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Genetic Diversity and Historical Demography of *Physalaemus centralis* (Anura, Leptodactylidae) in the Cerrado

Amanda Varago

Dissertação apresentada à Fundação Universidade Federal de Mato Grosso do Sul, como requisito à obtenção do título de Mestre em Biologia Animal. Área de concentração: Zoologia.

Orientador: Diego José Santana Silva
Coorientadora: Sarah Mângia Barros

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Resumo

Compreender a história evolutiva das espécies é fundamental para o desenvolvimento de estratégias de conservação eficazes, especialmente em hotspots de biodiversidade como o Cerrado. Este bioma, conhecido por seu alto endemismo, enfrenta crescentes ameaças de perda de habitat e das mudanças climáticas. Os anfíbios, devido à sua sensibilidade às mudanças ambientais e limitada capacidade de dispersão, são especialmente vulneráveis, tornando-se modelos valiosos para o estudo da dinâmica da biodiversidade. A rã neotropical *Physalaemus centralis*, endêmica do Cerrado, oferece um caso ideal para investigar como flutuações climáticas históricas, eventos geológicos e barreiras ecológicas moldaram a diversidade genética e a estrutura populacional. Sua ampla distribuição no Cerrado abrange condições ecológicas diversas e potenciais barreiras, proporcionando um modelo representativo para compreender como a heterogeneidade ambiental influencia os processos evolutivos. Analisando os padrões filogeográficos de *P. centralis* com DNA mitocondrial (16S rRNA) de 66 amostras de toda sua distribuição, identificamos dois grupos genéticos distintos que divergiram há aproximadamente 4,99 milhões de anos, durante a transição Mioceno-Plioceno. Esses grupos apresentam contrastes na diversidade genética e na distribuição geográfica, influenciados por mudanças climáticas históricas e por restrições ecológicas. As análises do Bayesian Skyline Plot indicaram estabilidade populacional em ambos grupos, enquanto modelos de nicho ecológico destacaram o papel das mudanças climáticas passadas na conectividade e nos refúgios de habitat. Os resultados evidenciam o papel duplo do Cerrado como refúgio e motor de diversificação, enfatizando sua importância na história evolutiva de *P. centralis*. Este estudo fornece insights fundamentais sobre os processos que impulsionam e mantêm a biodiversidade neste bioma ameaçado, formando uma base para esforços de conservação direcionados à preservação da resiliência genética e ecológica de *P. centralis* e outras espécies do Cerrado.

Palavras-chave: Filogeografia, modelagem de nicho ecológico, diversificação, biodiversidade do Cerrado, DNA mitocondrial

Abstract

Understanding the evolutionary history of species is crucial for developing effective conservation strategies, particularly in biodiversity hotspots like the Cerrado. This biome, known for its high endemism, faces increasing threats from habitat loss and climate change. Amphibians, due to their sensitivity to environmental changes and limited dispersal abilities, are especially vulnerable, making them valuable models for studying biodiversity dynamics. The Neotropical frog *Physalaemus centralis*, endemic to the Cerrado, offers an ideal case to investigate how historical climatic fluctuations, geological events, and ecological barriers have shaped genetic diversity and population structure. Its wide distribution across the Cerrado encompasses diverse ecological conditions and potential barriers, providing a representative model for understanding how environmental heterogeneity influences evolutionary processes. By analyzing the phylogeographic patterns of *P. centralis* using mitochondrial DNA (16S rRNA) from 66 samples across its range, we identified two distinct genetic groups that diverged approximately 4.99 million years ago during the Miocene-Pliocene transition. These groups exhibit contrasting genetic diversity and geographic distributions, influenced by historical climatic shifts and ecological constraints. The Bayesian Skyline Plot indicated population stability in both groups, while ecological niche models highlighted the role of past climatic changes in shaping habitat connectivity and refugia. These findings underscore the Cerrado's dual role as both a refuge and a driver of diversification, emphasizing its importance in the evolutionary history of *P. centralis*. This study provides key insights into the processes driving and maintaining biodiversity in this threatened biome, forming a foundation for targeted conservation efforts to preserve the genetic and ecological resilience of *P. centralis* and other Cerrado species.

Key words: Phylogeography, ecological niche modeling, Diversification, Cerrado biodiversity, Mitochondrial DNA

Introdução Geral

O Cerrado, reconhecido por sua biodiversidade única e insubstituível, é uma das regiões ecológicas mais significativas do planeta, abrigando espécies endêmicas e uma complexa estrutura ecológica (Klink & Machado, 2005; Sano et al., 2019). Ao longo de sua história evolutiva, essa região experimentou profundas transformações geoclimáticas que desempenharam um papel crucial na formação de sua biodiversidade. Eventos geológicos, como o soerguimento do Planalto Central e a formação de grandes bacias hidrográficas, modificaram a conectividade ecológica, criando barreiras e corredores que influenciaram a distribuição de espécies e a estrutura genética das populações (Rossetti et al., 2005; Fonseca et al., 2021). Além disso, mudanças climáticas durante o Pleistoceno intensificaram esses processos, com ciclos de expansão e retração de habitats facilitando migrações, dispersões e, em alguns casos, o surgimento de novas linhagens (Guillory et al., 2024). Barreiras físicas, como rios e montanhas, frequentemente levaram ao isolamento geográfico, impulsionando a especiação alopátrica (Oliveira et al., 2018; de Oliveira et al., 2019). Ao mesmo tempo, o fluxo gênico entre populações adjacentes contribuiu para a manutenção da diversidade genética, enquanto a hibridização em zonas de contato gerou novas combinações genéticas, aumentando a variabilidade (Góes et al., 2019; Muniz et al., 2022).

Estudos sobre diferentes organismos destacam o impacto extensivo desses processos no Cerrado. Pesquisas em plantas (Diniz-Filho et al., 2015), mamíferos (Machado et al., 2018), aves (Cabanne et al., 2019), anfíbios (Vasconcellos et al., 2019) e répteis (Domingos et al., 2017) demonstraram como as mudanças ambientais e as barreiras geográficas influenciaram a evolução e diversificação das espécies. Os anfíbios, em particular, são considerados bons modelos para investigar dinâmicas evolutivas devido à sua alta sensibilidade às mudanças ambientais e estrutura genética complexa (Magalhães et al., 2020; Santana et al., 2024a). Entre as muitas espécies endêmicas do Cerrado, a rã *Physalaemus centralis* (Bokermann, 1962) serve como um valioso exemplo para estudar a diversificação dentro do bioma. Amplamente distribuída por toda a ecorregião (Valdujo et al., 2012; Frost, 2024), essa espécie representa um bom modelo para investigar como fatores históricos e geoclimáticos influenciaram sua dinâmica populacional.

