Francisco Humberto Dal Vechio Filho

Filogenia e revisão sistemática das espécies de *Bothrops* do grupo *jararacussu* (Serpentes, Crotalinae), com teste de hipóteses biogeográficas para a existência de contato florestal pretérito Amazônia-Mata Atlântica

Phylogeny and systematics of *jararacussu* species group of *Bothrops* (Serpentes, Crotalinae), with test of biogeographic hypothesis to the past connection Amazonia-Atlantic Forest

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Prof(a). Dr(a).

Prof(a). Dr(a).

Prof(a). Dr(a)

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Dedicatória

Dedico todo meu empenho e comprometimento
à minha amada esposa
ao meu precioso pai
e à minha fenomenal irmã.

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Índice

Introdução Geral

A família Viperidae é representada por três sub-famílias: Viperinae possuindo 102 espécies; Azemiopinae duas, e Crotalinae, a mais diversa, 243 espécies (Uetz, 2019). *Bothrops* é um dos 12 gêneros de crotalíneos com ocorrência no novo mundo, distribuíndo-se por diversos ambientes da America Central e do Sul, inclusive em ilhas oceânicas (Campbell & Lamar 2004). Quarenta e cinco espécies estão alocadas em seis grupos distintos: *alternatus*, *jararaca*, *neuwiedi*, *atrox*, *taeniatus* e *jararacussu* (*sensu* Wuster *et al*., 2002; Fenwick *et al*., 2009; Dal Vechio *et al*., 2018).

A literatura de *Bothrops* tem se mantido aquecida nos últimos anos, com hipóteses filogenéticas (Fenwick *et al*., 2009; Carrasco *et al*., 2012; Alencar *et al*., 2016), descrições de novas espécies (Da Silva & Rodrigues, 2008; Folleco-Fernandez, 2010; Barbo *et al*., 2012, 2016; Dal Vechio *et al*., em preparação) e estudos filogenéticos e biogeográficos (Machado *et al*., 2012; Dal Vechio *et al*., 2018; Dal Vechio *et al*., aceito). Contudo, os trabalhos genéticos têm, recorrentemente, recuperado diversidade críptica e dificuldades na delimitação de espécies para os grupos: *jararaca*, *neuwiedi*, *taeniatus* e *jararacussu* (Grazziotin *et al*., 2006; Machado *et al*., 2012; Dal Vechio *et al*., 2018; Dal Vechio *et al*., aceito), realçando a necessidade de um aprofundamento sistemático e estudos que investiguem sua diversificação.

Dentre as espécies em *Bothrops* cuja taxonomia e relações filogenéticas são ainda pouco conhecidas estão os membros do grupo *jararacussu*. Nesse grupo, o status taxonômico de populações isoladas na mata atlântica e populações com variação fenotípica pela Amazônia é incerto (Cunha & Nascimento, 1975, 1978, 1993;

Campbell & Lamar, 2004). Além disso, permanece como não testado o posicionamento filogenético de *B. pirajai*, *B. muriciensis* e *B. sanctaecrucis*, espécies alocadas no grupo com base em caracteres morfológicos (Amaral, 1923; Hoge, 1953; Ferrarezzi & Freire, 2001), enquanto que os dados genéticos confirmam a relação de irmandade entre *B. brazili* e *B. jararacussu* (Fenwick *et al*., 2009, Carrasco *et al*., 2012, Alencar *et al*., 2016; Dal Vechio *et al*., 2018).

As espécies do grupo *jararacussu* são caracterizadas por possuírem hábitos terrestres e serem exclusivas de áreas florestadas de baixas altitudes, até 700m (Campbell & Lamar, 2004). *Bothrops brazili* é amplamente distribuída pelas terras baixas da Amazônia cis-Andina, em ambientes de mata primária não alagada, apresentando marcada variação fenotípica entre populações ao norte e ao sul do Rio Amazonas (Cunha & Nascimento, 1975,1978, 1993).

Já *Bothrops jararacussu*, *B. pirajai* e *B. muriciensis* ocorrem na Mata Atlântica brasileira, sendo as duas últimas com distribuição restrita ao corredor central na Bahia (sul de Elísio Medrado até Ilhéus) e à Estação Ecológica de Murici, Alagoas, no corredor norte, respectivamente. *Bothrops jararacussu* distribui-se do extremo sul da Bahia (em áreas de planalto) até o norte do Rio Grande do Sul, ocorrendo nas florestas interioranas semideciduais de São Paulo, Paraná e Santa Catarina, adentrando continentalmente pelo nordeste da Argentina, em Missiones, leste do Paraguai e sul/oeste do Mato Grosso do Sul, com registro impreciso para o sul da Bolívia, ocorrendo também em algumas ilhas da plataforma continental de São Paulo. Por último, *Bothrops sanctaecrucis* é endêmica da Bolívia, das florestas úmidas de montanha de baixas altitudes (Ferrarezzi & Freire, 2001; Campbell & Lamar, 1989, 2004; Argôlo, 2004; Harvey *et al*., 2005, Freitas, 2008).

O grupo *jararacussu* ganha interesse biogeográfico a nível continental, por ter suas espécies distribuídas de forma disjunta Amazônia – Mata Atlântica. Conexão florestal pretérita entre esses dois domínios é citada para diferentes grupos da fauna e flora (Aves: Cabanne *et al.*, 2008, Batalha-Filho *et al*., 2013; Mamíferos: Vivo, 1997; Costa, 2003; Lagartos: Pellegrino *et al*., 2011, Prates *et al*., 2016a,b, 2017; Anuros: Fouquet *et al.*, 2012; Serpentes: Zamudio & Green, 1997; Dal Vechio *et al*., 2018; Plantas: Martini *et al.*, 2007) e que para alguns casos pode ser explicada no contexto da teoria de refúgios florestais (Haffer, 1969), associada com as mudanças climáticas do Quaternário. Essa teoria sugere que durante períodos frios e secos, as florestas Neotropicais se retrairiam, ao passo que nos períodos quentes e úmidos elas se expandiriam, possibilitando o contato físico entre Amazônia e a Mata Atlântica, favorecendo o intercâmbio faunístico entre elas (Haffer, 1969).

Entre os possíveis contatos florestais que poderiam ter ocorrido por diferentes rotas geográficas e períodos temporais é sugerido um contato mais antigo, durante o Mioceno (entre 23 – 5 milhões de anos atrás), pela parte sudoeste da Amazônia (Rondônia, norte do atual Chaco no estado do Mato Grosso, Mato Grosso do Sul, leste da Bolívia e norte-leste do Paraguai) com a região oeste da Mata Atlântica da Bacia do Paraná (Batalha-Filho *et al*., 2013, Ledo & Colli, 2017; Prates *et al*., 2017). Outro, mais recente (Plio/Pleistoceno – últimos 5 milhões de anos), teria ocorrido através da expansão de áreas florestadas pela Caatinga e pelo Cerrado norte, ou mesmo pela costa nordeste do Brasil (Maranhão, Piauí, Ceará, Rio Grande do Norte, Pernambuco) (Costa 2003, Batalha-Filho *et al*., 2013; Prates *et al*., 2016a,b; Dal Vechio *et al*., 2018). Ambas as vias de contato são plausíveis de terem ocorrido no caso do grupo jararacussu: (1) pelo nordeste brasileiro, uma vez que *Bothrops muriciensis* assemelha-se morfologicamente *B. brazili* e (2) pelo sudoeste amazônico devido às

semelhanças encontradas entre *B. sactaecrucis* e *B. brazili*. *Bothrops jararacussu* e *B. pirajai* se assemelham morfologicamente entre si e poderiam ter origem em qualquer uma das áreas geográficas ocupadas atualmente por essas duas linhagens.

Desse modo, para se investigar o dinamismo das paisagens Neotropicais ao longo do tempo, com teste de hipótese biogeográfica para conexão e fragmentação florestal pretérita entre Amazônia e Mata Atlântica, torna se crucial o estudo da demografia populacional histórica das espécies do grupo *jararacussu*. Além disso, uma análise comparativa com a história evolutiva de *Bothrops bilineatus* e *B. atrox-B.leucurus*, que ocorrem em simpatria com as espécies do grupo *jararacussu*, num padrão de distribuição geográfica semelhante, apresentando similar preferência ecológica por ambientes florestados (Campbell & Lamar, 2004), pode fornecer evidências acerca do tempo histórico, rotas geográficas e direcionalidade para o intercâmbio faunístico entre esses blocos florestados. Adicionalmente, o teste complementar, que considera a perda de conectividade florestal Amazônia-Mata Atlântica e estabilização da diagonal sul-americana de áreas abertas, dar-se-ia a partir da análise da assinatura genética da dinâmica populacional de *Crotalus durissus*, espécie exclusiva de ambientes abertos e amplamente distribuída no Cerrado e Caatinga.

Assim os objetivos gerais dessa tese são:

1. Testar a validade das espécies do grupo *jararacussu*, assim como a relação de parentesco entre elas, apresentando uma hipótese filogenética para o grupo, estimando a data de divergência entre as linhagens e elucidando seu padrão de diversificação;
2. Testar os efeitos da fragmentação de um suposto corredor florestal pretérito para a diversificação de linhagens que atualmente ocorram em distribuição relictual

Amazônia e Mata Atlântica, utilizando-se de datação molecular e dados filogeográficos para as espécies de crotalíneos exclusivos de ambientes florestados: *Bothrops bilineatus*, *Bothrops* do grupo *jararacussu*, *Bothrops* do grupo *atrox* (*B. atrox* e *B. leucurus*);

3. Testar complementarmente os efeitos da aridificação pretérita no clima regional para a expansão recente de *Crotalus durissus* pelas áreas abertas do Cerrado e da Caatinga, utilizando dados filogeográficos, parâmetros demográficos e datação molecular.

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Capítulo 1

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Rainforest shifts through time and riverine barriers shaped the diversification of terrestrial South American pit vipers (*Bothrops jararacussu* species group)

Neotropical pit viper biogeography

Francisco Dal Vechio[1*], Ivan Prates[2], Felipe G. Grazziotin[3], Hussam Zaher[4], Roberta Graboski[4], Miguel Trefaut Rodrigues[1]

[1]Departamento de Zoologia, Instituto de Biociências, Universidade de São Paulo, Rua do Matão, Trav. 14, nº 321, Cidade Universitária, CP 11461, CEP 05508-090, São Paulo, SP, Brazil.

[2]Department of Vertebrate Zoology, National Museum of Natural History, Smithsonian Institution, Washington, DC, 20560, USA.

[3]Laboratório de Coleções Zoológicas, Instituto Butantan, CP 42694, CEP 04299-970, São Paulo, SP, Brazil.

[4]Museu de Zoologia, Universidade de São Paulo, CP 42694, CEP 04299-970, São Paulo, SP, Brazil.

[*]Corresponding author. E-mail address: francisco.dalvechio@gmail.com

Key words: Forest refugia, phylogeography, biogeography, Crotalinae, *Bothrops pirajai*, *Bothrops muriciencis*.

ABSTRACT

Aim: To investigate (1) historical biogeographical connections and species interchange among rainforest habitats and (2) the role of riverine barriers on population divergence and speciation in the Neotropical region.

Location: Amazonia and Atlantic Forest in South America.

Taxon: *Bothrops jararacussu* species group (Serpentes: Viperidae)

Methods: We inferred phylogenetic relationships within *Bothrops* with an emphasis on the *jararacussu* species group under a Bayesian framework based on a multi-locus approach including six molecular loci. We also used genetic coalescent simulations and Approximate Bayesian Computation to infer historical demography within the *jararacussu* group based on tests of alternative scenarios.

Results: We found the *jararacussu* species group to be monophyletic. The Atlantic Forest species *B. pirajai* and *B. muriciensis* were inferred nested within this group, closely related to *B. jararacussu*, confirming that Atlantic Forest species form a clade. The historical demographic analyses support vicariant separation between populations of *B. brazili* north and south of the Amazon River during the late Miocene, as well as colonization of the Atlantic Forest by a northern Amazonian ancestor in the Pleistocene.

Main Conclusion: The evolutionary history of the *jararacussu* species group sheds light on the dynamism of Neotropical rainforests over time, with at least one event of forest expansion leading to faunal interchange between Amazonian and Atlantic forests in the Plestocene. Moreover, tests of alternative demographic scenarios suggest that the populations of *B. brazili* from north and south of the Amazon River originated from a vicariant event during the late Miocene, in agreement with the proposed age of establishment of the modern Amazon River drainage. Our results also have taxonomic

implications for these medically important venomous snakes, supporting unrecognized diversity at the species level.

Resumo

Objetivo: Investigar (1) as conexões biogeográficas ao longo do tempo histórico e o intercâmbio de espécies entre as florestas tropicais e (2) o papel dos rios como barreiras na diversificação e especiação de linhagens na região Neotropical.

Local: Amazônia e Mata Atlântica, América do Sul.

Taxon: Grupo *jararacussu* em *Bothrops* (Serpentes: Viperidae)

Métodos: Nós inferimos as relações filogenéticas em *Bothrops* com ênfase no grupo *jararacussu* sob perspectiva Bayesiana, baseando-se em *dataset* multi-locus com seis *loci*. Nós também usamos simulações coalescentes e ABC (*Approximate Bayesian Computation*) para inferior a história demográfica das espécies do grupo *jararacussu*, com base em teste de cenários alternativos.

Resultados: O grupo *jararacussu* foi recuperado como monofilético, com as espécies *B. pirajai* and *B. muriciensis* alinhadas dentro dele e filogeneticamente próximas a *B. jararacussu*, confirmando um clado Atlântico para o grupo. As análises de demografia histórica suportam vicariância entre as populações de *B. brazili* ao norte e sul do Rio Amazonas durante o Mioceno tardio, assim como colonização da Mata Atlântica por ancestral do norte do Rio Amazonas durante o Pleistoceno.

Conclusões: A história evolutiva do grupo *jararacussu* traz resultados sobre o dinamismo das florestas tropicais no Neotrópico ao longo do tempo, com pelo menos um evento de expansão florestal, levando ao intercâmbio de fauna entre a Amazônia e a Mata Atlântica durante o Pleistoceno. Adicionalmente, teste alternativo de cenários demográficos sugerem que as populações de *B. brazili* ao norte e sul do Rio Amazonas

se originaram através de um evento vicariante durante o Mioceno Tardio, em concordância com tempo proposto para a formação e estabilização da drenagem moderna do Rio Amazonas. Nossos resultados ainda têm implicações taxonômicas para essas serpentes venenosas de importância médica, suportando diversidade não reconhecida a nível de espécie.

1. INTRODUCTION

Since the 19th century, scientists have worked on the description of spatial patterns of biodiversity in the Neotropical region, and on uncovering the evolutionary and ecological factors that underlie these patterns. Questions such as "how have changes in landscape over time influenced the diversification of lineages?", "why are certain regions more diverse than others?", and "what are the factors that shape species' distributions?" have motivated inferences of the history of species and clades, providing insights on how environmental shifts through time have contributed to current species distributions. Among these environmental drivers, climate-driven changes in habitat distribution and the establishment of rivers and mountains have been considered major catalysts of speciation (Haffer, 2008; Hoorn *et al.*, 2010).

