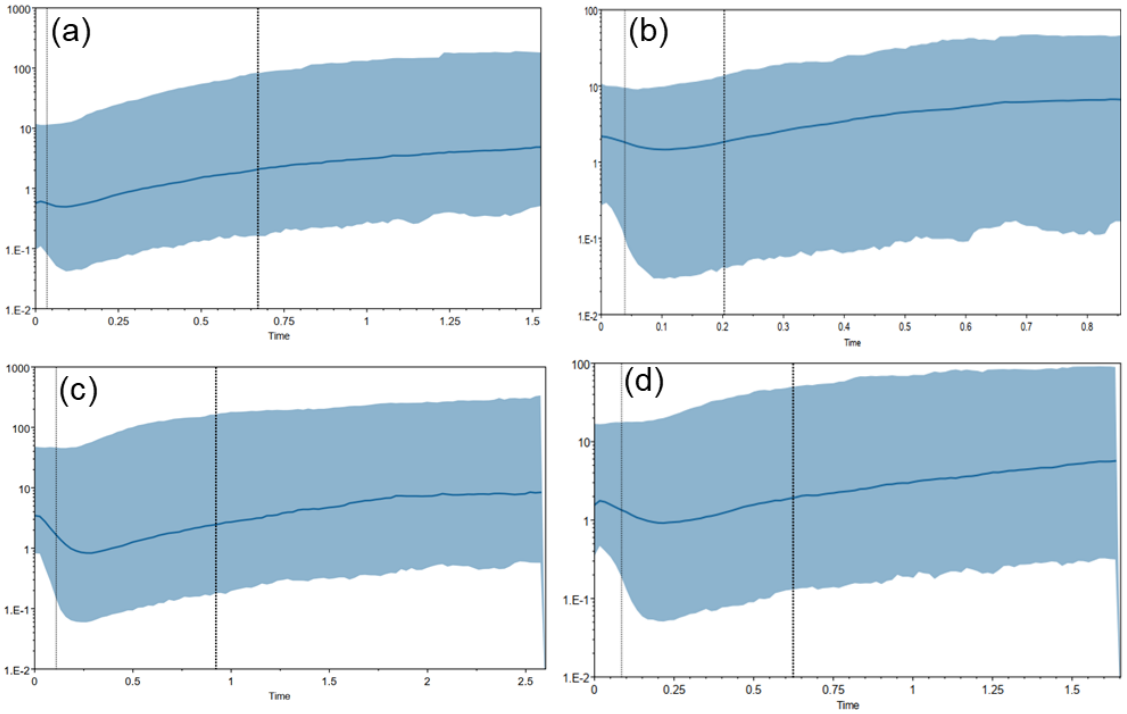

FIGURE S1 Estimates for historical population size dynamics in *Leptodactylus latinasus* from mitochondrial data. (a-d) Effective population size trajectory based on a Bayesian skyride analysis of cytb data. The median population size is shown with a blue line, and the 95% HPD is shown with blue shading (a) L1 lineage (b) L2 (c) L3 lineage (d) L5 lineage.

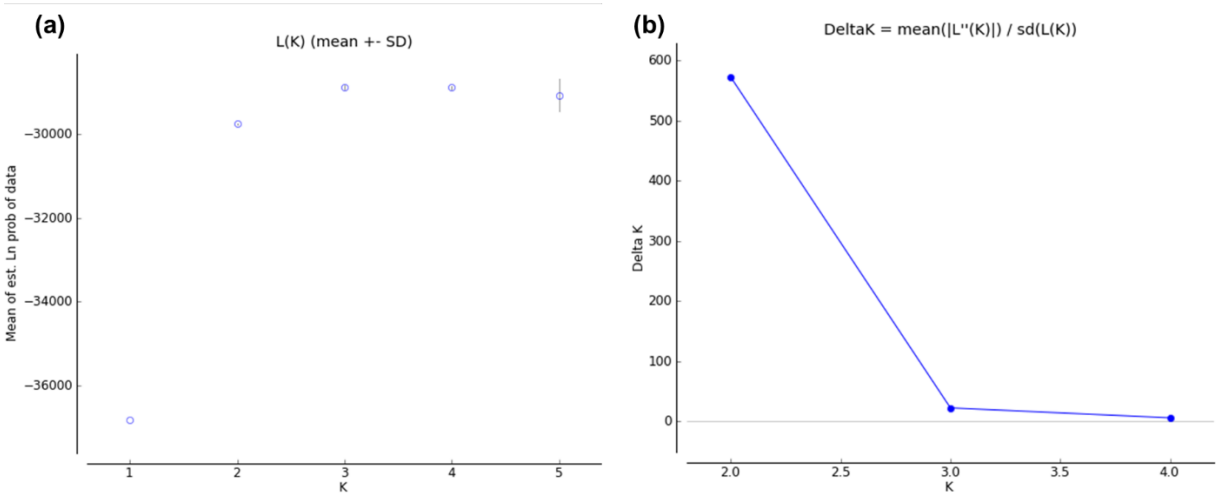

FIGURE S2 Plot of K values from STRUCTURE using the SNPs dataset containing 40 individuals of *Leptodactylus latinasus*. (a) Plot of mean likelihood L(K) and standard deviations per K value. (b) Value of ΔK as a function of K = 1–5.