A compartimentalização do Cerrado e regiões circundantes influenciou intensamente as trajetórias evolutivas de numerosas espécies. Estudos prévios fornecem evidências sobre essas dinâmicas, revelando como diferentes táxons responderam a fatores históricos e ambientais. Por exemplo, flutuações climáticas durante o Pleistoceno tiveram impactos significativos em espécies como *Boana lundii*, com mudanças na disponibilidade de habitat impulsionando padrões de expansão e retração populacional (Vasconcelos et al., 2019). Elementos topográficos, como cadeias montanhosas, influenciaram a diferenciação genética em *Dermatonotus muelleri*, onde o isolamento geográfico desempenhou um papel importante na promoção da especiação alopátrica (Oliveira et al., 2018). Além disso, sistemas fluviais também exercem influência dual, atuando como barreiras ao fluxo gênico em certas regiões enquanto facilitam a dispersão em outras, como demonstrado em estudos sobre a rã paradoxal *Pseudis bolbodactyla* (Santana et al., 2024b). Coletivamente, esses achados ilustram a complexa interação de processos históricos e características geográficas na formação de padrões de diversificação no Cerrado.

Com base no entendimento de que processos climáticos e geomorfológicos históricos no Cerrado moldaram as dinâmicas evolutivas dos organismos, este estudo investiga a diversificação genética de *Physalaemus centralis* utilizando marcadores mitocondriais. Nosso objetivo é desvendar os fatores históricos e ecológicos que direcionaram a história evolutiva da espécie por meio de: (1) avaliação da variação genética ao longo de sua distribuição, (2) identificação de populações distintas por meio de análises de estrutura populacional, (3) avaliação de padrões demográficos históricos e (4) análise da influência de mudanças no nicho climático sobre sua diversificação.

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Genetic Diversity and Demographic Stability of *Physalaemus centralis* (Anura, Leptodactylidae) in the Cerrado

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Running title: Genetic Diversity and Demographic Stability of *Physalaemus centralis*

Abstract.

Understanding the evolutionary history of species is crucial for developing effective conservation strategies, particularly in biodiversity hotspots like the Cerrado. This biome, known for its high endemism, faces increasing threats from habitat loss and climate change. Amphibians, due to their sensitivity to environmental changes and limited dispersal abilities, are especially vulnerable, making them valuable models for studying biodiversity dynamics. The Neotropical frog *Physalaemus centralis*, endemic to the Cerrado, offers an ideal case to investigate how historical climatic fluctuations, geological events, and ecological barriers have shaped genetic diversity and population structure. Its wide distribution across the Cerrado encompasses diverse ecological conditions and potential barriers, providing a representative model for understanding how environmental heterogeneity influences evolutionary processes. By analyzing the phylogeographic patterns of *P. centralis* using mitochondrial DNA (16S rRNA) from 66 samples across its range, we identified two distinct genetic groups that diverged approximately 4.99 million years ago during the Miocene-Pliocene transition. These groups exhibit contrasting genetic diversity and geographic distributions, influenced by historical climatic shifts and ecological constraints. The Bayesian Skyline Plot indicated population stability in both groups, while ecological niche models highlighted the role of past climatic changes in shaping habitat connectivity and refugia. These findings underscore the Cerrado's dual role as both a refuge and a driver of diversification, emphasizing its importance in the evolutionary history of *P. centralis*. This study provides key insights into the processes driving and maintaining biodiversity in this threatened biome, forming a foundation for targeted conservation efforts to preserve the genetic and ecological resilience of *P. centralis* and other Cerrado species.

Key words: Phylogeography, ecological niche modeling, Diversification, Cerrado biodiversity, Mitochondrial DNA

Introduction

The Cerrado, recognized for its unique and irreplaceable biodiversity, is one of the planet's most significant ecological regions, hosting endemic species and a complex ecological structure (Klink & Machado, 2005; Sano et al., 2019). Over its evolutionary history, this region has experienced profound geoclimatic transformations that played a crucial role in shaping its biodiversity. Geological events such as the uplift of the Central Plateau and the formation of major river basins have modified ecological connectivity, creating barriers and corridors that influenced species distribution and population genetic structure (Rossetti et al., 2005; Fonseca et al., 2021). Additionally, climatic changes during the Pleistocene intensified these processes, with cycles of habitat expansion and contraction facilitating migrations, dispersals, and, in some cases, the emergence of new lineages (Guillory et al., 2024). Physical barriers like rivers and mountains often led to geographic isolation, driving allopatric speciation (Oliveira et al., 2018; de Oliveira et al., 2019). Meanwhile, gene flow among adjacent populations contributed to the maintenance of genetic diversity, while hybridization in contact zones generated novel genetic combinations, increasing variability (Góes et al., 2019; Muniz et al., 2022).

Studies on different organisms highlights the extensive impact of these processes in the Cerrado. Research on plants (Diniz-Filho et al., 2015), mammals (Machado et al., 2018), birds (Cabanne et al., 2019), amphibians (Vasconcellos et al., 2019), and reptiles (Domingos et al., 2017) have demonstrated how environmental changes and geographic barriers have influenced species evolution and diversification. Amphibians, in particular, are considered good models for investigating evolutionary dynamics due to their high responsiveness to environmental changes and complex genetic structure (Magalhães et al., 2020; Santana et al., 2024a). Among the many Cerrado endemic species, the foam nest frog *Physalaemus centralis* (Bokermann, 1962) serves as a valuable example for studying the diversification within the biome. It is widely distributed across the entire ecoregion (Valdujo et al., 2012; Frost, 2024). Its wide distribution provides a good model for investigating how historical and geoclimatic factors have influenced its population dynamics.

The compartmentalization of the Cerrado and its surrounding regions has intensely influenced the evolutionary trajectories of numerous species. Previous studies provide insights into these dynamics, revealing how different taxa have responded to historical and environmental factors. For instance, climatic fluctuations during the

Pleistocene had significant impacts on species such as *Boana lundii*, with changes in habitat availability driving patterns of population expansion and contraction (Vasconcelos et al. 2019). Topographic features, including mountain ranges, have been shown to influence genetic differentiation in *Dermatonotus muelleri*, where geographic isolation played a key role in promoting allopatric speciation (Oliveira et al. 2018). Additionally, the river systems also exhibit a dual influence, acting as barriers to gene flow in certain regions while facilitating dispersal in others, as demonstrated in studies of the paradoxical frog *Pseudis bolbodactyla* (Santana et al. 2024). Collectively, these findings illustrate the complex interplay of historical processes and geographic features in shaping patterns of diversification across the Cerrado.

Based on the understanding that historical climatic and geomorphological processes in the Cerrado have shaped the evolutionary dynamics of organisms, this study investigates the genetic diversification of *Physalaemus centralis* using mitochondrial markers. We aim to uncover the historical and ecological factors driving the species' evolutionary history by: (1) assessing its genetic variation across its range, (2) identifying distinct populations through structure analyses, (3) evaluating historical demographic patterns, and (4) examining the influence of climatic niche shifts on diversification.

Material and Methods

We analyzed 66 sequences of *Physalaemus centralis* obtained from 33 locations across its known geographic range in the Cerrado biome (Figure 1). The sampling was designed to include both central and peripheral areas to capture genetic and geographic variability. To root the phylogenetic tree, we included one sequence of *Physalaemus kroyeri* (Reinhardt and Lütken, 1862) as an outgroup. The GenBank accession numbers and genetic vouchers for all specimens analyzed are provided in Table S1.