In the highly diverse Amazonian lowlands, large rivers such as the Amazon, Negro, Madeira, and Tapajós have been associated with species range limits and turnover, suggesting that these rivers act as biogeographic barriers (Wallace, 1852). More recently, the advancement of molecular techniques allowed tests of how rivers contribute to spatial patterns of genetic structure in Amazonian organisms, revealing that riverine barriers correlate with lineage turnover in several animal groups (Birds: Aleixo, 2004; Heyes & Sewlal, 2004; Ribas *et al.*, 2012; Pomara *et al.*, 2014; Primates: Ayres & Clutton-Brock, 1992; Boubli *et al.*, 2012; Rodents: da Silva &

Patton, 1998; Carnivores: Eizirik *et al.*, 1998; Frogs: Gascon *et al.*, 2000; Simões *et al.*, 2008). However, whether river formation triggered lineage diversification is still a contentious question (Smith *et al.*, 2014). Did Amazonian rivers promote vicariant separation of lineages at the time of their establishment, or have rivers simply acted as barriers limiting lineage distribution after colonizations across margins? Genetic tests of alternative historical demographic scenarios can help answering this question, because demographic events leave distinct signatures in the DNA of organisms. For instance, a history of colonization of a new region through a founder event predicts a signature of strong population bottleneck, while bottlenecks are not expected in the case of vicariant separation of large and wide-ranged populations (Prates *et al.*, 2016a).

Another influential hypothesis proposed to explain high species diversity in Amazonia is that of Pleistocene rainforest refugia (Haffer, 1969), in which pulses of aridification would have promoted vicariant speciation in isolated forest fragments. The original proposition of this theory has been largely rejected due to lack of evidence of Pleistocene aridity and forest fragmentation (Colinveaux *et al.*, 1996; Bush *et al.*, 2004). Moreover, crown-group ages based on molecular clocks largely predate the Pleistocene in Amazonian organisms (Moritz *et al.*, 2000; Hoorn *et al.*, 2010; Prates *et al.*, 2015). More recently, however, biogeographers have addressed the idea of “mega-refugia” that would correspond to presently disjunct Neotropical forested biomes. Under this new interpretation, habitats such as Amazonia and the Atlantic Forest have acted as historically linked refuge areas; pulses of rainforest expansion led to species interchange across regions, followed by lineage divergence and speciation after forest separation (Prates *et al.*, 2016a; Prates *et al.*, 2017; Dal Vechio *et al.*, 2018; Prates *et al.*, 2018).

The idea of past range expansions across South American rainforests has received increased attention. Proposed historical corridors between Amazonia and the Atlantic Forest include northern bridges through the dry Caatinga in present-day northeastern Brazil and along Brazil's northern coast (Mammals: Costa, 2003; Lizards: Pellegrino *et al.*, 2011; Prates *et al.*, 2016a, Prates *et al.*, 2016b; Birds: Batalha-Filho *et al.*, 2013; Snakes: Dal Vechio *et al.*, 2018), as well as southern bridges between southwestern Amazonia and the southern Atlantic Forest (Mammals: Costa, 2003; Lizards: Prates *et al.*, 2017; Birds: Batalha-Filho *et al.*, 2013; Snakes: Dal Vechio *et al.*, 2018). These forest corridors might have been established at different times in the past, with evidence of northern connections during the Pleistocene and southern ones over the Miocene (Batalha-Filho *et al.*, 2013; Prates *et al.*, 2016a, 2017; Ledo & Colli, 2017; Dal Vechio *et al.*, 2018). Colonizations may have been bidirectional, from Amazonia into the Atlantic Forest and vice versa (Rodrigues *et al.*, 2014; Prates *et al.*, 2016a; Dal Vechio *et al.*, 2018). As such, a general picture about faunal interchange across rainforest habitats in South America is yet to emerge. Tests of alternative historical demographic scenarios on the basis of genetic data can provide insight into the routes, directionality, and timing of former biotic interchange between these proposed "mega-refugia".

In this study, we investigate the role of former landscape changes on Neotropical diversification, using the *Bothrops jararacussu* species group as model. In this clade of South American snakes, both river establishment and climate-driven habitat shifts may have contributed to current species distributions and spatial patterns of genetic structure. These snakes are restricted to forested habitats, with a widespread Amazonian taxon (*B. brazili*) that shows pronounced phenotypic variation between populations from north and south of the Amazon River and an Atlantic Forest

assemblage composed of *B. jararacussu*, *B. pirajai* and *B. muriciensis* (Cunha & Nascimento, 1975, 1978, 1993; Campbell & Lamar, 2004). On the basis of comprehensive geographic sampling and multi-locus data, we perform phylogenetic and phylogeographic inference and tests of alternative historical demographic scenarios using genetic coalescent simulations and approximate Bayesian computation. Our approach sheds light on how past rainforest expansions favored dispersal and how river establishment contributed to speciation in South America. Moreover, our results have implications for the recognition of lineage diversity in this group of medically important venomous snakes.

2. MATERIALS AND METHODS

2.1. Sampling and molecular protocols

We generated new sequence data from 18 specimens in the *jararacussu* species group, including nine samples of *Bothrops brazili*, six *B. jararacussu*, two *B. muriciensis* and one *B. pirajai* (Table 1) (GenBank accession numbers XX-XX). We also used sequences available in GenBank from two *B. brazili* and two *B. jararacussu* individuals. While DNA data have confirmed that *B. jararacussu* and *B. brazili* cluster together in their own clade, the other three species in the *jararacussu* group - *B. pirajai*, *B. muriciensis* and *B. sanctaecrucis* - have been assigned to it based solely on morphological features (Amaral, 1923; Hoge, 1953; Ferrarezzi & Freire, 2001). For broader phylogenetic inference within *Bothrops*, our analysis also included 49 samples representing the other five species groups in the genus (*alternatus*, *atrox*, *jararaca*, *neuwiedi*, and *taeniatus*).

We followed molecular protocols and PCR reaction conditions used by Dal Vechio et al. (2018), sampling nine DNA markers corresponding to five loci (totaling 6,009 bp), as follows: the mitochondrial genes *16s ribosomal RNA* (*16s*, 534 bp; Palumbi, 1996), *12s ribosomal RNA* (*12s*, 394 bp; Palumbi, 1996), *cytochrome b* (*cytb*, 737 bp; Pook *et al.*, 2000), and *NADH dehydrogenase subunit 4* (*nd4*, 798 bp; Arévalo *et al.*, 1994); and the nuclear genes *megakaryoblastic leukemia 1* (*mkl1*, 787 bp; Townsend *et al.*, 2008), *recombination-activating gene 1* (*rag1*, 995 bp; Wiens *et al.*, 2008), *prolactin receptor* (*prlr*, 551 bp; Townsend *et al.*, 2008), *oocyte maturation factor* (*c-mos*, 575 bp; Godinho *et al.*, 2006), and *brain-derived neurotrophic factor* (*bdnf*, 639 bp; Townsend *et al.*, 2008).

Table 1. Sampled specimens and locality information.

Species	**Locality**	**Voucher**
Bothrops brazili	Venezuela	-
Bothrops brazili	Ecuador	-
Bothrops brazili	Igarapé Camaipi, Rio Maracá, Amapá, Brazil	MTR6306
Bothrops brazili	Lourenço, Calçoene, Amapá, Brazil	MTR13844
Bothrops brazili	UHE Jirau, Abunã, Porto Velho, Rondônia, Brazil	H2576
Bothrops brazili	Apiacás, Mato Grosso, Brazil	UFMT10195
Bothrops brazili	Santa Isabel do Rio Negro, Amazonas, Brazil	MSH12346
Bothrops brazili	Coari, Amazonas, Brazil	MPEG22277
Bothrops brazili	Rio Madeira, Ilha do Búfalo, Rondônia, Brazil	INPA5507
Bothrops brazili	Rio Madeira, Jaci, Rondônia, Brazil	INPA5570

Bothrops brazili	Rio Madeira, Jaci, Rondônia, Brazil	INPA5637
Bothrops jararacussu	Camburi, São Sebastião, São Paulo, Brazil	MTR11070
Bothrops jararacussu	Bertioga, São Paulo, Brazil	H569
Bothrops jararacussu	Viçosa, Minas Gerais, Brazil	1JASSU
Bothrops jararacussu	Santa Teresa, Espírito Santo, Brazil	ST1
Bothrops jararacussu	Santa Teresa, Espírito Santo, Brazil	ST2
Bothrops jararacussu	Santa Teresa, Espírito Santo, Brazil	MTR34735
Bothrops jararacussu	Cananéia, São Paulo, Brazil	MZUSP15220
Bothrops jararacussu	Cananéia, São Paulo, Brazil	IB55313
Bothrops muriciensis	Murici, Alagoas, Brazil	MZUFAL10751
Bothrops pirajai	Estação Ecológica Estadual de Wenceslau Guimarães, Wenceslau Guimarães, Bahia, Brazil	MTR22184

2.2. Phylogenetic inference

To investigate phylogenetic relationships among samples of *Bothrops* species with emphasis on the *jararacussu* group, we performed Bayesian inference using MrBayes v. 3.2 (Ronquist *et al.*, 2012) and the **Beast* tool (a coalescent-based method) in BEAST 1.8.4 (Drummond *et al.*, 2012) through the Cipres Science Gateway (Miller *et al.*, 2010). Sequences were edited in CodonCode Aligner v. 3.7.1.1 (www.codoncode.com) and aligned using MUSCLE (Edgar, 2004) as implemented in MEGA v. 6 (Tamura *et al.*, 2013) under default settings. The best-fit model of evolution for each aligned marker was GTR+G+I for all mitochondrial markers, HKY for *bdnf* and *c-mos*, GTR for *prlr*, GTR+I for *rag1*, and HKY+I for *mkl1*, as inferred

using jModelTest v. 2.1.3 (Darriba *et al.*, 2012) under the Akaike Information Criterion (AIC). For MrBayes analyses, two independent runs were performed with four Markov chains each, starting from a random tree; each run consisted of 10,000,000 generations, with trees sampled every 1,000 generations. For **Beast* analyses, two independent runs of 400 million generations each were used, with a sampling frequency of 40,000 generations. For both MrBayes and **Beast* runs, we discarded the first 25% of trees as burn-in; convergence of independent runs and stationarity of model parameters (effective sampling sizes > 200) were verified using Tracer v. 1.6 (Rambaut & Drummond, 2009). A 50% majority consensus tree was summarized from MrBayes runs, while a maximum clade credibility tree was summarized from **Beast* runs using TreeAnnotator (Drummond *et al.*, 2012). Resulting phylogenies were visualized using FigTree 1.4.0. (http://tree.bio.ed.ac.uk/).

2.3. Alternative scenarios of demographic history

To investigate the history of landscape occupation and population shifts over time in the *jararacussu* group, we tested alternative historical demographic scenarios using genetic coalescent simulations and approximate Bayesian computation (ABC). Our approach seeks to uncover the historical events (e. g. vicariance, colonization, population expansion) involved with the diversification of the major lineages identified by the phylogenetic analysis. To that end, our proposed scenarios (see below) test a history of colonization of Amazonia or the Atlantic Forest versus vicariant separation of a wide-ranged ancestor. While a scenario of colonization predicts genetic signatures of a population bottleneck as a result of spatially or temporally restricted connections across domains, a scenario of vicariance of a wide-ranged ancestor would not have involved pronounced shifts in population sizes during

divergence. Additionally, our proposed scenarios test the role of the establishment of the Amazon River on lineage divergence. Our phylogenetics analyses recovered two major lineages of *B. brazili*, which are geographically restricted to north and south of the Amazon River (see Results). We therefore test whether the establishment of the Amazon River promoted vicariant separation of a wide-ranged Amazonian ancestor versus the alternative scenario of colonization of the area north of the Amazon River from a southern ancestor (since the southern *B. brazili* population was recovered as sister to a clade including all others lineages within the group; see Results). This later scenario predicts a population bottleneck associated with the colonization event. Based on these hypothesized events, we simulated and confronted four scenarios (described in detail below; see also illustrations in Fig. S1.1). In all scenarios, the topological relationships between lineages were set based on the highly supported results from phylogenetic analyses based on MrBayes and **Beast* (see Results).

In scenario A, a founding population colonized the Atlantic Forest from south of the Amazon River (central and western Amazonian region) during a forest expansion event, followed by population expansion in the Atlantic Forest; subsequent forest expansion would have led to colonization of the Guiana Shield region from the Atlantic Forest, followed by population expansion in the Guiana Shield (Fig. S1 in Appendix S1).

In scenario B, a founding population colonized Amazonian lowlands north of the Amazon River (Guiana Shield region) from an ancestor from south of the Amazon River (central and western Amazonian region), followed by population expansion in the Guiana Shield; a subsequent forest expansion event led to colonization of the Atlantic Forest from a Guiana Shield ancestor, followed by population expansion in the Atlantic Forest (Fig. S1 in Appendix S1).

In scenario C, a wide-ranged ancestral population occurring north (Guiana Shield region) and south (central and western Amazonian region) of the Amazon River would be split through a vicariant event, with subsequent colonization of the Atlantic Forest from a Guiana Shield ancestor during a forest expansion, followed by population expansion in the Atlantic Forest (Fig. 4 and Fig. S1 in Appendix S1).

Lastly, in scenario D, a wide-ranged ancestral population occurring south of the Amazon River (central and western Amazonian region) and in the Atlantic Forest would be split through vicariance due to expansion of open and dry habitats; subsequently, a founder population colonized northern Amazonia (Guiana Shield region) from an Atlantic Forest ancestor during a forest expansion event (Fig. S1 in Appendix S1).

We performed historical demographic analyses based on five loci: one mitochondrial locus (unphased data) comprising four genes (*12s*, *16s*, *cytb* and *nd4*), and four independent nuclear loci (*mkl1*, *rag1*, *prlr* and *bdnf*; phased data). The *c-mos* gene was not used in these analyses due to lack of data for one of the lineages of *B. brazili*. Following Prates et al. (2016a), we performed four million coalescent simulations (one million simulations under each of the four demographic scenarios) followed by ABC inference with *DIYABC* 2.1 (Cornuet *et al.*, 2014). We chose summary statistics based on preliminary runs that confirmed that Tajima's D (Tajima, 1989), number of private segregating sites, mean numbers of the rarest nucleotide at segregating sites, variance of the number of the rarest nucleotide at segregating sites, and pairwise Fst (Hudson *et al.*, 1992) are appropriate for discrimination among simulated scenarios. We also confirmed if the observed genetic data were contained within the space of simulated data by performing a principal component analysis (PCA) based on the summary statistics chosen (Cornuet *et al.*, 2014).

Prior distributions of model parameters were set as per Dal Vechio *et al.* (2018), as follows: 1) divergence times between independently-evolving lineages with a uniform distribution ranging between 0.05-4 million years, assuming a generation time of three years to convert number of generations into years (see Dal Vechio *et al.*, 2018); 2) effective population size in each independently-evolving lineage with a uniform distribution ranging from 0.01-5 million of individuals; 3) effective population size during a founder event with a uniform distribution ranging from 0.001-0.1 million of individuals; and 4) effective population size prior to vicariant events with a uniform distribution ranging from 0.01-5 million of individuals. We set mutation rates based on the number of substitutions and previously estimated divergence times among Crotalinae species (Dal Vechio *et al.*, 2018), with a uniform prior distribution ranging from 10^{-9} to 10^{-8} substitutions per site per generation for the mitochondrial locus and 10^{-10} to 10^{-8} substitutions per site per generation for the nuclear loci. A *Tamura-Nei* (Tamura & Nei, 1993) substitution model was implemented for each locus.

To determine the best-fit historical scenario, we estimated the statistical support of each model based on Euclidean distances between the simulated data and the observed data. For this, we selected the 1,000 simulated datasets (0.025% of the total simulations) that were closest to the observed data. We then estimated posterior distributions of model parameters under the best-fit scenario, using the 1,000 simulated datasets that were closest to the observed data.