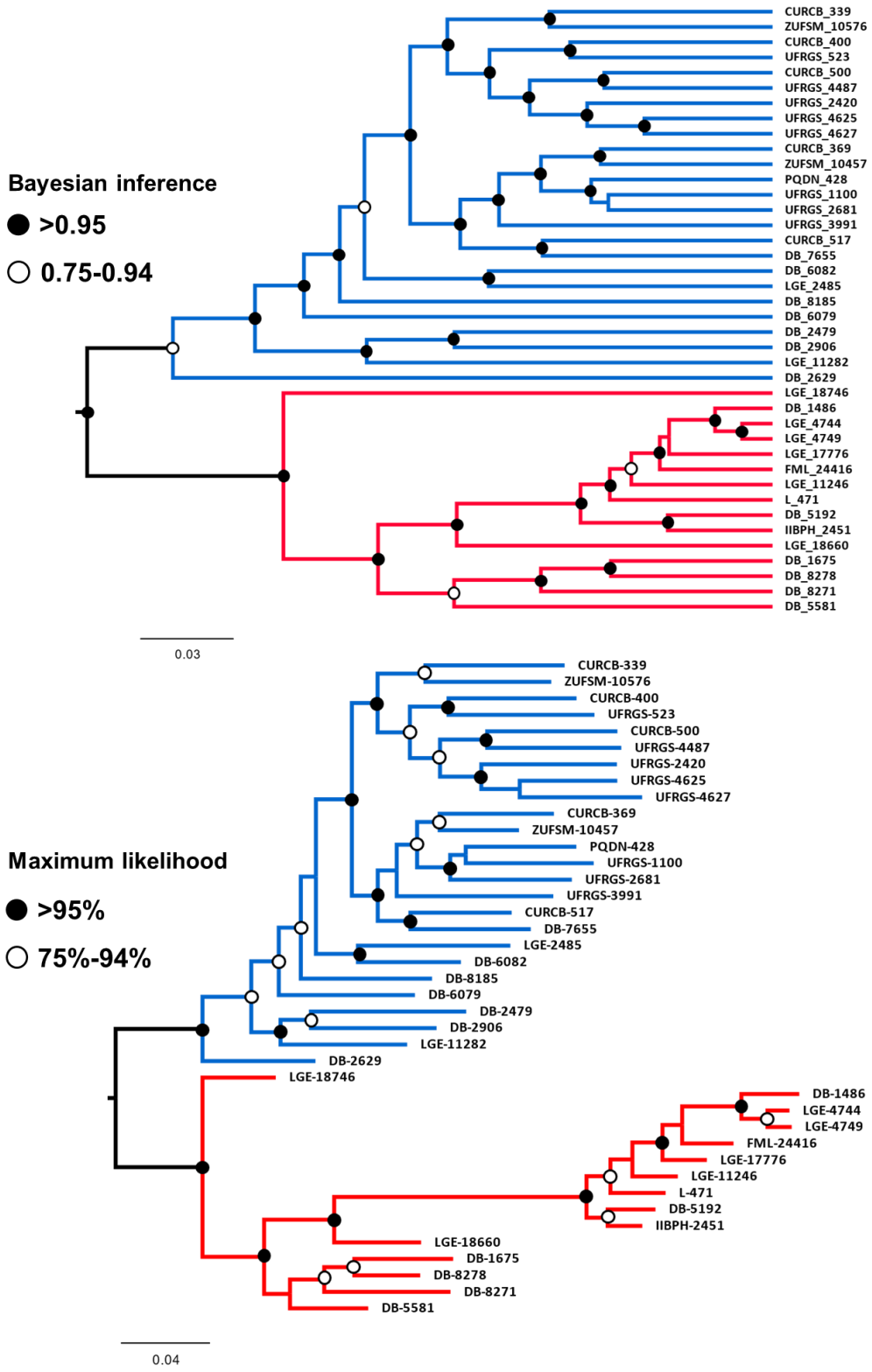

FIGURE S3 Phylogenomic trees using 3047 SNPs of 40 individuals. The top to bottom: Bayesian tree and Maximum Likelihood tree. Circles on nodes indicate statistical support in Bayesian (PP = Posterior probability) and maximum likelihood analyses (SH-aLRT = approximate Likelihood Ratio Test). Colours represent to the two identified nuclear clusters, cluster 1 (blue) and cluster2 (red).