Tissue samples were obtained through loans or donations from herpetological collections and research laboratories. Mitochondrial DNA was extracted using the Blood & Tissue DNA Mini Kit® (Ludwig Biotecnologia, Brazil). We amplified the mitochondrial 16S rRNA gene using specific primers, 16sAR (CGCCTGTTTATCAAAAACAT) and 16sBR (CTCCGGTTTGAACTCAGATCA) (Palumbi et al., 1991). PCR reactions were prepared with 11.25 μL of GoTaq G2 Hotstart DNA polymerase (Promega), 7.05 μL of Milli-Q® water, and 0.6 μL of each primer, following thermal cycling conditions as described by Costa et al. (2016). PCR products were purified using the Ammonium Acetate technique (Irwin et al., 2003), and their quality was assessed with a NanoDrop® ND-1000 spectrophotometer (Thermo Scientific, USA). Sequencing was performed by ACTGene Molecular Analyses, and the resulting sequences were edited using Geneious® R9 9.1.3 (Biomatters Ltd, Auckland, New Zealand).

Phylogenetic Analysis

We combined our newly generated 16S sequences with all comparable 16S sequences of *Physalaemus centralis* available on GenBank. We aligned the 16S gene fragments using MAFFT (Katoh et al. 2002) implemented in Geneious v.9.0.5 with the algorithm set as auto, which selects an appropriate strategy from L-INS-i, FFT-NS-I, and FFT-NS-2. The final alignment comprised 66 sequences of a 376 base-pairs (bp). All GenBank accession numbers and genetic vouchers used here are listed in Table S1. To explore evolutionary relationships among *P. centralis* populations, we estimated a Bayesian phylogenetic tree based on the 16S rRNA gene using BEAST 2.7 software (Bouckaert et al., 2019). The optimal substitution model, HKY+G, was determined using jModelTest (Darriba et al., 2012). A Yule speciation model was applied and the clock rate was set to 0.0026 substitutions per million years (Evans et al., 2004; Lemmon et al., 2007; Miranda et al., 2019). We ran the analysis for 50 million generations, sampling every 5000 generations, with a 10% burn-in discarded. The maximum clade credibility tree, including median node ages, was calculated using TreeAnnotator v.1.8 (Drummond et al., 2012). Effective sample sizes (ESS) and parameter stationarity were assessed using Tracer 1.7.1 software (Rambaut et al., 2018), ensuring all ESS values exceeded 200.

Table S1. Species, specimen voucher numbers, GenBank accession numbers for sequence data, collecting locality information, GPS coordinates, Group of *Physalaemus centralis*, and references for all samples included in molecular analyses. G1 = Group 1; G2 = Group 2. * Sequences awaiting inclusion in Genbank.

Species	Voucher	Genbank Accession	Locality	Latitude	Longitude	Group	Reference
P. centralis	AS0193	JF789845	Santa Cruz, San Sebastian, BOL	-16.3596	-62.0001	G1	Jansen et al. 2011
P. centralis	AS0268	JF789846	Santa Cruz, Caparu, BOL	-14.9121	-61.0825	G1	Jansen et al. 2011
P. centralis	AS0517	JF789848	Beni, Trinidad, BOL	-12.7720	-65.8109	G1	Jansen et al. 2011
P. centralis	AS0545	JF789844	Santa Cruz, San Sebastian, BOL	-16.3596	-62.0001	G1	Jansen et al. 2011
P. centralis	AS0566	JF789847	Santa Cruz, San Sebastian, BOL	-16.3596	-62.0001	G1	Jansen et al. 2011
P. centralis	AS281	KF723148	Santa Cruz, Caparu, BOL	-14.9121	-61.0825	G1	Schulze et al. 2013
P. centralis	AS476	KF723151	Beni, Trinidad, BOL	-12.7720	-65.8109	G1	Schulze et al. 2013
P. centralis	AS521	KF723152	Beni, Trinidad, BOL	-12.7720	-65.8109	G1	Schulze et al. 2013
P. centralis	CFBHT02825	MW201198	Tocantins, Caseara, BRA	-10.4000	-49.9650	G1	Loebmann et al. 2017
P. centralis	CFBHT03525	MW201201	Tocantins, Wanderlândia, BRA	-6.8850	-47.9270	G1	Loebmann et al. 2017
P. centralis	CFBHT04709	MW201199	Mato Grosso, Chapada dos Guimarães, BRA	-15.4510	-55.8430	G1	Loebmann et al. 2017
P. centralis	CFBHT09127	MW201202	Bahia, Caetite, BRA	-14.2610	-42.5350	G1	Loebmann et al. 2017
P. centralis	CFBHT09160	MW201196	Bahia, Caetite, BRA	-14.2610	-42.5350	G1	Loebmann et al. 2017
P. centralis	CFBHT11311	MW201193	Piauí, Piracuruca, BRA	-3.9520	-41.6810	G1	Loebmann et al. 2017
P. centralis	CFBHT11785	MW201205	Minas Gerais, Jaboticatubas, BRA	-19.4880	-43.6130	G1	Loebmann et al. 2017
P. centralis	DS3914	*	Mato Grosso, Santa Cruz do Xingu, BRA	-9.7607	-52.2609	G1	present work
P. centralis	DS3915	*	Mato Grosso, Santa Cruz do Xingu, BRA	-9.7607	-52.2609	G1	present work

P. centralis	DS3923	*	Tocantins, Caseara, BRA	-9.3505	-49.9475	G1	present work
P. centralis	DS3924	*	Tocantins, Porto Nacional, BRA	-10.7040	-48.3072	G1	present work
P. centralis	DS3925	*	Tocantins, Lagoa da Confusão, BRA	-10.6625	-49.8858	G1	present work
P. centralis	DS3926	*	Mato Grosso, Rondonópolis, BRA	-16.4595	-54.5789	G1	present work
P. centralis	DS3931	*	Mato Grosso, Rondonópolis, BRA	-16.4612	-54.5804	G1	present work
P. centralis	DS3932	*	Mato Grosso, Rondonópolis, BRA	-16.4601	-54.5816	G1	present work
P. centralis	DS3933	*	Mato Grosso, Rondonópolis, BRA	-16.4592	-54.5810	G1	present work
P. centralis	DS3938	*	Goiás, Terezópolis de Goiás, BRA	-16.4195	-49.0941	G1	present work
P. centralis	DS3940	*	Goiás, Silvania, BRA	-16.6439	-48.6513	G1	present work
P. centralis	DS3941	*	Mato Grosso, Sinop, BRA	-11.4426	-55.5709	G1	present work
P. centralis	DS3943	*	Mato Grosso, Sinop, BRA	-11.4426	-55.5709	G1	present work
P. centralis	DS3944	*	Mato Grosso, Santa Cruz do Xingu, BRA	-9.7607	-52.2609	G1	present work
P. centralis	DS3945	*	Mato Grosso, Sinop, BRA	-11.4426	-55.5709	G1	present work
P. centralis	DS3952	*	Tocantins, Lagoa da Confusão, BRA	-10.6625	-49.8858	G1	present work
P. centralis	DS3966	*	Tocantins, Pium, BRA	-9.4184	-50.0003	G1	present work
P. centralis	DS3970	*	Tocantins, Caseara, BRA	-9.44627	-49.9095	G1	present work
P. centralis	DS3971	*	Minas Gerais, Buritizeiro, BRA	-17.3571	-44.9766	G1	present work
P. centralis	DS3974	*	Mato Grosso, Guiratinga, BRA	-16.2111	-53.7312	G1	present work
P. centralis	DS3975	*	Mato Grosso, Santa Cruz do Xingu, BRA	-9.7607	-52.2609	G1	present work
P. centralis	DS3976	*	Mato Grosso, Santa Cruz do Xingu, BRA	-9.7607	-52.2609	G1	present work
P. centralis	DS3977	*	Mato Grosso, Santa Cruz do Xingu, BRA	-9.7607	-52.2609	G1	present work
P. centralis	MAP3542	*	Tocantins, Caseara, BRA	-9.3505	-49.9475	G1	present work