Lastly, we tested the accuracy of the model selection procedure by examining whether the different simulated scenarios can be discriminated based on the summary statistics used. For this, we simulated 100 pseudo-observed datasets under each scenario, such that the true scenario was known for each pseudo-observed dataset. We

then performed ABC inference on each pseudo-simulated dataset, thus calculating the proportion of pseudo-observed datasets that were correctly assigned to its generating model.

3. RESULTS

3.1. Phylogenetic relationships, molecular dating and species delimitation

Phylogenetic analyses within *Bothrops* based on both a concatenated dataset (using MrBayes; Fig. 1) and a coalescent approach (using **Beast*; Fig. 2) support that *Bothrops* is monophyletic (Posterior Probability [PP] = 1).

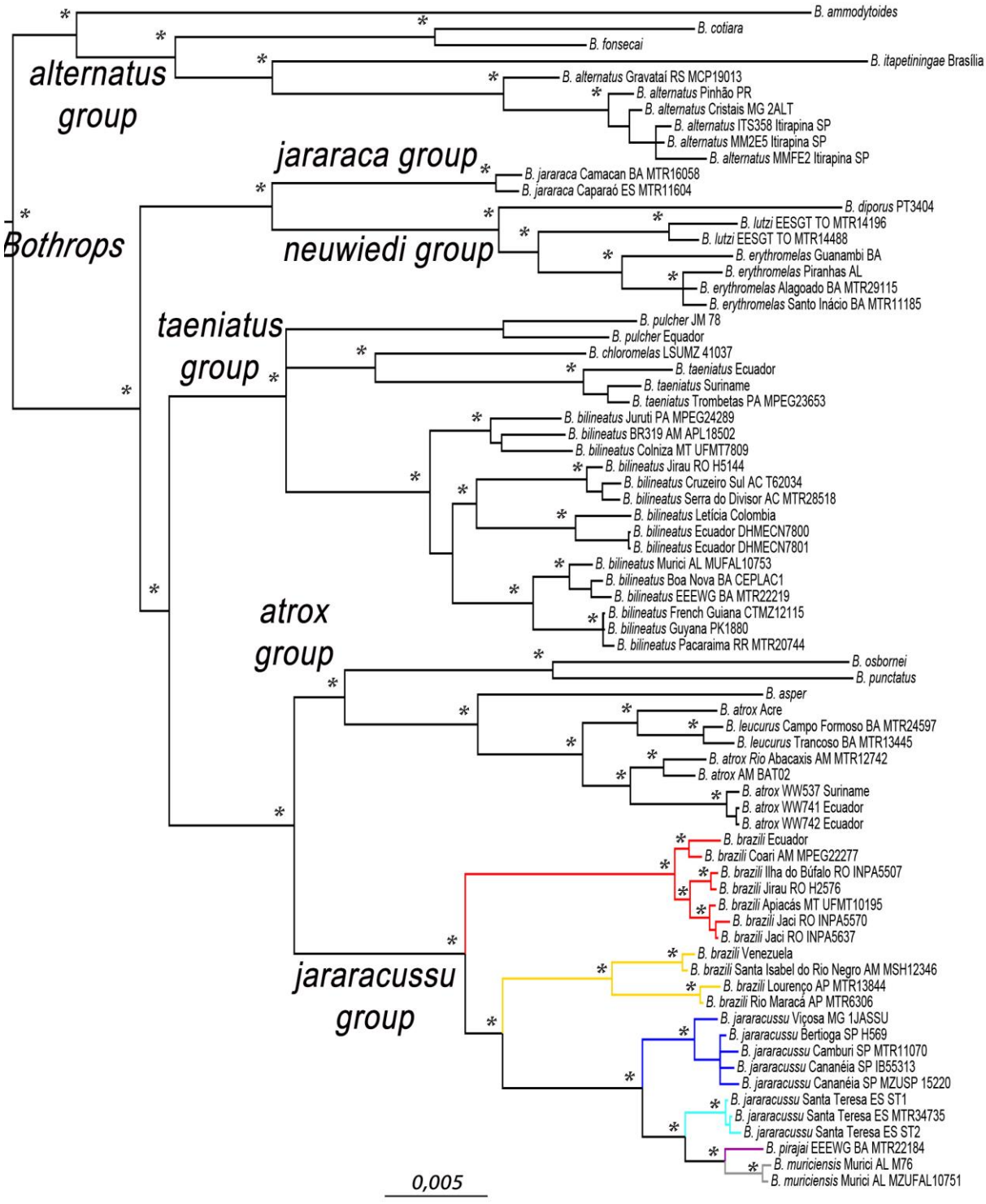

Figure 1. Phylogenetic relationships within *Bothrops* based on a Bayesian analyses using a concatenated dataset of nine molecular markers (five nuclear and four mitochondrial genes). The six species groups within *Bothrops* are shown (*alternatus*,

neuwiedi, *jararaca*, *taeniatus*, *atrox* and *jararacussu*). Asterisks on nodes indicate posterior probabilities > 0.95. Scale bar indicates substitutions per site.

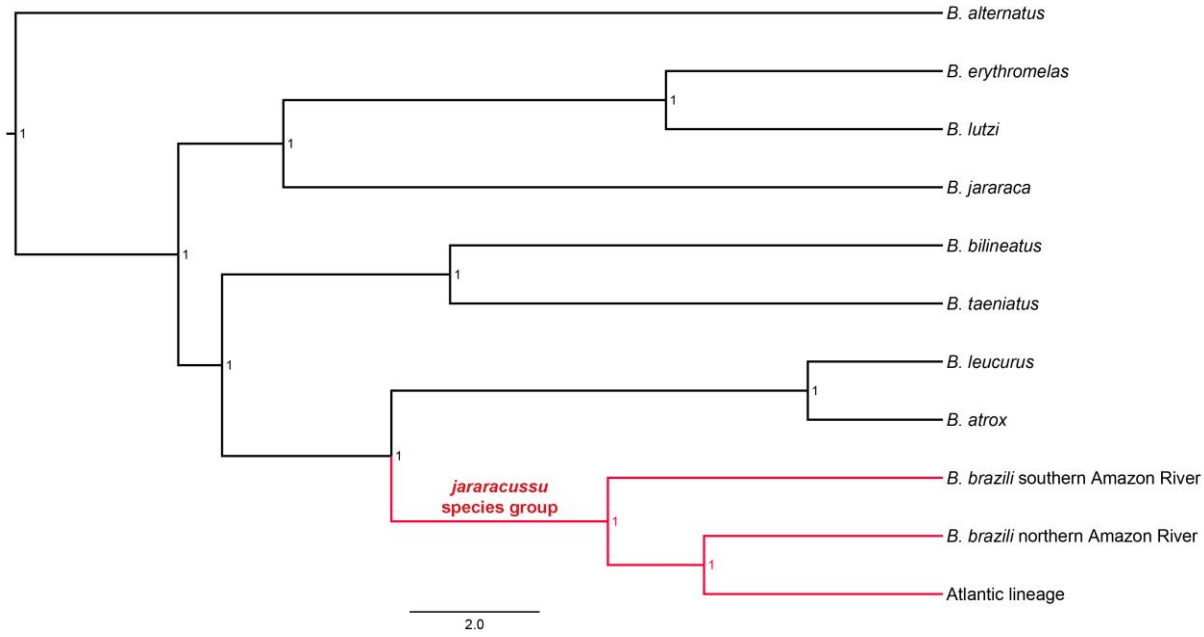

Figure 2. Species-tree for *Bothrops* based on coalescent analysis using **Beast* implemented in BEAST. The Atlantic lineage within the *jararacussu* group is composed of *B. jararacussu*, *B. pirajai* and *B. muriciensis*. Numbers on nodes represent values for posterior probability. Scale bar indicates substitutions per site.

The *jararacussu* species group was inferred as monophyletic (PP = 1), with an Atlantic Forest clade composed of *B. jararacussu*, *B. pirajai* and *B. muriciencis* (PP = 1), confirming *B. pirajai* and *B. muriciensis* as nested within the *jararacussu* species group (Figs. 1, 2 and 3). However, we found *B. brazili* to be paraphyletic, forming two highly supported non-sister clades that are restricted in geographic space: one clade that occurs north of the Amazon River (= Guiana Shield clade), which is sister to the Atlantic Forest clade (composed by *B. jararacussu*, *B. pirajai*, and *B. muriciensis*) (PP > 0.95); and another *B. brazili* clade that occurs south of the Amazon River (= central

and western Amazonian clade), which was recovered as sister to all other species in the *jararacussu* species group (PP = 1) (Fig. 2 and 3). Similar to the case of *B. brazili*, we recovered *B. jararacussu* in paraphyly, forming two highly supported non-sister lineages: one from the Santa Teresa municipality, state of Espírito Santo in Brazil, which grouped with *B. pirajai* and *B. muriciencis* (PP = 1); and another clade formed by samples from the state of Minas Gerais and São Paulo in Brazil (Fig. 3), sister to the remaining Atlantic Forest clades.

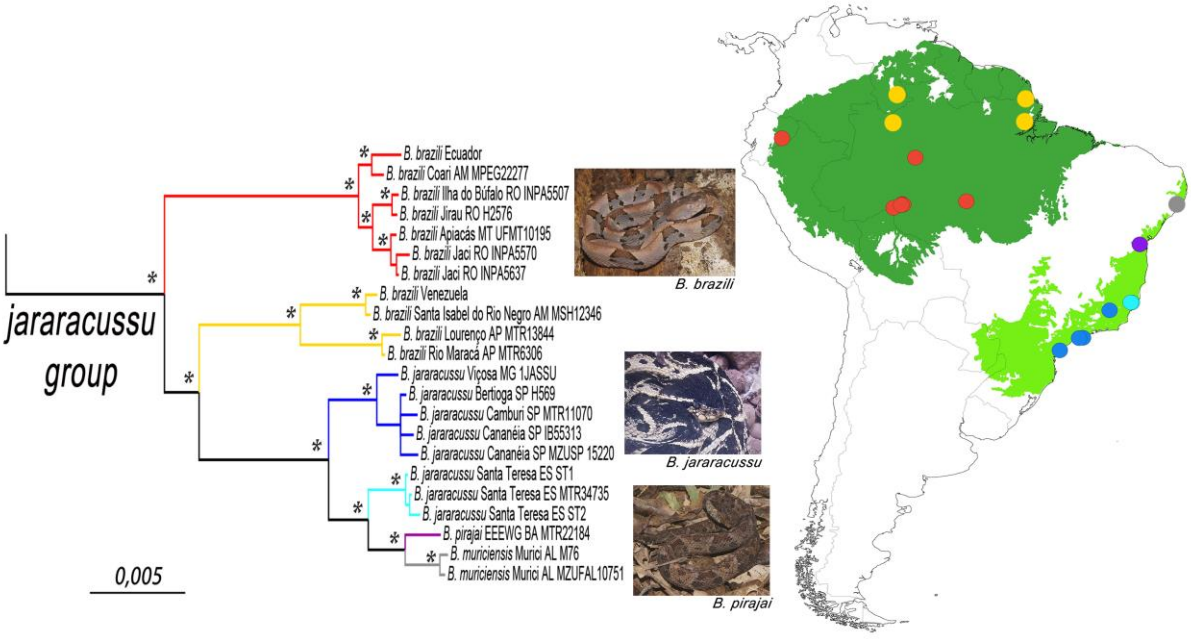

Figure 3. Phylogenetic relationships within the *jararacussu* species group of *Bothrops* based on a concatenated dataset composed of nine molecular markers (five nuclear and four mitochondrial genes). Colors on map correspond to the major lineages. Red and yellow: *B. brazili*; light and dark blue: *B. jararacussu*; purple: *B. pirajai*; gray: *B. muriciensis*. Asterisks on nodes indicate posterior probabilities > 0.95. Scale bar indicates substitutions per site.

3.2. Best-fit historical scenario

The best-fit historical scenario recovered by coalescent simulations and approximate Bayesian computation (PP = 0.99) supports a history of a vicariant split between populations of *B. brazili* north and south of the Amazon River, with no genetic signatures of pronounced population size shifts (Fig. 4). The divergence between the two *B. brazili* clades dates back to the Miocene, (median = 4.65 mya, C.I. = 4.5-6.1 mya). The best-fit scenario also includes colonization of the Atlantic Forest from an ancestor from Amazonia north of the Amazon River (Guiana Shield region) during the Pleistocene (median = 0.36 mya, C.I. = 0.1-1.7 mya) (Fig. 4).

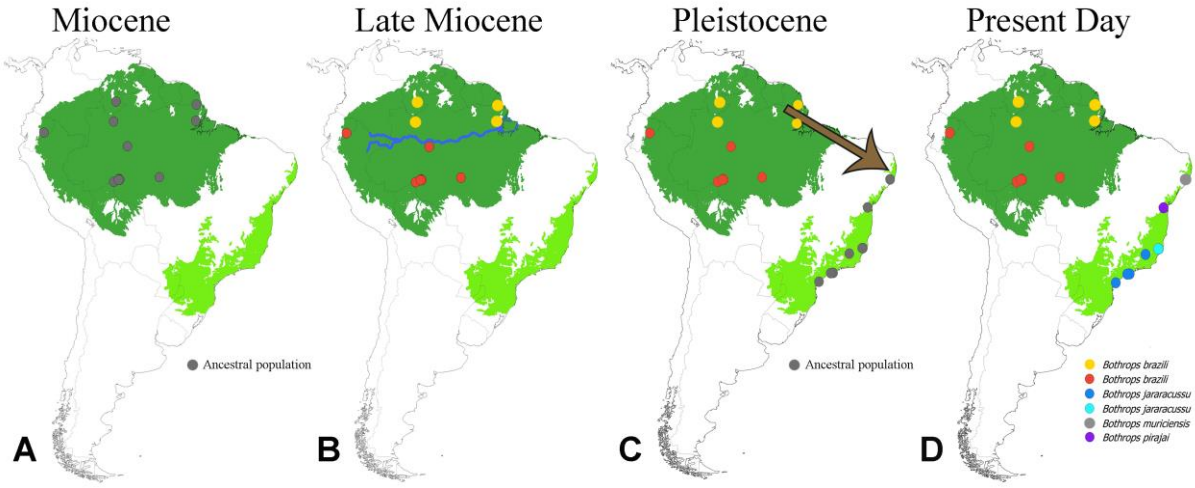

Figure 4. Best-fit scenario supported by historical demographic analyses of the *jararacussu* species group of *Bothrops* using DIYABC based on eight markers (four mitochondrial and four nuclear genes). The model includes a vicariant split during the Late Miocene (A and B) between populations of *B. brazili* from north and south of the Amazon River, and a subsequent colonization event (brown arrow) also during the Pleistocene (C) of the Atlantic Forest from a northern Amazonian ancestor. D represents the current distribution of the species in the *jararacussu* species group.

Gray circles in A and C represent hypothesized ancestral populations. For details, see text.

Effective population size estimates suggest that the initial pool of Atlantic Forest colonizers was composed of about 27,000 individuals (ca. 1.5% of the Guiana Shield source population), supporting a population bottleneck associated with dispersal into the Atlantic Forest. Posterior median substitution rates were estimated as 1.3×10^{-8} substitutions per site/year for the mitochondrial locus (C.I. = $0.6–2.3 \times 10^{-8}$ substitutions per site/year) and $0.11–0.19 \times 10^{-8}$ substitutions per site/year for the nuclear loci (C.I. = $0.42–1.1 \times 10^{-9}$ substitutions per site/year).

Plots based on a principal component analysis of the summary statistics confirmed that the observed data was contained within the space of simulated data. The posterior error rate for demographic model testing based on pseudo-observed datasets was estimated as 0.28.

4. DISCUSSION

4.1. Riverine barriers and diversification

Our historical demographic analyses support a scenario of vicariant separation between populations of *B. brazili* north and south of the Amazon River, a divergence that dates to the Late Miocene. Although the history of the Amazon basin is still an active area of research, the origin of the modern Amazon River has been largely associated with the final uplift of the Andes, which led to draining of the *Pebas* lacustrine system and eastward river flow towards the Atlantic Ocean starting in the Late Miocene, around 7-9 mya (Hoorn *et al.*, 2010, 2017). These major

paleogeological changes may have had major effects on the evolutionary history of the Amazonian biota, for instance promoting vicariant separation between many lineages (Ayres & Clutton-Brock, 1992; Boubli *et al.*, 2012; Ribas *et al.*, 2012; Pomara *et al.*, 2014; Naka & Brumfield, 2018). Our historical demographic analyses of *B. brazili* are consistent with the idea that the establishment of the Amazon River has favored divergence and disrupted gene flow between populations, promoting diversification.

It has been suggested that the Amazon River and other major rivers in the Amazon basin delimit areas of endemism (Cracraft, 1985; Silva *et al.*, 2005; Ribas *et al.*, 2012). These areas have been proposed on the basis of the distribution of closely related species, particularly in birds and mammals (Aleixo, 2004; Heyes & Sewlal, 2004; Ribas *et al.*, 2012; Boubli *et al.*, 2012; Pomara *et al.*, 2014), although such areas not always hold in other groups, such as lizards and frogs (Antonelli *et al.*, 2010; Souza *et al.*, 2013). In the case of snakes, the data are still scarce. Dal Vechio *et al.* (2018) studied the demographic history of the arboreal pit viper *Bothrops bilineatus* (*taeniatus* species group), which is sympatric in Amazonian and Atlantic forests with species from the *jararacussu* group. That study inferred a lineage geographically restricted to the Guiana Shield (north of the Amazon River) as the sister of an Atlantic clade, a pattern that the current investigation found also in the *jararacussu* group. Despite this similarity, these two groups of snakes show differences in their demographic histories. For example, the Amazon River did not act as a vicariant barrier between northern and southern Amazonian populations of *B. bilineatus*, although this river may currently help limit the distribution of populations (Dal Vechio *et al.*, 2018).

4.2. Atlantic Forest colonization

Our phylogenetic analyses found that the three Atlantic Forest species in the *jararacussu* group (*B. jararacussu, B. pirajai* and *B. muriciencis*) form a clade, corroborating previous suggestions based on morphological data that *B. pirajai* and *B. muriciencis* belong to this species group (Amaral, 1923; Hoge, 1953; Ferrarezzi & Freire, 2001; Fenwick *et al.*, 2009). This Atlantic clade is sister to a clade of *B. brazili* that occurs in Amazonia north of the Amazon River. A similar pattern was found in the congeneric species *B. bilineatus*, in which Atlantic Forest samples were more closely related to samples from the Guiana Shield (Dal Vechio *et al.*, 2018). Both in that study and in the present one, best-fit historical scenarios support a Pleistocene faunistic interchange between Amazonian and Atlantic forests through northern bridge, likely due to former rainforest expansions in northeastern South America. Moreover, the best-fit historical scenarios support colonization events for both species complex, but in opposite direction, in the case of *B. jararacussu* group there was a colonization flow toward Atlantic Forest.

The hypothesis of northern forest bridges through present-day northeastern Brazil is supported by the presence of the "Brejos Nordestinos", relictual forest patches on mountains in the semi-arid Caatinga, which harbor a mixture of species with both Atlantic Forest and Amazonian affinities (Pôrto *et al.*, 2004). Moreover, paleopalynological, speleothem and fossil data (Dever *et al.*, 1987; Hartwing & Cartelle, 1996; Czaplewski & Cartelle, 1998; Oliveira *et al.*, 1999; Auler & Smart, 2001; Auler *et al.*, 2004, Wang *et al.*, 2004) support the idea of higher levels of rainfall and replacement of Caatinga vegetation with rainforest tree species in the past. Former forest corridors connecting northern Amazonia and the Atlantic Forest agree with the patterns of genetic structure that we inferred in the *jararacussu* species group of *Bothrops*.

A history of past forest connections and separation has been invoked to explain presently disjunct distributions in a variety of organisms (Birds: Batalha-Filho et al. 2013; Mammals: Costa, 2003; Nascimento *et al.*, 2008; Pavan, Martins, Santos, Ditchfield, & Redondo, 2011; Lizards: Pelegrino *et al.*, 2011; Rodrigues *et al.*, 2014; Prates *et al.*, 2015, 2016a,b, 2017, 2018; Snakes: Zamudio & Green, 1997; Dal Vechio *et al.*, 2018). However, studies based on genetic data have found incongruent times, spatial routes, and directionalities of forest colonization. For instance, within squamates, Prates *et al.* (2016a) found that colonization of the Atlantic Forest by arboreal anole lizards occurred from an Amazonian ancestor through northeastern South America, a pattern similar to that inferred in the *jararacussu* species group during the Pleistocene, yet happening little older (middle Pleistocene). Based on *Enyalius* lizards, Rodrigues *et al.* (2014) found a similar spatial route of colonization, yet in the opposite direction (from the Atlantic Forest to the Amazonia) and at a different time (middle Miocene). Lastly, Dal Vechio *et al.* (2018) found a similar route and directionality of colonization as that found by Rodrigues *et al.* (2014), yet much more recently (early Pleistocene), based on the arboreal pit viper *B. bilineatus*. Taken together, these studies support a picture of multiple rainforest connections over time in South America, which promoted faunal interchange in both directions - leaving aside the idea of a major migratory event in a single direction.

4.3. Concluding remarks

Using a multi-locus approach to perform phylogenetic inference and tests of alternative historical demographic scenarios on the basis of genetic coalescent simulations and approximate Bayesian computation, this study supports the proposed role of river establishment in speciation through vicariance in the Amazonian

lowlands. Moreover, our results shed light on the historical dynamism of the Neotropical forests, supporting that pulses of rainforest expansion led to physical contact and faunal interchange between Amazonian and Atlantic forests. This study invites the investigation of other snake taxa, several of which have similar distribution patterns, helping to clarify how organismal attributes contribute to species responses to habitat shifts through time. Lastly, by pointing to paraphyletic species in the *jararacussu* group, our work has implications for cryptic species diversity in these medically important venomous snakes, revealing the need of dedicated taxonomic studies. These studies will benefit from an increased geographic sampling and the incorporation of morphological evidence.

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ORCID

Francisco Dal Vechio http://orcid.org/0000-0003-1328-0243

Ivan Prates https://orcid.org/0000-0001-6314-8852

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BIOSKETCH

Francisco Dal Vechio is PhD candidate in the Herpetology Lab in the Biosciences Institute of the University of Sao Paulo. His main research interests are the systematics and biogeographic history of Neotropical pit vipers.

Data accessibility

All sequence data will be deposited in the GenBank (pending acceptance).

Author contributions

F.D.V. gathered the molecular data, performed analyses, and wrote the manuscript; I.P. contributed to the molecular analyses and wrote the manuscript; M.T.R. funded the research, supervised and contributed intellectually to the project, and revised the text; F.G.G., R.G. and H.Z. contributed intellectually to the project and revised the text.

Journal of Biogeography

SUPPORTING INFORMATION

Rainforest shifts through time and riverine barriers shaped the diversification of the terrestrial South American pit vipers in the *Bothrops jararacussu* species group

Francisco Dal Vechio, Ivan Prates, Felipe G. Grazziotin, Hussam Zaher, Roberta Graboski, Miguel Trefaut Rodrigues

Appendix S1: Historical models used to test the evolutionary history of *Bothrops jararacussu* species group based on coalescent simulations performed on DIYABC.

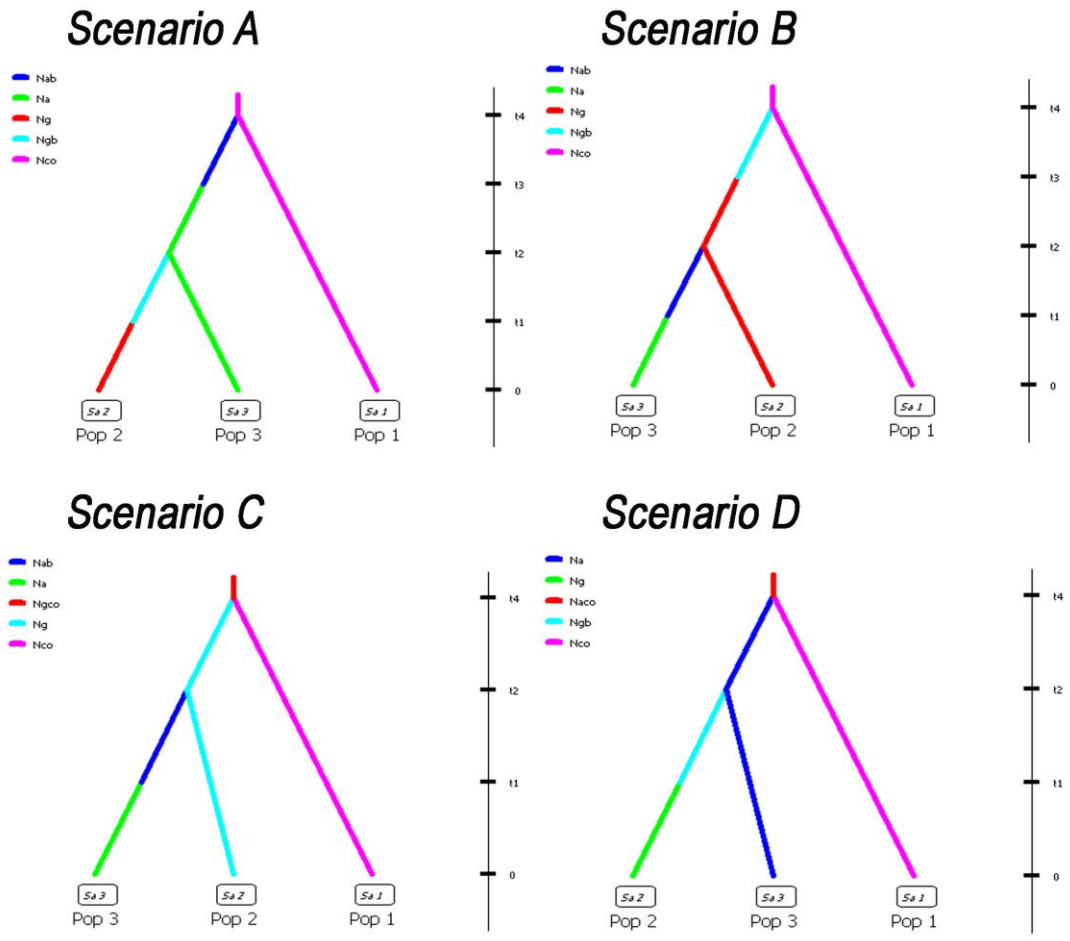

Figure S1. Four historical models used to test the evolutionary history of *Bothrops jararacussu* species group based on a dataset of eight molecular genes (four mitochondrial and four nuclear) using DIYABC. All scenarios have the same topology which is based on results from the phylogenetic analyses (*Beast and MrBayes), where central-western Amazonia is sister of remaining samples and Guiana shield is sister to the Atlantic Forest clade. Scenarios A test for a colonization of the Atlantic Forest by an ancestor from the central-western Amazonia, and a second event colonization, but now of the Amazon, at Guiana Shield, by an ancestor from Atlantic Forest. Scenarios B test for colonization of north of the Amazon River by an ancestor from south of the river, and for a colonization of the Atlantic Forest by an ancestor from the Guiana Shield. Scenarios C test for a vicariant processes between samples

from south and north of the Amazon River, and a colonization of the Atlantic Forest by an ancestor from the Guiana Shield (samples from north of Amazon River). Scenarios D test for a vicariant processes between central-western Amazonia and Atlantic Forest samples, and a colonization of the Amazon, at Guiana Shield, by an ancestor from Atlantic Forest. Each colonization event is followed by a population expansion. Na: present population in the Atlantic Forest; Nco: present population in central-western Amazonia; Ng: present population in the Guiana Shield; Nab: hypothetical historical population that colonized the Atlantic Forest; Ngb: hypothetical historical population that colonized the Guiana Shield; Ngco: hypothetical historical population ancestor of central-western Amazonia (south Amazonia River) + Guiana Shield (North Amazon River). T1-T4 are time in the past, with T4 older than T3, T2 and T1; T3 older than T2 and T1; T2 older than T1; and T1 older than the present day.

Capítulo 2

Manuscrito formatado a ser submetido para revista Zootaxa

Molecular and phenotypic data reveal a new Amazonian species of pit vipers (Serpentes: Viperidae: *Bothrops*)

Francisco Dal Vechio[1*], Ivan Prates[2], Felipe G. Grazziotin[3], Hussam Zaher[4], Miguel Trefaut Rodrigues[1]

[1]Departamento de Zoologia, Instituto de Biociências, Universidade de São Paulo, Rua do Matão, Trav. 14, nº 321, Cidade Universitária, CP 11461, CEP 05508-090, São Paulo, SP, Brazil.

[2]Department of Vertebrate Zoology, National Museum of Natural History, Smithsonian Institution, Washington, DC, 20560, USA.

[3]Laboratório de Coleções Zoológicas, Instituto Butantan, CP 42694, CEP 04299-970, São Paulo, SP, Brazil.

[4]Museu de Zoologia, Universidade de São Paulo, CP 42694, CEP 04299-970, São Paulo, SP, Brazil.

[*]Corresponding author. E-mail address: francisco.dalvechio@gmail.com

Running Title: A new species from the *Bothrops jararacussu* group

Key words: Amazonia, Amazon River, *Bothrops brazili*, *B. jararacussu B. pirajai*, *B. muriciencis*, Crotalinae.

Abstract

Recent genetic studies have found unclear species boundaries and evidence of undescribed diversity in the poorly studied *jararacussu* species group within *Bothrops*. In this contribution, we investigate phenotypic and genetic diversity in the Amazonian snake *Botrops brazili* to test previous assertions of unrecognized species diversity within this taxon. Our phylogenetic results and inferences of independently evolving lineages based on molecular data recover two divergent clades within *B. brazili*, one restricted to areas north and another to areas south of the Amazon River. Phylogenetic relationships between these lineages and other species in the *jararacussu* species group reveal *B. brazili* to be paraphyletic, with the northern clade inferred as sister to a clade composed of Atlantic Forest taxa (*B. jararacussu*, *B. muriciensis*, *B. pirajai*). External morphology (number of ventral and subcaudal scales) and coloration patterns (lateral trapezoidal marks) consistently separate the two lineages of *B. brazili*. We therefore recognize and describe the northern lineage as a new species of *Bothrops*, improving our knowledge of species diversity within a medically important clade of venomous South American snakes.

Resumo

Estudos recentes têm encontrado evidências de diversidade críptica e problemas no reconhecimento específico entre as linhagens pertencentes ao grupo *jararacussu* em *Brothrops*. Assim, nós investigamos a variação fenotípica e genética da jararaca amazônica *Bothrops brazili*, com a finalidade de testar proposições anteriores sobre presença de diversidade não reconhecida para a espécie. Nossos resultados filogenéticos e de inferências de linhagens evolutivas independentes, com base em dados moleculares, recuperam dois clados divergentes em *B. brazili*, um restrito as

áreas ao norte do Rio Amazonas e outro ao sul do rio. As análises filogenéticas entre essas linhagens a as outras espécies pertencentes ao grupo *jararacussu* revelam *B. brazili* como parafilético, com o clado ao norte do Rio Amazonas inferido como irmão do clado Atlântico, composto por *B. jararacussu*, *B. muriciensis*, *B. pirajai*. Dados de morfologia externa (números de escamas ventrais e subcaudais) e padrões de coloração (manchas laterais), consistentemente, separa as duas linhagens de *B. brazili*. Desse modo, nós reconhecemos e descrevemos a linhagem ao norte do Rio Amazonas como uma nova espécie de *Bothrops*, melhorando o nosso conhecimento sobre a diversidade de espécies nesse grupo sul-americano de serpentes venenosas com importância médica.