P. centralis	MAP4928	*	Tocantins, Caseara, BRA	-9.3505	-49.9475	G1	present work
P. centralis	MAP5110	*	Tocantins, Caseara, BRA	-9.3505	-49.9475	G1	present work
P. centralis	MJ1159	JF789840	Santa Cruz, San Sebastian, BOL	-16.3596	-62.0001	G1	Jansen et al. 2011
P. centralis	MJ1233	JF789841	Santa Cruz, San Sebastian, BOL	-16.3596	-62.0001	G1	Jansen et al. 2011
P. centralis	MJ1245	JF789842	Santa Cruz, San Sebastian, BOL	-16.3596	-62.0001	G1	Jansen et al. 2011
P. centralis	MJ1270	JF789843	Santa Cruz, San Sebastian, BOL	-16.3596	-62.0001	G1	Jansen et al. 2011
P. centralis	MJ1338	JF789850	Beni, Trinidad, BOL	-12.7720	-65.8109	G1	Jansen et al. 2011
P. centralis	MJ1343	JF789849	Beni, Trinidad, BOL	-12.7720	-65.8109	G1	Jansen et al. 2011
P. centralis	MNKA 9280	KF723149	Beni, Trinidad, BOL	-12.7720	-65.8109	G1	Schulze et al. 2013
P. centralis	MNKA 9727	KF723147	Santa Cruz, Caparu, BOL	-14.9121	-61.0825	G1	Schulze et al. 2013
P. centralis	SMF 94359	KF723150	Beni, Trinidad, BOL	-12.7720	-65.8109	G1	Schulze et al. 2013
P. centralis	CFBHT00295	MW201194	São Paulo, Itirapina, BRA	-22.2420	-47.9070	G2	Loebmann et al. 2017
P. centralis	CFBHT00567	MW201200	São Paulo, Itirapina, BRA	-22.2420	-47.9070	G2	Loebmann et al. 2017
P. centralis	CFBHT00576	MW201195	São Paulo, Itirapina, BRA	-22.2420	-47.9070	G2	Loebmann et al. 2017
P. centralis	CFBHT00959	MW201203	São Paulo, Nova Itapirema, BRA	-21.0740	-49.5400	G2	Loebmann et al. 2017
P. centralis	CFBHT11571	MW201197	Mato Grosso do Sul, Três Lagoas, BRA	-20.7700	-51.7100	G2	Loebmann et al. 2017
P. centralis	CFBHT11589	MW201204	Mato Grosso do Sul, Três Lagoas, BRA	-20.7700	-51.7100	G2	Loebmann et al. 2017
P. centralis	DS3918	*	Mato Grosso do Sul, Campo Grande, BRA	-20.5381	-54.7507	G2	present work
P. centralis	DS3919	*	Mato Grosso do Sul, Campo Grande, BRA	-20.5381	-54.7507	G2	present work
P. centralis	DS3954	*	Mato Grosso do Sul, Selvira, BRA	-20.3513	-51.4228	G2	present work
P. centralis	DS3957	*	Mato Grosso do Sul, Costa Rica, BRA	-18.2365	-53.2989	G2	present work
P. centralis	DS3962	*	Mato Grosso do Sul, Coxim, BRA	-18.5365	-54.7862	G2	present work

P. centralis	DS3964	*	Mato Grosso do Sul, Costa Rica, BRA	-18.2365	-53.2989	G2	present work
P. centralis	DS3967	*	Mato Grosso do Sul, Costa Rica, BRA	-18.2365	-53.2989	G2	present work
P. centralis	DS3969	*	Mato Grosso do Sul, Alcinópolis, BRA	-18.1613	-53.4033	G2	present work
P. centralis	DS3972	*	Mato Grosso do Sul, Campo Grande, BRA	-20.5381	-54.7507	G2	present work
P. centralis	JQ627210	JQ627210	São Paulo, São Carlos, BRA	-21.9800	-47.8700	G2	Rodriguez et al. 2012
P. kroyeri	CFBHT13935	MW201208	-	-	-	-	Loebmann et al. 2017

Lineage Delimitation

To test hypotheses regarding genetic differentiation, we conducted a lineage delimitation analysis using the Generalized Mixed Yule Coalescent (GMYC) method (Pons et al., 2006) on the online GMYC platform. This method distinguishes between speciation (Yule model) and coalescent processes. Mitochondrial 16S rRNA sequences were prepared by aligning them in Geneious® R9. Redundant sequences were removed using the Biostrings package in R (Pagès et al., 2023) to avoid artifacts in phylogenetic inference. The resulting alignment was used to construct an ultrametric tree in BEAST v2.5 with the HKY+G substitution model and a Yule speciation process.

Genetic Diversity and Haplotype Network

We constructed a haplotype network using the Median-Joining (MJ) method in PopART (Leigh et al., 2015) to investigate genetic relationships and lineage structure. The network was grouped according to GMYC results.

Based on the groups defined through the GMYC method, we calculated summary statistics for each group using DNAsp v.5 (Librado and Rozas, 2009). The analyzed metrics included: sequence length (in base pairs), number of samples (N), number of polymorphic sites (S), number of haplotypes (H), haplotype diversity (Hd), nucleotide diversity (π), and the P-values for Tajima's D test.

Demographic History

Bayesian Skyline Plots (BSPs) were analyzed for each group using BEAST 2.6, with the aim of inferring historical changes in effective population size (Ne). This approach is essential for understanding how climate fluctuations and geoclimatic events over time have influenced the species' population dynamics. Analyses employed a substitution rate of 0.0026 substitutions per million years and the HKY model, running for 50 million generations. Stationarity and ESS values were assessed using Tracer to ensure robust demographic reconstructions.

Ecological Niche Modeling (ENM)

To assess the possible impact of past climatic oscillations on the genetic structure of *Physalaemus centralis*, we employed an Ecological Niche Modeling (ENM) approach to model the species' potential distribution under different past climate change scenarios. The scenarios included current and historical periods such as the Mid

Holocene (6kya), Last Glacial Maximum (21kya), Last Interglacial (130kya), Marine Isotope Stage MIS19 (790kya), Mid Pliocene Warm (3.2Mya), and Late Pliocene (3.3Mya), using 19 standard Bioclim variables with a 5 arc minutes resolution (Fick & Hijmans, 2017). We analyzed 317 occurrence records before filtering, based on sequences obtained in this study.