Introduction

Venomous snakes in the genus *Bothrops* (family Viperidae) have been the focus of several phylogenetic and diversification studies over recent years (Wuster *et al*., 2008; Fenwick *et al*., 2009; Carrasco *et al*., 2012; Alencar *et al*., 2016), with an equally active taxonomy reveling unrecognized diversity at the species level (Ferrarezzi & Freire, 2001; Marques *et al*., 2002; Da Silva & Rodrigues, 2008; Folleco-Fernandez, 2010; Barbo *et al*., 2012, 2016). Despite these efforts, conflicting topologies, taxonomic uncertainty, and underestimation of species diversity persist (Machado *et al*., 2014; Dal Vechio *et al*., 2018, Dal Vechio *et al*., 2019 *in press*). Among the *Bothrops* species whose taxonomy is poorly known are the members of the *jararacussu* species group. In this group, the phylogenetic relationships and taxonomic status of populations from the coastal Atlantic Forest (corresponding to three taxa, *B. jararacussu*, *B. muriciensis*, and *B. pirajai*), as well as of phenotypically distinct

populations in Amazonia (under the name *B. brazili*), remain unclear (Cunha & Nascimento, 1975, 1978, 1993; Campbell & Lamar, 2004).

Similar to the other members of the *jararacussu* group, *Bothrops brazili*, the Brazil's lancehead, is characterized by a robust body and terrestrial habit. Despite its broad distribution in Amazonian lowlands, these reddish pit vipers went unnoticed by 19th century naturalists, possibly as a consequence of their rarity in most localities. Only in 1953 Hoge recognized and described the species, named in honor of Dr. Vital Brazil, the founder of the Butantan Institute in São Paulo. This description was performed on the basis of two specimens, a male and a female, from a single locality that lies south of the Amazon River: "Tomé Assu, Acará Mirim River, state of Pará, Brazil". Subsequently, Cunha & Nascimento (1975, 1978, 1993) recognized pronounced phenotypic variation between populations of *B. brazili* that occur north and south of the Amazon region, suggesting well defined "geographic races".

Recently, Dal Vechio *et al.* (2019 *in press*) studied the demographic history of the *Bothrops jararacussu* species group on the basis of molecular data, recovering two allopatric lineages under the taxon name of *Bothrops brazili*. One such lineages is restricted to areas north of the Amazon River, while the other occurs south to the river on its right bank. Surprisingly, that study recovered northern and southern lineages to be non-sister, with samples from the northern population clustering with a clade composed of specimens from the Atlantic Forest. This Atlantic Forest clade included populations recognized as three distinct taxa: *B. muriciensis*, *B. pirajai* and *B. jararacussu* (Dal Vechio *et al*., 2019 *in press*).

Evidence of *B. brazili* as a paraphyletic species, coupled with previously suggested morphological differences between populations north and south of the Amazon River, indicate the need of an evaluation of unrecognized species diversity

under this taxon name. In this contribution, we perform a comprehensive assessment of phenotypic variation across the distribution of *B. brazili* under the light of patterns of spatial genetic structure. This assessment reveals a new species of Amazonian pit viper, which we formally describe.

Materials and methods

External morphological data

We examined 41 specimens of *Bothrops brazili* spanning the taxon's entire geographic distribution. These specimens are housed in the collection of the Laboratório de Herpetologia at the Universidade de São Paulo (MTR, Miguel Trefaut Rodrigues acronym), Museu de Zoologia da Universidade de São Paulo, Brazil (MZUSP), Instituto Butantan, Brazil (IB), Museu Paraense Emilio Göeldi, Brazil (MPEG), Museu Nacional, Rio de Janeiro, Brazil, (MNRJ), Instituto, Nacional de Pesquisas da Amazônia, Brazil, (INPA), Museo de Zoología Pontificia Universidad Católica del Ecuador, Ecuador (QCAZ), Fundación Herpetologica Gustavo Orces, Equador (FHGO), Museo Ecuatoriano de Ciencias Naturales, Ecuador (MECN), and Museo de Historia Natural de la Universidad Nacional de Colombia, Colombia (ICN). We complemented this examination with data available in the literature (Hoge, 1953, 1962; Hoge & Lancini, 1962; Roze, 1966;Cunha & Nascimento, 1975, 1978, 1993; Gasc & Rodrigues, 1980; Chippaux, 198; Dixon & Soini, 1986; Campbel & Lamar, 1989, 2004; Duellman, 2005) (Appendix 1).

We follow Campbell (1985) in the description of scale attributes and counts. Morphometric measurements were taken using a solid metallic ruler to the nearest 10 mm or a caliper to the nearest 0.1 mm. Measurements were: body length (SVL), from the tip of the snout to the cloaca; tail length (TL), from the posterior border of the

cloaca to the tip of the tail; head length (HL), from the snout to the posterior edge of the lower jaw. Observation of scale attributes and scale counts were performed under a Zeiss stereomicroscope. Traits were: number of ventrals (V), subcaudals (SC), supra (SL) and infralabials (IL), and number of lateral trapezoidal marks (LTM) from the neck to the cloaca region (blotches on the tail were not considered).

Hemipenial preparation

We prepared the left hemipenis of the holotype of the new species (MZUSP XXX) following Manzani & Abe (1998) and Zaher (1999). The retractor muscle was manually separated and the everted organ filled with stained petroleum jelly. The organ was immersed in an alcoholic solution of Alizarin Red for 24 hours to stain possible calcified structures as proposed by Nunes *et al*. (2012). The terminology of hemipenial structures follows Dowling and Savage (1960) and Zaher (1999).

Molecular data

For phylogenetic inference, we used sequences from 18 samples from the *jararacussu* species group generated by Dal Vechio *et al*. (2019 *in press*), as follows: nine specimens of *Bothrops brazili*, six *B. jararacussu*, two *B. muriciensis*, and one *B. pirajai*. We also included in our alignments two additional samples of *B. brazili* and two of *B. jararacssu* available in the Genbank. Lastly, we added previously published sequences from 49 samples representing species from other *Bothrops* groups. Our final molecular dataset was a concatenated matrix including nine molecular markers, four of which mitochondrial and five nuclear (total alignment length 6,009 bp), as follows: the mitochondrial genes *16s ribosomal RNA* (*16s*, 534 bp; Palumbi, 1996), *12s ribosomal RNA* (*12s*, 394 bp; Palumbi, 1996), *cytochrome b* (*cytb*, 737 bp; Pook *et al.*, 2000), and

NADH dehydrogenase subunit 4 (*nd4*, 798 bp; Arévalo *et al*., 1994), and the nuclear genes *megakaryoblastic leukemia 1* (*mkl1*, 787 bp; Townsend *et al*., 2008), *recombination-activating gene 1* (*rag1*, 995 bp; Wiens *et al*., 2008), *prolactin receptor* (*prlr*, 551 bp; Townsend *et al*., 2008), *oocyte maturation factor* (*c-mos*, 575 bp; Godinho *et al*., 2006), and *brain-derived neurotrophic factor* (*bdnf*, 639 bp; Townsend *et al*., 2008).

We performed Bayesian inference for phylogenetic reconstruction using MrBayes v. 3.2 (Ronquist *et al*., 2012), through the Cipres Science Gateway (Miller *et al*., 2010). Sequences were aligned using MUSCLE (Edgar 2004) as implemented in MEGA v. 6 (Tamura *et al*., 2013) under default settings. The best-fit model of evolution for each aligned marker was determined using jModelTest v. 2.1.3 (Darriba *et al*., 2012) under the Akaike Information Criterion (AIC). Best-fit models were as follows: GTR+G+I for all mitochondrial markers, HKY for *bdnf* and *c-mos*, GTR for *prlr*, GTR+I for *rag1*, and HKY+I for *mkl1*. For the Bayesian analyses, two independent runs were performed with four Markov chains each, starting from a random tree; each run consisted of 10,000,000 generations, with trees sampled every 1,000 generations. We discarded the first 25% of trees as burn-in; convergence of independent runs and stationarity of model parameters (effective sampling sizes > 200) were verified using Tracer v. 1.6 (Rambaut & Drummond, 2009). A 50% majority consensus tree was summarized from MrBayes analyses. Resulting phylogenies were visualized using FigTree 1.4.0 (http://tree.bio.ed.ac.uk/). Uncorrected genetic distances (p-distances) were calculated using MEGA v. 6 (Tamura *et al*., 2013).

Inferring independently evolving lineages

To support an evaluation of unrecognized species diversity in the *jararacussu* species group, we estimated independently evolving lineages using the molecular species delimitation algorithm *Generalized Mixed Yule-Coalescent* (GMYC; Pons *et al*., 2006), using its Bayesian implementation (bGMYC; Reid & Carstens, 2012). This method interprets splits on a phylogeny as either species divergences or intra-specific coalescent events. As GMYC assumes that the frequency of coalescent events within species is higher than the rate of divergences between species, attempting to find a threshold that distinguishes these two branching types on a target phylogeny, it is important that the dataset bears samples representative of intra and inter specific splits. An ultrametric target tree was inferred based on the mitochondrial dataset using BEAST 1.8.4 (Drummond *et al*., 2012), with two independent runs of 20 million generations each sampling every 2,000 generations. bGMYC was applied based on 500 posterior trees from BEAST runs using the *bgmyc.multiphyl()* function in *R* 3.3.2, with 50,000 MCMC steps (40,000 steps as burn-in) sampling every 100 steps. We used a probability threshold of 0.7 because the default value (0.5) failed to recognize well established species based on morphological and genetic data within the *neuwiedi*, *alternatus*, and *taeniatus* species groups of *Bothrops*.

Results

Phylogenetic relationships

Our molecular phylogenetic analysis inferred the *Bothrops jararacussu* species group as monophyletic (PP = 1) and sister to the *Bothrops atrox* group (Fig. 1). We found *B. brazili* to be paraphyletic, forming two highly supported non-sister clades. One such clade occurs north of the Amazon River; this clade is sister to a clade composed of the Atlantic Forest species *B. jararacussu*, *B. pirajai*, and *B. muriciensis*

(PP =1). A second *B. brazili* clade included samples from south of the Amazon River; this clade was inferred as the sister of the ancestor of all other samples in the *jararacussu* species group (PP = 1) (Fig. 1).

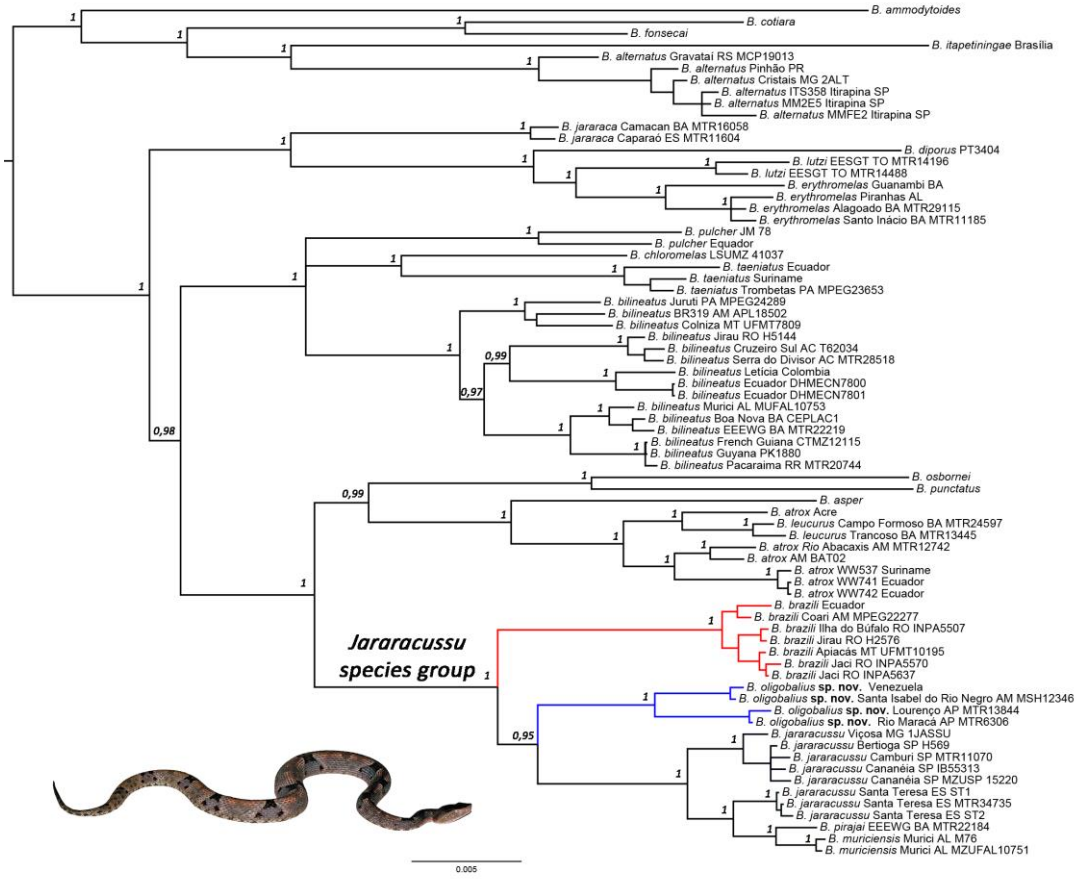

Figure 1. Phylogenetic relationships within *Bothrops* highlighting the placement of *B. oligobalius* **sp. nov.** (blue) and *B. brazili* (red) in the *jararacussu* species group. Posterior probabilities are shown above nodes. Scale bar indicates substitutions per site. Photo: *B. oligobalius* **sp. nov.** from French Guiana, by Maël Dewynter.

Like *B. brazili*, we inferred *B. jararacussu* as paraphyletic. Samples from the coastal state of Espírito Santo in Brazil form a clade that is sister to the ancestor of *B. pirajai* and *B. muriciensis* (PP = 1). In turn, samples of *B. jararacussu* from the states

of São Paulo and Minas Gerais in Brazil's southeast compose a clade that is sister to the ancestor of all remaining Atlantic Forest samples (PP = 1) (Fig. 1).

Uncorrected pairwise genetic distances between the northern clade of *B. brazili* and species in the Atlantic Forest clade ranged from 3.9 to 4.9% for the mitochondrial markers *nd4*. Genetic distances between the two non-sister clades of *B. brazili* ranged from 5.6 to 7.1%.

Inferences of independently evolving lineages

Our molecular species delimitation analysis based on bGMYC identified three independently evolving lineages within the *B. jararacussu* species group. One of these lineages is composed of samples of *B. brazili* from south of the Amazon River; one is composed of samples of *B. brazili* from north of the Amazon River; and another one is composed of samples from the Atlantic Forest species *B. jararacussu*, *B. pirajai* and *B. muriciencis* (the Atlantic clade) (Figs. 1).

Morphological examination of specimens demonstrates that the non-sister northern and southern clades of *B. brazili* have consistent phenotypic differences (see below). Based on these differences, coupled with the phylogenetic and molecular delimitation results, we describe the northern clade as a new species, as follows.