To avoid overprediction and low specificity, we restricted the environmental layers to latitudes between -67° and -36° and longitudes between -28° and -1°. Spatial filtering of locality data was performed at a 2 km^2 resolution using the R package *spThin* (Aiello-Lammens et al., 2015) to remove spatial clusters of localities, resulting in 222 independent occurrence points for modeling. To prevent bias related to multicollinearity among environmental explanatory variables, we calculated the Variance Inflation Factor (VIF) for the variables. All highly correlated variables (VIF > 5) were removed through a stepwise procedure, using the R package *usdm* v.1.1-18 (Naimi, 2017). Thus, seven of the 19 bioclimatic variables were retained for this study: Bio4 - Temperature Seasonality (standard deviation × 100), Bio8 - Mean Temperature of Wettest Quarter, Bio13 - Precipitation of Wettest Month, Bio14 - Precipitation of Driest Month, Bio15 - Precipitation Seasonality (Coefficient of Variation), Bio18 - Precipitation of Warmest Quarter, and Bio19 - Precipitation of Coldest Quarter.

The distribution modeling of *P. centralis* was carried out using nine different algorithms implemented in the *biomod2* package (Thuiller et al., 2016) in R 4.1 (R Core Team, 2021), including three regression methods [GAM: Generalized Additive Model (Hastie & Tibshirani, 1990), GLM: Generalized Linear Model (McCullagh & Nelder, 1989), MARS: Multivariate Adaptive Regression Splines (Friedman, 1991)]; three machine learning methods [GBM: Generalized Boosted Model (Ridgeway, 1999), MAXENT: Maximum Entropy (Phillips et al., 2006), RF: Random Forest (Breiman, 2001)]; two classification methods [CTA: Classification Tree Analysis (Breiman, 1984), FDA: Flexible Discriminant Analysis (Hastie et al., 1994)]; and an envelope model [SRE: Surface Range Envelope (Booth et al., 2014)].

The performance of each model was evaluated using two metrics: True Skill Statistic (TSS) and the area under the Receiver Operating Characteristic (ROC) curve, both implemented in the *biomod2* package. TSS is calculated as "sensitivity + specificity - 1" and ranges from -1 to +1, where +1 indicates perfect agreement, 0 indicates agreement expected by chance, and values less than 0 indicate agreement worse than expected by chance. Models with high prediction accuracy (TSS > 0.8) were used to project the distribution of *P. centralis*. Ensemble maps were constructed based on the median of two runs from all selected models with individual TSS values equal to or greater than 0.8. Habitat stability regions over time were identified by overlapping and averaging the projected paleoclimatic ENMs, with areas highlighted in these projections inferred as climatic refugia over time.

Results

Phylogenetic reconstruction revealed two geographically structured groups (Figures 1 and 2). The first group encompasses individuals from Bolívia (San Sebastian, Caparu, and Beni) and the Brazilian states of Mato Grosso, Goiás, Minas Gerais, Bahia, Tocantins, and Piauí (hereafter referred to as Group 1). The second group is composed of individuals from São Paulo and Mato Grosso do Sul (hereafter referred to as Group 2). The estimated divergence between these two groups occurred approximately 4.99 million years ago, with a 95% highest posterior density (HPD) interval of 3.17–7.24 million years (Figure 2).

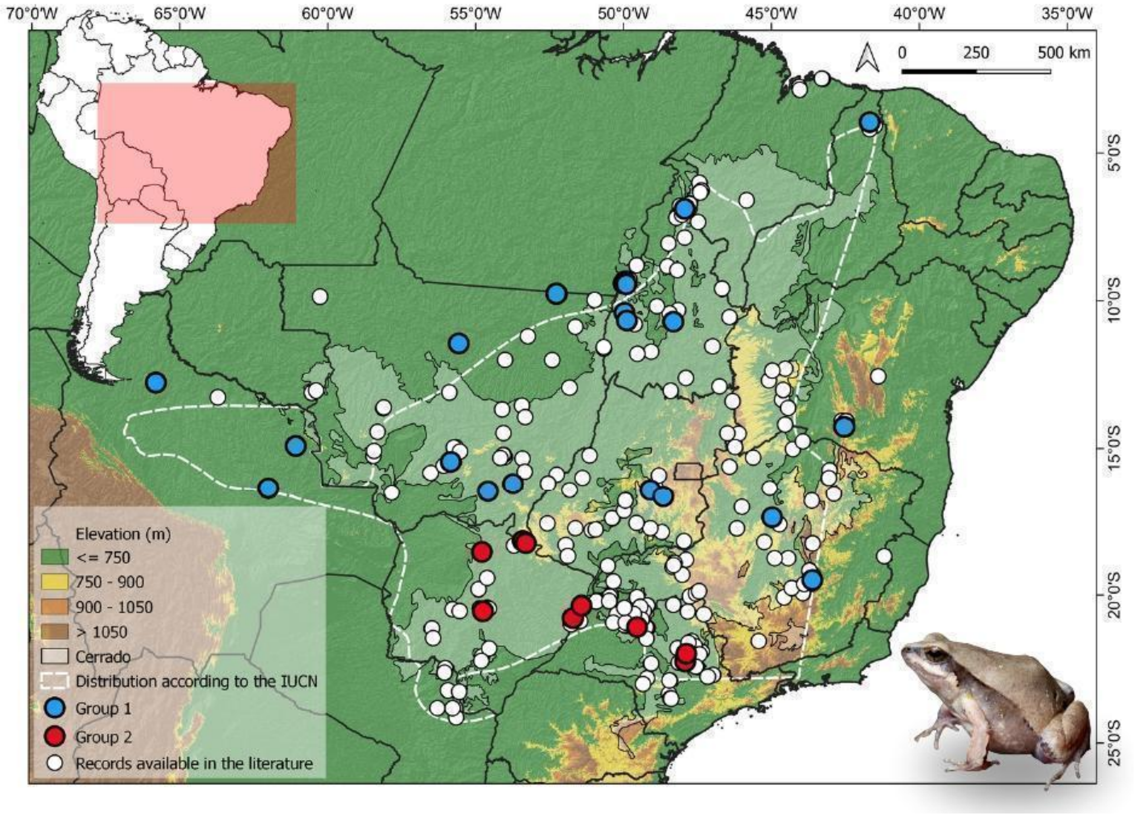

Figure 1. Geographic distribution of *Physalaemus centralis* and samples used in this study. White dots represent records available in the literature, blue dots indicate individuals from Group 1, and red dots represent Group 2, as identified by the GMYC analysis of 16S rRNA mitochondrial gene sequences. The photograph shows a live specimen of *Physalaemus centralis* from the municipality of Guiratinga, state of Mato Grosso, Brazil (Photo by L.A. da Silva).