***Bothrops oligobalius* sp. nov.**

(Figs. 2-6)

Bothrops neglecta, Amaral 1923: 100–102; in part;

Bothrops neglecta, Hoge & Lancini, 1962: 16;

Bothrops brazili, Hoge, 1962: 63;

Bothrops neglecta, Roze, 1966: 286;

Bothrops brazili, Hoge *et al*., 1972: 231; in part;

Bothrops brazili, Cunha & Nascimento, 1975: 12–17; in part;

Bothrops brazili, Gasc & Rodrigues, 1980: 591;

Bothrops brazili, Chippaux, 1986: 50;

Bothrops brazili, Campbell & Lamar, 1989;

Bothrops brazili, Campbell & Lamar, 2004.

Bothrops brazili "northern clade", Dal Vechio *et al*., 2019

Figure 2. *Bothrops oligobalius* **sp. nov.** from French Guiana. Photo by Maël Dewynter.

Holotype. A male, MZUSP XXX (MTR 13844) collected by Miguel Trefaut Rodrigues and Antoine Fouquet on April 22, 2007, under a leaf of *Cecropia* sp. in *terra firme* (non-flooded) Amazonian lowland forests, at the Lourenço municipality (00°41'48.2"S, 57°42'45.1"W), state of Amapá, Brazil (Fig. 3).

Figure 3. Holotype of *Bothrops oligobalius* **sp. nov.** (MZUSP XXX, field number MTR 13844), from Lourenço Municipality, State of Amapá, Brazil. A: Dorsal view; B: Ventral view. Scale bar = 1cm.

Paratypes. Ten specimens: **Brazil:** MNRJ 10050-51 (females) from São Gabriel da Cachoeira municipality, state of Amazonas; MPEG 3274 (female) from Almeirim municipality, state of Pará; MZUSP 11719 (male) from Rio Maracá, Mazagão municipality, state of Amapá; **Colombia**: ICN 2155 (male) from Puerto Santander, Caquetá river, Araracuara, Amazonas; ICN 10000 (female), ICN 10001-02 (male) from Puré river, Letícia, Amazonas (Fig. 4); ICN 8176 (female) from Caparú, Taraira lake, Vaupés; ICN 10404 (female) from Mosiro Itajura biological research, Taraira lake, Vaupés.

Etymology. The specific name derives from the Greek *oligos* (few) and *balios* (spotted, dappled). The name is a reference to the smaller number of lateral trapezoidal marks on the body compared to *Bothrops brazili*, a name now restricted to the populations south of the Amazon River (see below).

Diagnosis. (1) A robust species, SVL = 245-805 mm, (2) short tail , TL = 35-113 mm; (3) 23-27 dorsals on the first third of the body; (4) 23-25 dorsals at midbody; (5) 18-21 dorsals on the last third of the body; (6) 156-164 ventrals in females (median=160) and 154-159 in males (median=156) ; (7) anal scale not divided; (8) 42-48 paired subcaudals in females (median= 44,5) and 47-52 in males (median=48,8); (9) 9-13 lateral trapezoidal marks in each side (median=11,01 and 11,7 on the right and left side, respectively) ; (10) postorbital stripe absent or poorly marked; (11) eight supralabials; (12) 10-12 infralabials; (13) second supralabial fused to the prelacunal; (14) belly checkered; (15) absence of vertebral stripe.

Figure 4. Paratype of *Bothrops oligobalius* **sp. nov.** (ICN10002) from Leticia, Amazonas, Colombia. A: Dorsal view; B: Ventral view. Scale bar = 1cm.

Comparisons with other species. Comparisons for meristic characters are presented in Table 1. Attributes from other species are presented in parenthesis. The new species can be promptly assigned to the *jararacussu* group due its morphological similarities with *B. brazili*, with whom the new species shared its specific epithet for over 60 years. Like other members in the *jararacussu* group, the new species presents the second labial scale fused to the prelacunal (separate in the *alternatus* and *neuwiedi* groups), postorbital stripe absent or faint (present in the *alternatus*, *neuwiedi*, *atrox* and *jararaca* groups), dorsal skin of the head with homogeneous coloration (blotched or patterned in the *alternatus* and *neuwiedi* groups and generally in the *atrox* group as well), stout and cylindrical bodies and terrestrial habit (slender, laterally compressed and arboreal habit in the *taeniatus* group), reddish and grayish background skin coloration and reddish eye (greenish background color in the *taeniatus* group, skin generally brownish in the *atrox*, *jararaca*, *alternatus* and *neuwiedi* groups), eight supralabials and lower number of ventrals (generally 7 supralabials and higher number of ventrals in *atrox* group). Assignment of *B. oligobalius* **sp. nov.** to the *jararacussu* group is further supported by our molecular approach (Fig. 1).

Within the *jararacussu* species group, *Bothrops oligobalius* **sp. nov.** can be distinguished from *B. jararacussu* and *B. pirajai* by the absence or a faint postorbital stripe (present in both species), grayish and reddish background skin coloration (yellowish or brownish background with black trapezoidal marks in both species), reddish eye (yellowish or brownish in both species). A higher number of ventrals in *Bothrops oligobalius* **sp. nov.** (156-164 in females, 154-159 in males) distinguishes it from *B. muriciensis* (151-155 in females, 148-150 in males) and *B. jararacussu* (170-186 in females, 166-181 in males) (Table 1). Recovering of *Bothrops oligobalius* **sp. nov.** sister to a clade assembling *B. jararacussu*, *B. pirajai* and *B. muriciensis* (which

correspond to the Atlantic Forest lineage in the *jararacussu* group) is further supported by high genetic divergence and highly disjunct distributions (Fig. 1).

Bothrops oligobalius **sp. nov.** is similar to *B. brazili* in background skin coloration and general external morphology features. However, the species can be promptly distinguished from *B. brazili* due to a smaller number of lateral trapezoidal marks (9-13; median=11,01 and 11,7 on the right and left side, respectively; in *B. brazili*, 13-21 median=17.1 and 16,3 on the right and left side, respectively) and absence of a vertebral stripe (present in *B. brazili*) (Table 1, Figs 2, 3, 4 and 7). Moreover, the low number of ventrals 156-164 in females and 154-159 in males, and subcaudals 42-48 in females and 47-52 in males in *Bothrops oligobalius* **sp. nov.** also distinguish from *B. brazili* (ventrals 174-186 in females and 175-184 in males, and subcaudals 45-56 in females and 54-61 in males) (Table 1). Molecular analysis recovered these two species as non-sister, with pronounced genetic distances between them. Moreover, the new species occurs north of the Amazon River, while *B. brazili* is restricted to areas south of the river, with no sympatry reported (Figs. 5, 7).

Lastly, the new species is distinguished from *B. sanctaecrucis*, a poorly known member of the *jararacussu* group from Bolivia, by the lower number of ventrals and caudals (171-185 and 56-57 respectively in B. *sanctaecrucis*), lower number of lateral trapezoidal marks (18-21), and grayish to reddish background skin coloration (brownish to yellowish).

Table 1. Meristic and qualitative features of species within *jararacussu* group of *Bothrops*. LTM: lateral trapezoidal marks; POS: posocular stripe; SC: subcaudal V: ventrals; VS: vertebral stripe.

	LTM	V	SC	POS	VS	Distribution
B. oligobalius **sp. nov.**	9-13	156-164 (F) 154-159 (M)	42-48 (F) 47-52 (M)	Absent or faded	Absent	Amazonia
B. brazili	13-21	174-186 (F) 175-184 (M)	45-56 (F) 54-61 (M)	Absent or faded	Present	Amazonia
B. muriciensis	12-15	151-155 (F) 148-150 (M)	45-51 (F) 51-55 (M)	Absent or faded	Absent	Atlantic Forest
B. pirajai	12-18	157-168 (F) 155-167 (M)	45-58 (F) 43-54 (M)	Present	Absent	Atlantic Forest
B. jararacussu	16-22	170-186 (F) 166-181 (M)	44-55 (F) 50-68 (M)	Present	Absent	Atlantic Forest
B. sactaecrucis	18-21	171-185	56-57	Absent or faded	Absent	Montains in Bolivia

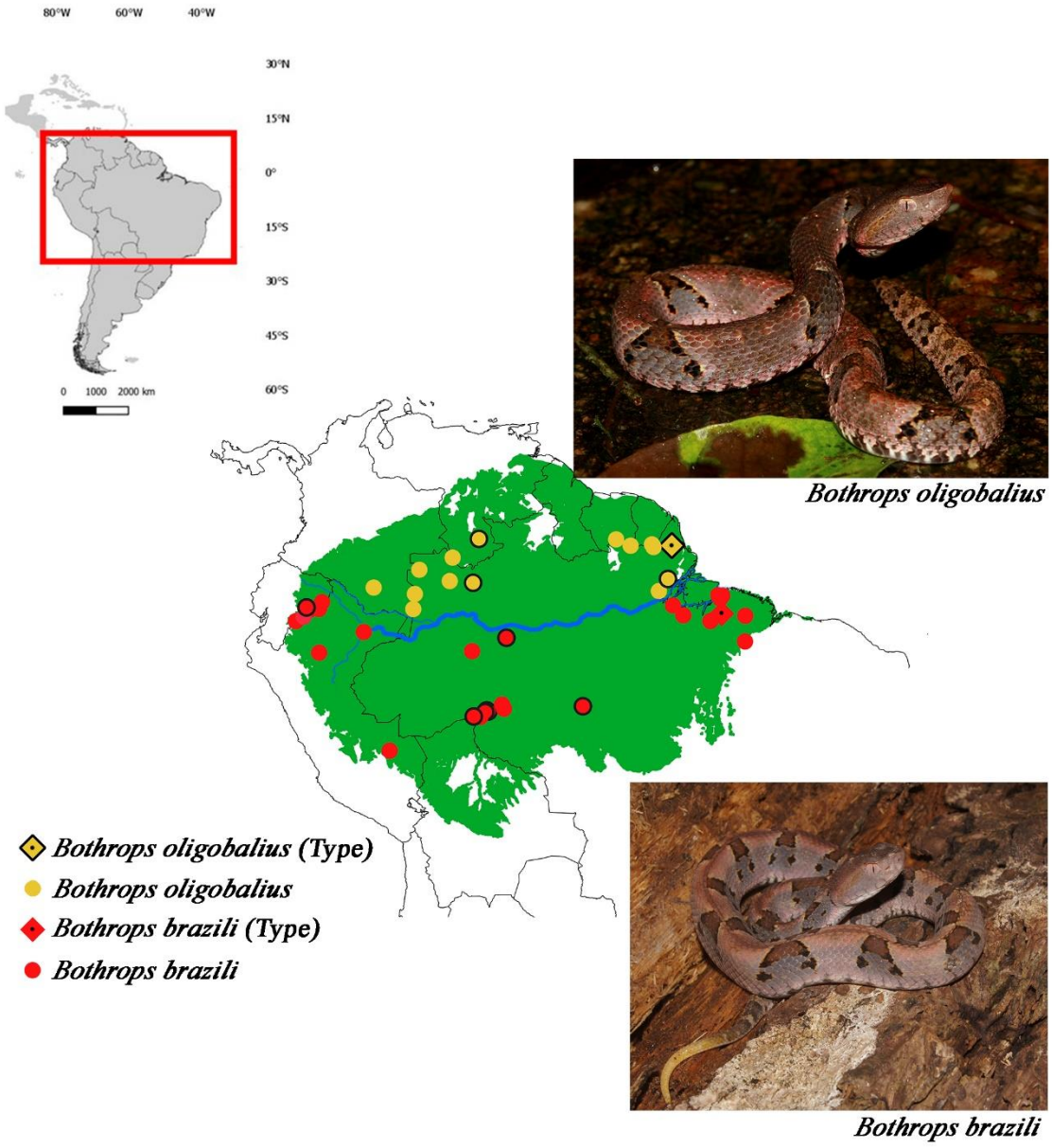

Figure 5. Geographic map of South America showing the distribution of *Bothrops oligobalius* **sp. nov.** (yellow) and *B. brazili* (red). Outlined diamonds and circles represent sites with genetic samples available. Diamonds with an inner dot indicate the type locality of each both species. The Amazonian biome is highlighted in green; the Amazon River is also indicated. Photos: *B. oligobalius* **sp. nov.** from French Guiana, by Maël Dewynter; *B. brazili* from Porto Velho, RO, Brazil, by FDV.

Description of the holotype. Subadult male (Figs. 3), preserved in ethanol with the left hemipenis prepared; SVL 594 mm; TL 95 mm (13.7% of total length); head length 32.1 mm; head width 20.7 mm; rostral 4.6 mm high; nasals divided; 2/1 prefoveals; 2/2 postfoveals; prelacunal fused with second supralabial in both sides of head; 1/1 preocular; 2/2 postoculars; 8/8 supralabials; 7/7 interoculabials; 5/5 circumorbitals; 10/11 infralabial; mental longer than broad, contacting the first two infralabials anteriorly (on each side); five gulars between the chin shield and the first ventral scale; 4/5 rows of gulars separating the first ventral scales from the infralabials; 2/1 canthals; 8 posterior intercanthals; 4 intersupraoculars; cephalic scales with no or weak keels; 25/24/19 dorsals; 159 ventrals; anal scale not divided; 48 divided subcaudals. Posterior cephalic scales longer than wide and strongly keeled; temporal scales keeled; internasals, canthals and supraoculars smooth. At midbody, eight rows of paravertebral scales between two adjacent lateral trapezoidal marks. In preservation, the background skin coloration of the holotype is grayish, with 12/11 brownish/grayish lateral trapezoidal marks lighter on the central part; marks usually meet on the dorsum. Dorsal skin of the head homogeneously grayish, without distinctive blotches. Ventral skin of the head light gray, with the center of the scales cream and scale edges light gray; Postorbital stripe absent to faint. Tail grayish/brownish dorsally with small dark brown lateral blotches; posterior part of tail dark brown; subcaudals speckled on anterior portion and brownish posteriorly. Venter irregularly speckled with cream and grayish; speckles reaching the middle of the subcaudal scales.

Hemipenial morphology. Hemipenis bilobed, bicalyculate, and bicapitate (with thinning toward the tip of the capitulum); sulcus spermaticus extending to the tip of each lobe (Fig. 6). Large calcified spines present on the body of the hemipenis on the

sulcate and asulcate faces, reaching the capitulum. On the asulcate face, region from the base of the hemipenis to the fork smooth and without spines. Intrasulcar region with many ossified spines; sulcus spermaticus bordered by small calcyfied spines up to the calyce (Fig. 6).