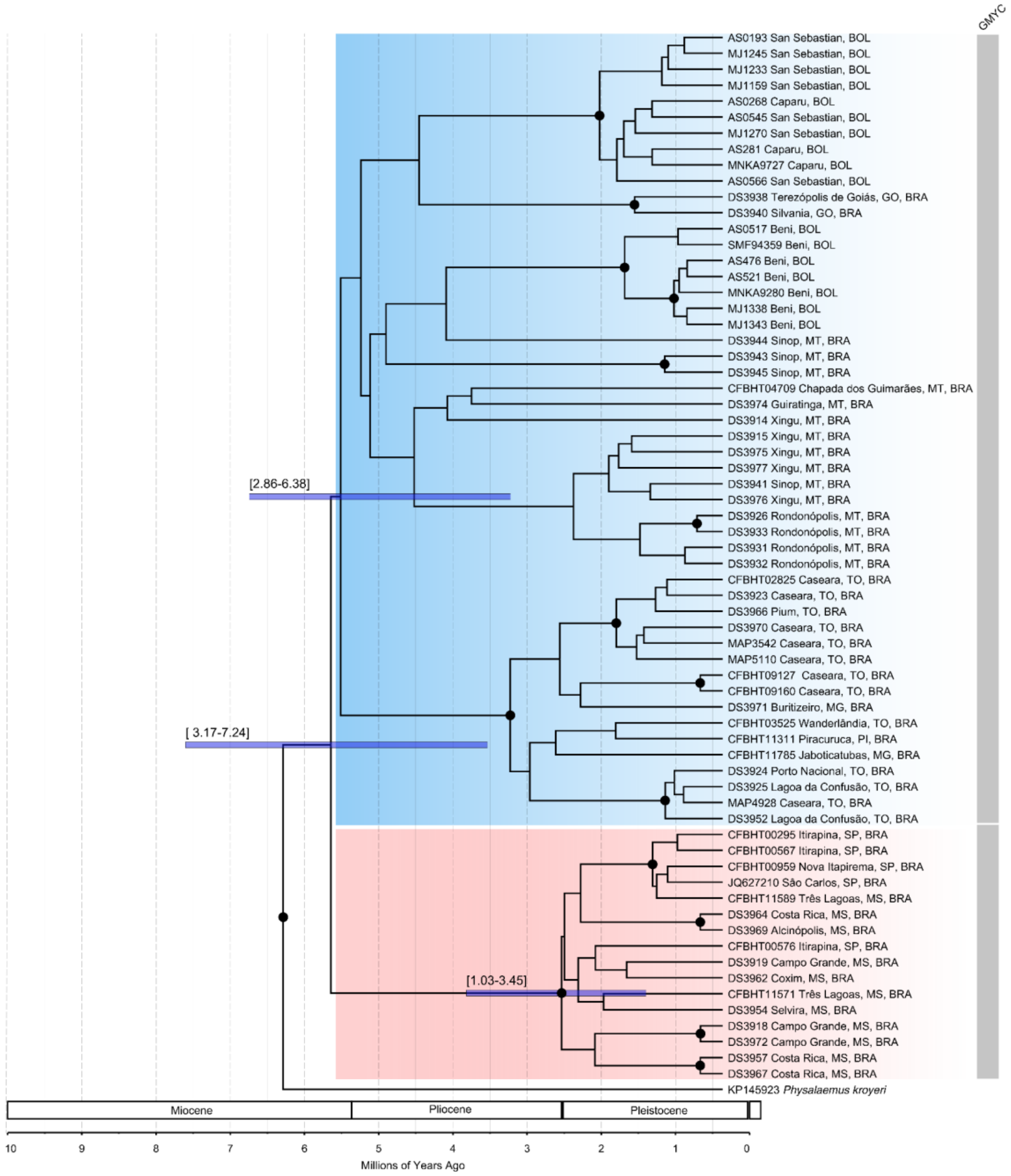

Figure 2. Bayesian phylogeny and divergence times of *Physalaemus centralis* based on analysis of the mitochondrial 16S rRNA gene. Circles on nodes denote significant posterior probability (pp = 0.95–1.0). Average ages in millions of years are shown on nodes and 95% HPDs of node ages are indicated by horizontal blue bars. Vertical gray bars on the right represent the groups delimited by GMYC (Generalized Mixed Yule Coalescent).

The haplotype diversity showed high values for both groups analyzed, ranging from 0.962 in Group 1 to 0.911 in Group 2 (Table 1). Although both groups exhibit high genetic variability, the haplotype network constructed based on the Generalized Mixed Yule Coalescent (GMYC) model visually highlights the absence of haplotype sharing between the two groups (Figure 3).

The Bayesian Skyline Plot (BSP) analyses indicated relatively stable population sizes over time for both groups (Figure 4). Group 2 showed a more recent coalescence point, approximately 300,000 years ago, while Group 1 exhibited a coalescence point around 3 million years ago. Despite these differences, both groups maintained largely stable effective population sizes, with no clear evidence of significant demographic events, such as population bottlenecks or rapid expansions.

Table 1. Summary statistics for each group of the *Physalaemus centralis*. Length in base pairs; N – number of samples; S – polymorphic sites; H – number of haplotypes; Hd – haplotype diversity; π – nucleotide diversity; P-values of the Tajima's D test.

Group	Length (pb)	N	S	H	Hd	π	Tajima's D	p-value
1	376	50	36	32	0,972	0,01771	-0,55108	P>0,10
2	375	16	9	10	0,911	0,00461	-0,69762	P>0,10

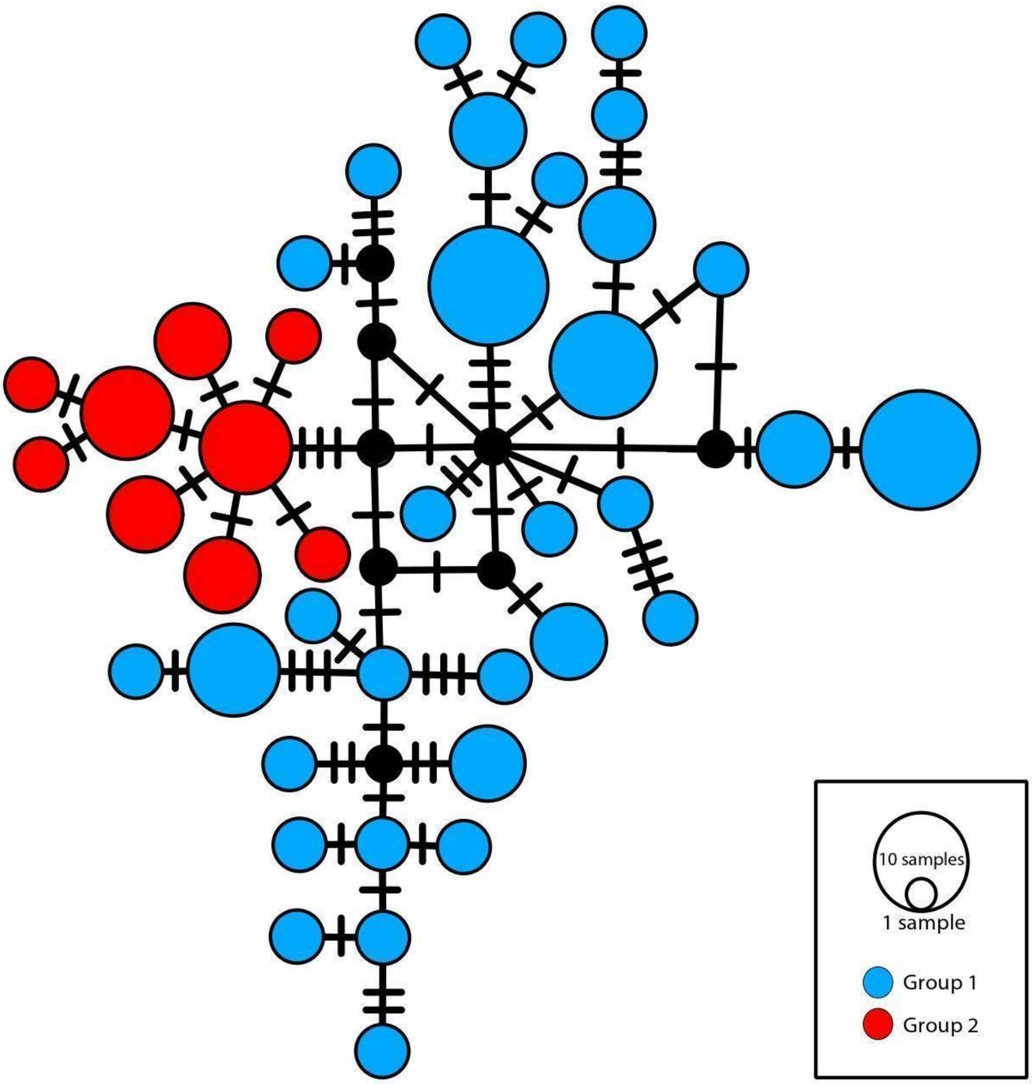

Figure 3. Haplotype networks for the 16S rRNA generated using the software PopArt (Leigh & Bryant, 2015). Circle size represents the frequency of each haplotype, with groups depicted in blue (Group 1) and red (Group 2) recovered by the GYMC analyses. The black dots are median vectors (hypothesized sequences).

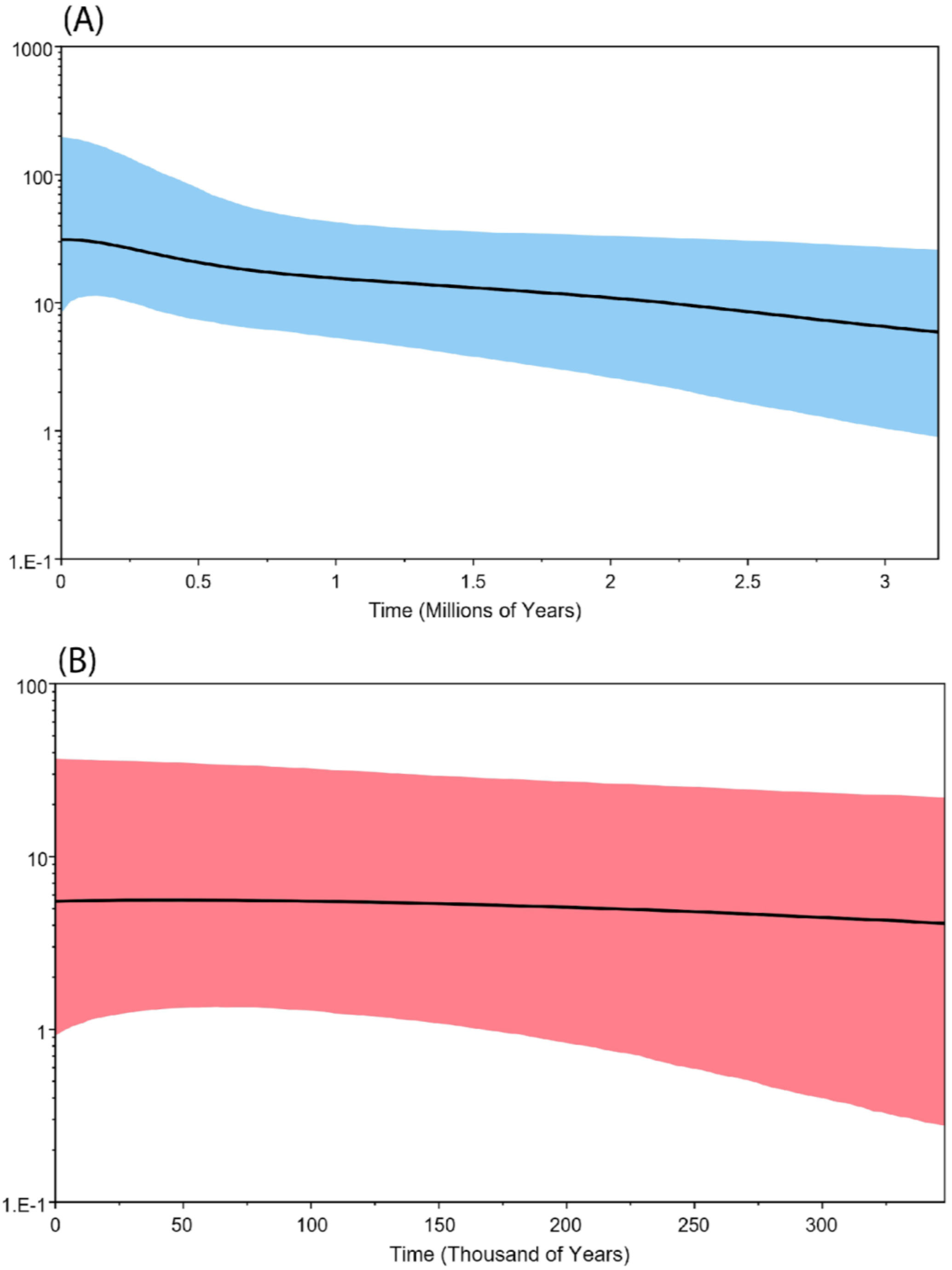

Figure 4. Demographic reconstructions over time using Bayesian Skyline Plots (BSP) for each group of *Physalaemus centralis*. *Ne* represents the effective population size over time (in years). (A) Group 1 and (B) Group 2. The black line indicates the median population size, with the 95% highest posterior density interval shown by the outer blue and red lines.

Ecological niche modeling revealed significant changes in the species’ potential distribution over time in response to climatic fluctuations (Figure 5). During the Late Pliocene, around 3.3 million years ago, the species niche occupied a broad range with highly suitable habitats extending into eastern and northern Brazil. In the Middle Pliocene, approximately 3.2 million years ago, suitable habitats became fragmented, reflecting a more restrictive climate. By Marine Isotope Stage 19, about 790,000 years ago, the species’ niche range expanded, but high-suitability areas remained concentrated in the central and northwestern Cerrado. The Last Interglacial period, around 130,000 years ago, showed a further reduction in suitable areas, with habitats primarily restricted to the central Cerrado. In contrast, during the Last Glacial Maximum, approximately 21,000 years ago, the species niche experienced a significant expansion of highly suitable areas, particularly in the central Cerrado, which enhanced habitat connectivity. The Holocene distribution closely mirrors the present day, with high-suitability areas concentrated in central Brazil and slight expansions into adjacent regions.