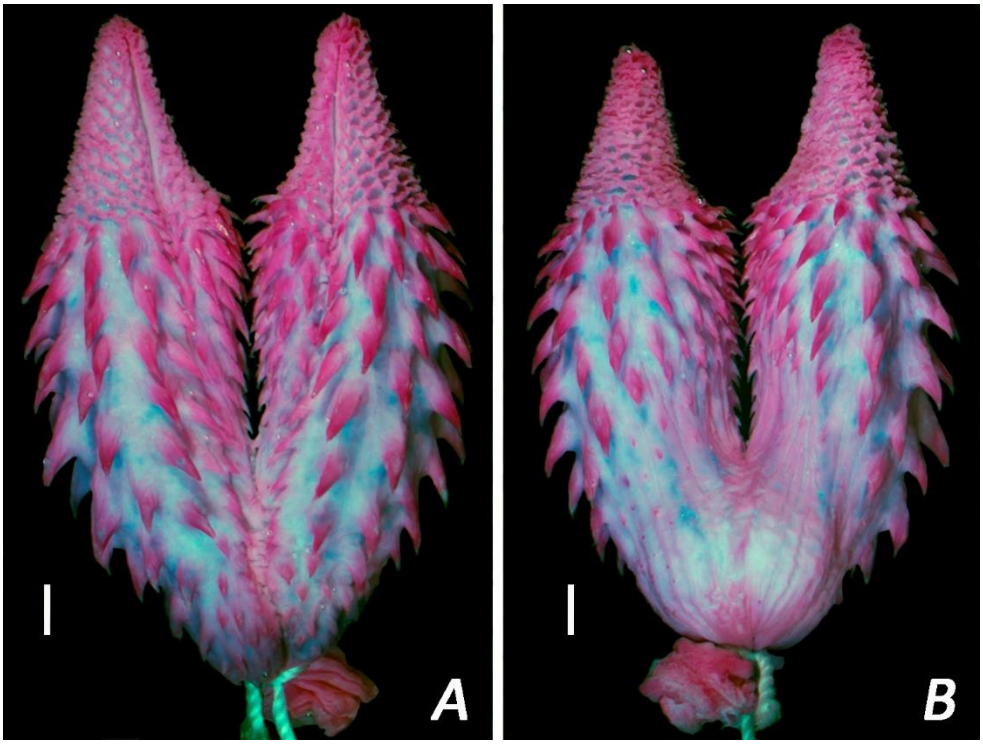

Figure 6. Left Hemipenis from the holotype of *Bothrops oligobalius* **sp. nov.** (MZUSP XXX, field number MTR 13844). A: Sulcade face; B: Asulcade face. Scale bar = 1mm.

Variation. The type series is fairly homogeneous in coloration pattern and pholidosis. The specimen ICN10001 shows nine supralabials on the right side, probably as a result of split of the fifth supralabial into two. Chin coloration can vary from totally cream to presenting the central part of scales cream with a grayish border. One specimen (MZUSP 11719) shows 13 lateral trapezoidal marks on each side of the

body; one (ICN8176) has 13 marks on the left side; all other specimens have less than 13 dorsal trapezoidal marks.

Distribution and natural history. The new species is known from areas north of the Amazon River in the Guiana Shield region extending into western Amazonia, an area that includes Brazil (states of Amapá, Roraima, and the northern parts of Pará and Amazonas), French Guiana, Surinam, Guyana, Venezuela and Colombia. Its currently known range limit to the west lies around the Putumayo River, although the species may also occur in the Putumayo-Napo interfluve. The new species presents stout body and terrestrial habit, being found mainly in *terra firme* (non-flooded) Amazonian lowland forests. The species has rarely been sampled in the *várzea* (flooded) forests.

Figure 7. *Bothrops brazili* in dorsal view. A- DHMECN3035 from Ecuador B- QCAZ4827 from Pastaza, Ecuador. Scale bar = 1cm.

Discussion

A new Amazonian species of Bothrops

The systematics and taxonomy of *Bothrops* snakes have been highly active over the last two decades, with several new species descriptions (e.g., *B. marmoratus* Da Silva & Rodrigues, 2008; *B. ayerbei* Folleco-Fernandez, 2010; *B. otavioi* and *B. sazimai* Barbo *et al*., 2012, 2106) and phylogenetic studies failing to fully resolve relationships within the *neuwiedi*, *atrox*, *taeniatus* and *jararacussu* species groups (Machado *et al*., 2014; Saldarriaga-Córdoba *et al*., 2017; Dal Vechio *et al*., 2018; Dal Vechio *et al*., 2019 *in press*). These studies have revealed persistent knowledge gaps about species diversity and evolutionary relationships in *Bothrops*, in spite of the medical significance of these ubiquitous venomous snakes.

These knowledge gaps involve broadly distributed taxa, which is the case of the two lineages that we recovered under the name *Bothrops brazili*. These snakes are easily distinguished from the other *Bothrops* species by the presence of eight supralabial scales, reddish coloration of the eye and body, stout bodies, and terrestrial habit (Campbel & Lamar, 2004). Since its description by Hoge (1953), *B. brazili* has been treated as a single taxon, despite previous suggestions of differences in scale traits between populations north and south of the Amazon River (Cunha & Nascimento, 1975, 1978, 1993).

In agreement with these previous morphological evidences, we found consistent differences in the number of ventral and subcaudal scales between populations of *B. brazili* that occur north and south to the Amazon River. Moreover, we found that the number of lateral trapezoidal marks is a distinguishing feature between these two geographical groups. Lastly, our molecular analyses inferred high genetic divergence between the northern and southern lineage of *B. brazili*, which were also found to be non-sister. Based on these results, we describe the northern lineage as a new taxon, which we name as *Bothrops oligobalius*. The name *B. brazili*

is now restricted to those populations that occur south of the Amazon River, given the original type locality of *B. brazili* in "Tomé Assú" (Tomé Açu municipality), Brazilian state of Pará, south of the Amazon River.

Specimens of *B. oligobalius* may have been previously treated under the name *Bothrops neglecta*, presently a synonym of *B. pirajai*. Amaral (1932) described *B. neglecta* based on two specimens: one from "southeastern Bahia", the holotype, and one paratype from "British Guyana", a specimen reported in Boulenger's snake catalogue (1896). These specimens caught the attention of Amaral because of their low number of ventral and caudal scales. While we did not have access to the specimen from "British Guyana", the locality and morphological features described by Amaral (1923) suggest that it corresponds to *B. oligobalius*. The name *B. neglecta*, however, is tied to the holotype, a specimen from an Atlantic Forest population referred earlier to *B. pirajai*, and is therefore not available.

Evolutionary relationships in the Bothrops jararacussu species group

Our phylogenetic results agree with previous suggestions based on morphological attributes that *B. pirajai* and *B. muriciensis* are nested within the *jararacussu* species group of *Bothrops*. These two species clustered with *B. jararacussu*, forming a clade that occurs in the coastal Atlantic Forest. In turn, we inferred this Atlantic Forest clade as sister to an Amazonian lineage that occurs north of the Amazon River, previously referred to as *B. brazili*. We inferred northern and southern Amazonian lineages of *B. brazili* as not sister, revealing that *B. brazili* is a paraphyletic taxon, a finding that supports the recognition of *B. oligobalius*. Like *B. brazili*, we inferred *B. jararacussu* as a paraphyletic species, with samples from the Brazilian state of Espírito Santo clustering with *B. pirajai* and *B. muriciensis*, while

samples from São Paulo and Minas Gerais composed a clade that is sister to the ancestor of all remaining Atlantic Forest samples.

These phylogenetic results recapitulate the biogeographic investigation of Dal Vechio *et al*. (2019 *in press*). Based on inference of historical demography, that study found support for a history of vicariant separation between the ancestor of *Bothrops oligobalius* **sp. nov.** and *B. brazili* during the Late Miocene, presumably as a result of the establishment of the modern Amazon River drainage (Dal Vechio *et al*., 2019 *in press*). This vicariant event was followed by the colonization of the Atlantic Forest during the Late Pleistocene as a result of a rainforest expansion event that would connected eastern Amazonia and the Atlantic Forest through the Caatinga in present-day Brazil's northeast. Our phylogenetic results support that this proposed colonization event triggered a new radiation of snakes in the Atlantic Forest region, a radiation that now includes *B. muriciensis*, *B. pirajai*, and the two non-sister lineages within *B. jararacussu*.

Concluding remarks

On the basis of morphological and genetic data, we found unrecognized species diversity and paraphyletic taxa within the *jararacussu* species group of *Bothrops*. We therefore assign a new taxon name, *B. oligobalius*, to the populations that occur north of the Amazon River. The new species occurs in Amazonian lowlands in Colombia, Venezuela, French Guiana, Surinam, Guyana, and Brazil. We inferred *B. oligobalius* as sister to a clade composed of Atlantic Forest species, supporting the idea that climate-driven changes in habitat distribution contributed to the diversification of South American lanceheads. Our results also reveal additional taxonomic issues that require attention, particularly the paraphyletic nature of the Atlantic Forest taxon

Bothrops jararacussu. Resolving these issues will rely on comprehensive sampling of phenotypic and genetic variation in this fascinating and mysterious clade of venomous snakes.

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APPENDIX I.

Examined specimens

MTR: Field numbers acronyms of specimens housed at Laboratório de Herpetologia do Instituto de Biociências, Universidade de São Paulo, São Paulo, Brazil, under the care of Miguel Trefaut Rodrigues; **MZUSP:** Museu de Zoologia da Universidade de São Paulo, São Paulo, Brazil; **IB:** Instituto Butantan, São Paulo, São Paulo, Brazil; **MPEG:** Museu Paraense Emilio Göeldi, Belém, Pará, Brazil; **MZUFAL:** Museu de Zoologia, Universidade Federal de Alagoas, Maceió, Alagoas, Brazil; **MNRJ:** Museu Nacional, Rio de Janeiro, Rio de Janeiro, Brazil; **MZUESC:** Museu de Zoologia, da Universidade Estadual de Santa Cruz; **CEPLAC:** Comissão Executiva de Planejamento da Lavoura Cacaueira, Brazil; **INPA:** Instituto Nacional de Pesquisas da Amazônia, Amazonas, Brazil, **MZML:** Museu de Zoologia Melo Leitão, Santa Tereza, Espírito Santo, Brazil, **MECN:** Museo Equatoriano de Ciencias Naturales, Quito, Equador; **QCAZ:** Museo de Zoología, Pontificia Universidad Católica del Ecuador, Quito, Equador; **FHGO:** Fundación Herpetologica Gustavo Orces, Quito, Equador; **ICN:** Museo de Historia Natural, Universidad Nacional de Colombia, Bogotá, Colombia.

Bothrops atrox: (*n* = 7) BRAZIL: **Acre:** Cruzeiro do Sul: MTR 28211, MTR 28473; **Amazonas:** Beruri: MTR 18742, MTR 18745, MTR 18849, MTR 18979-80, MTR 19304; Japurá: MTR33835; **Rondônia**: Guajará Mirim: MTR 25508, MTR 25759.

Bothrops brazili: (*n* = 30) BRAZIL: **Amazonas:** Coari: MZUSP 10534 (INPA326); **Pará**: Belém: MPEG 3274; Benevides: MPEG 6854; Canindé: MZUSP 4260; Melgaço: MPEG 20914; Portel: MPEG 22703; Santo Antônio do

Tauá: MPEG 5450, Tailândia: MZUSP 18128; **Rondônia:** Porto Velho: MPEG 17824-25, MZUSP 20563 (H2576); Mutum Paraná: MZUSP 4895. ECUADOR: DHMECN 3035, FHGO 96, FHGO 232, FHGO 6501, FHGO 8666, FHGO 9155, FHGO 10753, FHGO 10760, FHGO 11512, FHGO 12031, FHGO 12509, FHGO 12513-14, FHGO 12555; **Morona Santiago:** QCAZ 5169; **Pastaza:** QCAZ 4827, QCAZ 4992, QCAZ 11766.

Bothrops oligobalius **sp. nov.**: (*n* = 11) BRAZIL: **Amapá:** Lourenço: MZUSP XXX (Holotype), Mazagão: MZUSP 11719; **Amazonas:** São Gabriel da Cachoeira: MNRJ 10050-51; **Pará**: Almerim: MPEG 3274. COLOMBIA: **Amazonas**: Araracuara: ICN 2155; Leticia: ICN 10000-02; **Vaupés**: Taraira: ICN 8176I, CN10404.

Bothrops jararacussu: (*n* = 25) BRAZIL: **Espírito Santo:** MBML 625, MBML 1398; Cariacica: MBML 1715; Domingos Martins MBML 2725; Santa Leopoldina: MBML 447, MBML 466, MBML 1116; Santa Maria de Jetibá: MBML 90, MBML 515; Santa Teresa: MBML 19, MBML 21, MBML 32, MBML 268, MBML 342, MBML 398, MBML 400, MBML 436, MBML 490, MBML 501, MBML 515, MBML 532, MBML 645, MBML 1222, MBML 1265, MBML 2730.

Bothrops leucurus: (*n* = 2) BRAZIL: **Alagoas:** Marechal Deodoro: MZUFAL 5562; Rio Largo: MZUFAL 10764.

Bothrops muriciensis: (*n* = 5) BRAZIL: **Alagoas:** Murici: MZUFAL 379, MZUFAL10751, MZUFAL 10958, MZUFAL11094, MZUESC 509.

Bothrops pirajai: (*n* = 6) BRAZIL: **Bahia:** CEPLAC 1171, CEPLAC 4316, CEPLAC 5345, CEPLAC 5471, CEPLAC 5125; Wenceslau Guimarães: MTR 22184.

Capítulo 3

Artigo publicado na Revista Journal of Biogeography, 2018, 1–12. Anexo 1.

Phylogeography and historical demography of the arboreal pit viper *Bothrops bilineatus* (Serpentes, Crotalinae) reveal multiple connections between Amazonian and Atlantic rainforests

Historical demography of *Bothrops bilineatus*

Francisco Dal Vechio[1*], Ivan Prates[2], Felipe G. Grazziotin[3], Hussam Zaher[4], Miguel Trefaut Rodrigues[1]

[1]Departamento de Zoologia, Instituto de Biociências, Universidade de São Paulo, Rua do Matão, Trav. 14, nº 321, Cidade Universitária, CP 11461, CEP 05508-090, São Paulo, SP, Brazil.

[2]Department of Vertebrate Zoology, National Museum of Natural History, Smithsonian Institution, Washington, DC, 20560, USA.

[3]Laboratório de Coleções Zoológicas, Instituto Butantan, CP 42694, CEP 04299-970, São Paulo, SP, Brazil.

[4]Museu de Zoologia, Universidade de São Paulo, CP 42694, CEP 04299-970, São Paulo, SP, Brazil.

[*]Corresponding author. E-mail address: francisco.dalvechio@gmail.com

Key words: *Bothriopsis*, DIYABC, forest refuge, historical biogeography, molecular dating, Neotropics

Abstract

Aim: To examine historical connections between rainforest habitats in the Neotropics based on the evolutionary history of the arboreal pit viper *Bothrops bilineatus*.

Location: Amazonia and Atlantic Forest in South America.

Methods: We inferred phylogenetic relationships within *Bothrops* and phylogeographic relationships within *B. bilineatus* using both concatenated and species tree approaches under a Bayesian framework based on four mitochondrial and five nuclear genes. We inferred demographic history within *B. bilineatus* based on tests of alternative historical scenarios using coalescent simulations and Approximate Bayesian Computation.

Results: Phylogenetic analyses recovered four major clades within *Bothrops bilineatus*. The best-fit historical scenario involved colonization of the Atlantic Forest by an Amazonian ancestor around 2 Mya, followed by range expansion in the Atlantic Forest. Subsequently, there was a colonization event into the Guiana Shield from an Atlantic Forest ancestor, dating back to around 0.3 Mya.

Main Conclusion: The complex demographic history of *Bothrops bilineatus* shows multiple connection events between Amazonia and the Atlantic Forest in the last 2 million years. Two previously-hypothesized forest bridges, southern and northeastern, may have been involved in range expansions in this species. These results shed light on the historical processes involved in the diversification of this venomous South American snake, providing information about the historical dynamism of forested habitats in the Neotropics.

Resumo

Objetivos: Examinar as conexões históricas entre os ambientes florestados do Neotrópico, baseando-se na história evolutiva da jararaca verde *Bothrops bilineatus*.

Local: Amazônia e Floresta Atlântica na América do Sul.