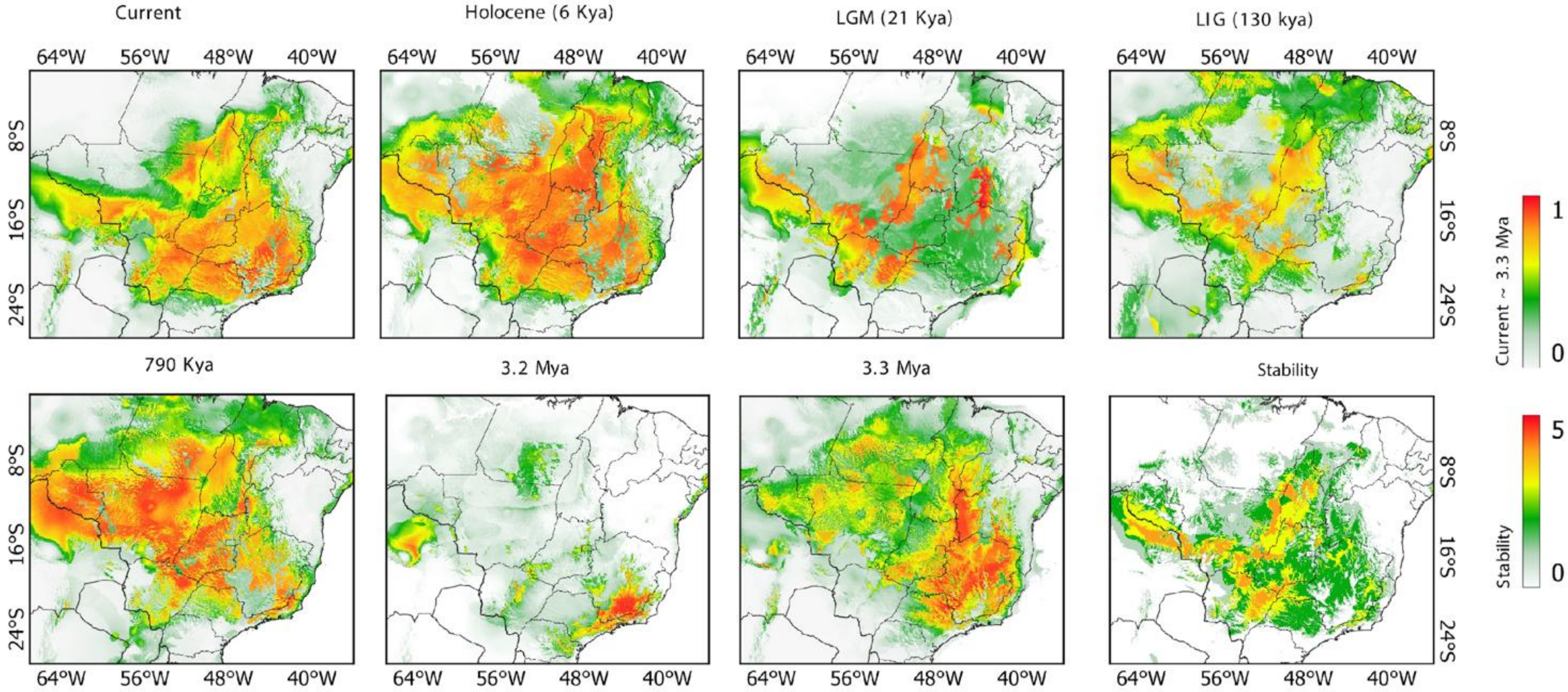

Figure 5. Ecological Niche Models (ENMs) for *Physalaemus centralis*. The top row displays ENMs under current climate conditions, Mid-Holocene (~6 kya), Last Glacial Maximum (LGM, ~21 kya), and Last Interglacial (LIG, ~130 kya). The bottom row shows models for Marine Isotope Stage 19 (MIS19, ~790 kya), Mid Pliocene Warm Period (~3.2 Mya), Late Pliocene (~3.3 Mya), and regions of climate suitability over time based on all projections.

Discussion

Our results highlight the influence of historical geological and climatic events on the diversification of *Physalaemus centralis* across its distribution and emphasize the role of ecological and environmental barriers in shaping the species' genetic structure. The analysis of geographic structuring revealed that the divergence between the two groups of *P. centralis* occurred approximately 4.99 million years ago, during the transition between the Miocene and Pliocene periods. This interval was marked by significant events, such as the final uplift of the Central Brazilian Plateau (Del'Arco & Bezerra, 1989), which contributed to the compartmentalization of the Cerrado and surrounding biomes. These changes profoundly impacted regional biodiversity, shaping the evolutionary trajectories of various taxa, including amphibians (Oliveira et al., 2018), plants (Diniz-Filho et al., 2015), mammals (Almeida et al., 2007), and reptiles (Werneck & Colli, 2006).

The genetic structuring detected by GMYC analysis reflects the effects of these historical changes. Two genetically distinct groups were identified, exhibiting contrasting patterns of geographic distribution and genetic diversity. Group 1, which is widely distributed across central-northern Brazil and Bolivia, and Group 2, restricted to São Paulo and Mato Grosso do Sul, suggest prolonged isolation with limited gene flow. This population separation is likely explained by the Central Brazilian Plateau acting as a geographic barrier—a phenomenon previously documented in other vertebrates from the region, such as *Rhinella marina*, *Boana albopunctata*, and *Dermatonotus muelleri* (Maciel et al., 2010; Prado et al., 2012; Oliveira et al., 2018). These examples underscore how the compartmentalization of the Cerrado and historical climatic changes have shaped the evolutionary patterns of numerous species, including the divergence observed in *P. centralis*.

Historical demographic patterns, evaluated through Bayesian Skyline Plots (BSP), further complement the genetic structuring results, revealing that *P. centralis* populations have maintained stable population sizes over time. This stability suggests a degree of resilience to environmental changes, even during periods marked by significant climatic oscillations, such as those of the Miocene-Pliocene transition and the Pleistocene. These demographic findings align with the proposed evolutionary history of the species and reinforce the role of ecological and geographic barriers in preserving the observed genetic diversity.

Ecological niche models provide a spatial perspective that complements these findings, emphasizing the shift of suitable habitats towards the central Cerrado over time. This dynamic ultimately led to the consolidation of current *P. centralis* populations in regions of higher suitability since the Holocene, consistent with patterns documented for squamate reptiles in the Cerrado (Werneck et al., 2012). The interplay between environmental suitability and historical climatic and geological events played a crucial role not only in the genetic structuring of the species but also in the stabilization of its populations.

In conclusion, our findings reinforce the importance of the Cerrado as a biodiversity hotspot, where physical and ecological barriers, combined with historical climatic fluctuations, have collectively shaped the diversification patterns of the regional biota. The study of *P. centralis* highlights how historical and ecological processes can interact to generate and maintain the rich genetic and ecological diversity of amphibians within this biome.

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