Métodos: Nós inferimos sob perspectiva Bayesiana, as relações de parentesco em *Bothrops* e as relações filogeográficas em *B. bilineatus* usando *dataset* concatenado e árvore de espécies, com base em quatro genes mitocondriais e cinco nucleares. Nós, também, inferimos a história demográfica em *B. bilineatus*, baseando-se em teste de cenários históricos alternativos, usando simulações coalescentes e ABC (*Approximate Bayesian Computation*).

Resultados: As análises filogenéticas recuperaram quarto clados principais em *Bothrops bilineatus*. O melhor cenário histórico envolve colonização da Mata Atlântica por ancestral amazônico, entorno de 2 milhões de anos atrás, seguido de expansão populacional pelo ambiente atlântico. Subsequentemente, houve um outro evento de colonização em direção ao Escudo das Guianas com ancestralidade na Mata Atlântica, datado de 0.3 milhão de anos.

Conclusões: A complexa história demográfica de *Bothrops bilineatus* mostra múltiplas conexões entre a Amazônia e a Mata Atlântica nos últimos 2 milhões de anos. As duas hipotéticas pontes florestais, do nordeste e a do sudeste, parecem estar envolvidas na diversificação dessa serpentes venenosas da América do Sul, provendo informação sobre o dinamismo histórico dos ambientes florestados do Neotrópico.

INTRODUCTION

Several biogeographic hypotheses have been proposed to explain patterns of species richness and distribution in Amazonia, one of the most species-rich ecosystems on the planet. Restricted species ranges and composition turnover across this rainforest domain have been associated with contemporary and former landscape features (Bass *et al.*, 2010), such as present-day Amazon Basin rivers as dispersal barriers (Wallace, 1852), vicariance related to the formation of these major rivers, and tectonism in western South America linked to final uplift of the Andean chain during the Miocene (Hoorn *et al.*, 2010). Another influential hypothesis is that of Pleistocene forest refugia, which proposes cycles of forest fragmentation and expansion as a result of former climatic shifts in South America (Haffer, 1969). More recently, divergence time estimates between Amazonian taxa based on molecular data (Moritz *et al.*, 2000; Prates *et al.*, 2015, Marques-Souza *et al.*, 2018) have suggested pre-Pleistocene diversification in Amazonia, which, together with paleopalynological and speleothem data, contested the temporal framework implied by the refugia hypothesis (Colinvaux *et al.*, 2001; Bush & Oliveira, 2006; Bush, 2017). Nevertheless, the idea that pulses of forest expansion and contraction during the Pleistocene have affected the demographic history of Amazonian species has found support in studies based on genetic data (Prates *et al.*, 2016). For instance, the possibility of past forest bridges connecting Amazonia and the coastal Atlantic Forest has received attention in the last few years. These connections may explain the present-day ranges of a multitude of species whose ranges span both forest regions, but not the open areas of the dry diagonal of South America that separate them (the Caatinga, Chaco, and Cerrado domains).

The advance of molecular bioinformatic such as divergence time estimation, reconstruction of ancestral areas, and historical demographic inference has enabled

new interpretations about spatial routes and the timing of former biogeographic connections between Amazonia and the Atlantic Forest. Studies with bird taxa proposed a Pleistocenic forest bridge between Amazonia and the Atlantic Forest along the northern coast of northeastern South America (Batalha-Filho *et al.*, 2013). Such connection is also supported based on genetic data from small mammals (Costa, 2003), lizards (Prates *et al.*, 2016; Prates *et al.*, 2016) and snakes (Zamudio & Green, 1997). Pellegrino *et al.* (2011) and Rodrigues *et al.* (2014) suggested a similar but much older (Miocene) route based on lizard taxa. The idea of a northeastern forest connection along the northern Brazilian coast is consistent with the current presence of the "Brejos Nordestinos" in this region: relictual forest patches, surrounded by the semi-arid Caatinga, harboring a mixture of Amazonian and Atlantic Forest animals and plants. On the other hand, paleopalynological (Oliveira *et al.*, 1999), speleothem (Dever *et al.*, 1987, Auler *et al.*, 2001, 2004, Wang *et al.*, 2004) and paleontological data (Hartwing & Cartelle, 1996; Czaplewski & Cartelle, 1998) point to a wetter climate in the semi-arid Caatinga during the Pleistocene, suggesting the possibility of preterit forest corridors extending through the central Caatinga, as opposed to along the northeastern coast. Lastly, studies have also proposed a southern forest corridor between southwestern Amazonia and the southern Atlantic Forest. Batalha-Filho *et al.* (2013) found evidence of a southern Miocene bridge for birds through this route, while Costa (2003) found it to be consistent with phylogenetic patterns among small mammal taxa. Recently, Prates *et al.* (2017) found close relationships between southern Atlantic Forest anole lizards and species from western Amazonia and the Andes, suggesting a southwestern forest corridor that dates back to about 11 million years ago. Because studies to date have found evidence of distinct times, magnitudes, and routes of presumed forest corridors, a general picture of biotic interchange

between South American rainforests is yet to emerge. Since species responses to former habitat shifts may be affected by the biological attributes of organisms (Paz *et al.*, 2015; Zamudio *et al.*, 2016; Prates *et al.*, 2016a, b), studies that target organisms with distinct ecologies and life histories can contribute to a better understanding of species interchange across regions over time.

In South America, snakes of the subfamily Crotalinae are a promising model to investigate historical changes in the distribution of rainforests, including the possibility of former connections between Amazonia (including the Guiana Shield region) and the Atlantic Forest, because several Crotalinae species show strong associations with dense closed-canopy wet forests. This is the case of *Bothrops bilineatus*, an arboreal pitviper of the *taeniatus* group of *Bothrops* that occurs in both Amazonia and the Atlantic Forest, but not in the intervening open and dry habitats. In this study, based on genetic data sampled throughout the distribution of this species, we infer the routes, directionality, and timing of former range expansions. Our approach combines phylogenetic reconstruction and historical demographic inference based on genetic coalescent simulations and Approximate Bayesian Computation. Beyond shedding light on the history of biotic interchange among highly diverse Neotropical rainforests, our study also has implications for lineage diversity and taxonomy in these medically-important arboreal venomous snakes.

MATERIALS AND METHODS

Sampling and molecular protocols

We generated genetic data from 47 specimens of *Bothrops bilineatus* representing the entire range of this species, as well as of one *B. taeniatus*. We also used sequences available on GenBank of two *B. taeniatus*, two *B. pulcher*, and one *B. chloromelas*, all

of which members of the *taeniatus* species group within *Bothrops* (Fig. 1, 2 and 3 and Table 1). For broader phylogenetic inference, we also included 41 samples (22 from GenBank and 19 unpublished) of other *Bothrops* species as outgroups (Table 1). All samples used in this work were deposited in Genbank (accession numbers MH697889 - MH698321).

Table 1. Tissues samples used on phylogenetic and phylogeographic inferences.

Voucher	Species	Locality
288	Bothrops bilineatus	Olivença, Bahia, Brazil
MTR22219	Bothrops bilineatus	EEEWG, Bahia, Brazil
MUFAL10753	Bothrops bilineatus	Murici, Alagoas, Brazil
CEPLAC1	Bothrops bilineatus	Boa Nova, Bahia, Brazil
M176	Bothrops bilineatus	Murici, Alagoas, Brazil
M75	Bothrops bilineatus	Murici, Alagoas, Brazil
M277	Bothrops bilineatus	Murici, Alagoas, Brazil
M221	Bothrops bilineatus	Murici, Alagoas, Brazil
M222	Bothrops bilineatus	Murici, Alagoas, Brazil
AAGarda9598	Bothrops bilineatus	Serra da Jibóia, Bahia, Brazil
AF765	Bothrops bilineatus	French Guiana
MTR20744	Bothrops bilineatus	Pacaraima, Roraima, Brazil
PK1880	Bothrops bilineatus	Lower slope of Maringma-tepui, Guyana
CTMZ12115	Bothrops bilineatus	Potaro-Siparuni, Guyana
-	Bothrops bilineatus	Petit Saut, French Guiana
MPEG22694	Bothrops bilineatus	Juruti, Pará, Brazil
MPEG24289	Bothrops bilineatus	Juruti, Pará, Brazil
UFMT8908	Bothrops bilineatus	Cotriguaçu, Mato Grosso, Brazil
UFMT-R7809	Bothrops bilineatus	Colniza, Mato Grosso, Brazil
DT3831	Bothrops bilineatus	Tapajós, Pará, Brazil
CGTA-C0043	Bothrops bilineatus	Médio Rio Purus, Amazonas, Brazil
APL18499	Bothrops bilineatus	Br-319, Amazonas, Brazil
APL18502	Bothrops bilineatus	Br-319, Amazonas, Brazil
BBFLONA	Bothrops bilineatus	Tapajós, Pará, Brazil
BB3	Bothrops bilineatus	Tapajós, Pará, Brazil
DT4309	Bothrops bilineatus	Tapajós, Pará, Brazil
LSUMZ14437	Bothrops bilineatus	Santarém, Pará, Brazil
LSUMZ17922	Bothrops bilineatus	Parque estadual Guajará-Mirim, Rondônia, Brazil
LSUMZ17923	Bothrops bilineatus	Parque estadual Guajará-Mirim, Rondônia, Brazil

LSUMZ17924	**Bothrops bilineatus**	**Parque estadual Guajará-Mirim, Rondônia, Brazil**
BB1	**Bothrops bilineatus**	**UHE Santo Antônio, Rondônia, Brazil**
T62034	**Bothrops bilineatus**	**Cruzeiro do Sul, Acre, Brazil**
MTR21460	**Bothrops bilineatus**	**UHE Santo Antonio, Rondônia, Brazil**
MTR28518	**Bothrops bilineatus**	**Serra do Divisor, Acre, Brazil**
MTR28528	**Bothrops bilineatus**	**Rio Branco, Acre, Brazil**
MTR28529	**Bothrops bilineatus**	**Rio Branco, Acre, Brazil**
APL16357	**Bothrops bilineatus**	**Rondônia, Brazil**
APL17803	**Bothrops bilineatus**	**Rondônia, Brazil**
BBLUIS	**Bothrops bilineatus**	**Porto Velho, Rondônia, Brazil**
H4259	**Bothrops bilineatus**	**UHE Jirau, Rondônia, Brazil**
H5144	**Bothrops bilineatus**	**UHE Jirau, Rondônia, Brazil**
IB5823	**Bothrops bilineatus**	**UHE Jirau, Rondônia, Brazil**
MTR37170	**Bothrops bilineatus**	**ESEC Rio Acre, Acre, Brazil**
DHMECN7800	**Bothrops bilineatus**	**Ecuador**
DHMECN7801	**Bothrops bilineatus**	**Ecuador**
FHGO 983	**Bothrops bilineatus**	**Morona Santiago, Macuma, Ecuador**
-	**Bothrops bilineatus**	**Letícia, Colombia**
MVZ 223514	**Bothrops ammodytoides**	**Neuquen, Argentina**
IB 55543	**Bothrops fonsecai**	**Campos do Jordão, São Paulo, Brazil**
ITS 427	**Bothrops itapetiningae**	**Itirapina, São Paulo, Brazil**
WWW	**Bothrops cotiara**	**Brazil**
MCP19013	**Bothrops alternatus**	**Gravataí, Rio Grande do Sul, Brazil**
2ALT	**Bothrops alternatus**	**Cristais, Minas Gerais, Brazil**
MMFE2	**Bothrops alternatus**	**Itirapina, São Paulo, Brazil**
ITS358	**Bothrops alternatus**	**Itirapina, São Paulo, Brazil**
MM2E5	**Bothrops alternatus**	**Itirapina, São Paulo, Brazil**
-	**Bothrops alternatus**	**Pinhão, Paraná, Brazil**
MTR16058	**Bothrops jararaca**	**Serra Bonita, Camacan, Bahia, Brazil**
MTR11604	**Bothrops jararaca**	**Caparaó, Espírito Santo, Brazil**
PT3404	**Bothrops diporus**	**Argentina**
MTR14196	**Bothrops lutzi**	**EESGT, Tocantins, Brazil**
MTR14488	**Bothrops lutzi**	**EESGT, Tocantins, Brazil**
MTR11185	**Bothrops erythromelas**	**Santo Inácio, Bahia, Brazil**
MTR29115	**Bothrops erythromelas**	**Alagoado, Bahia, Brazil**

IB55541	**Bothrops erythromelas**	**Guanambi, Bahia, Brazil**
RG829	**Bothrops erythromelas**	**Piranhas, Alagoas, Brazil**
H569	**Bothrops jararacussu**	**Bertioga, São Paulo, Brazil**
1JASSU	**Bothrops jararacussu**	**Viçosa, Minas Gerais, Brazil**
MTR6306	**Bothrops brazili**	**Rio Maracá, Amapá, Brazil**
MTR13844	**Bothrops brazili**	**Lourenço, Amapá, Brazil**
FHGO live 2142	**Botrops punctatus**	**-**
FHGO live 2166	**Bothrops osbornei**	**Ecuador**
MZUCR 11152	**Bothrops asper**	**Costa Rica**
-	**Bothrops atrox**	**Acre, Brazil**
MTR13445	**Bothrops leucurus**	**Trancoso, Bahia, Brazil**
MTR24597	**Bothrops leucurus**	**Campo Formoso, Bahia Brazil**
MTR12742	**Bothrops atrox**	**Rio Abacaxis, Amazonas, Brazil**
BAT02	**Bothrops atrox**	**Amazonas, Brazil**
-	**Bothrops atrox**	**Suriname**
WW741	**Bothrops atrox**	**Ecuador**
WW742	**Bothrops atrox**	**Ecuador**
JM78	**Bothrops pulcher**	**Peru**
FHGO live 2142	**Bothrops pulcher**	**Zamora Chinchipe, Ecuador**
LSUMZ41037	**Bothrops chloromelas**	**Pasco, Peru**
MPEG23653	**Bothrops taeniatus**	**FLONA Trombetas, Pará, Brazil**
FHGO live 1407	**Bothrops taeniatus**	**Morona Santiago, Macuma, Ecuador**
-	**Bothrops taeniatus**	**Suriname**

Genomic DNA was extracted from tissue samples (liver, muscle, scale or shed skin) and submitted to standard PCR protocols (see below). Nine DNA markers were amplified and sequenced (totaling 6,009 bp): the mitochondrial genes (matching available datasets for *Bothrops*) *16s ribosomal RNA* (*16s*, 534 bp; Palumbi, 1996), *12s ribosomal RNA* (*12s*, 394 bp; Palumbi, 1996), *cytochrome b* (*cytb*, 737 bp; Pook *et al.*, 2000), and *NADH dehydrogenase subunit 4* (*nd4*, 797 bp; Arévalo *et al.*, 1994), as well as the nuclear genes *megakaryoblastic leukemia 1* (*mkl1*, 787 bp; Townsend *et*

al., 2008), *recombination-activating gene 1* (*rag1*, 995 bp; Wiens *et al.*, 2008), *prolactin receptor* (*prlr*, 551 bp; Townsend *et al.*, 2008), *oocyte maturation factor* (*c-mos*, 575 bp; Godinho *et al.*, 2006), and *brain-derived neurotrophic factor* (*bdnf*, 639 bp; Townsend *et al.*, 2008). PCR reaction conditions were: an initial denaturation step at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 40 sec, annealing at X°C for 40 sec, and extension at 72°C for 40 sec, and a terminal extension cycle at 72°C for 7 min, in which X is the temperature for each set of primers, as follows: 51ºC for *16s*, *12s*, *cytb*, *nd4*, *prlr* and *rag1*; 52ºC for *c-mos*; 54ºC for *mkl1*; and 55ºC for *bdnf*.

Phylogenetic inference

To reconstruct the historical relationships among samples, we used Bayesian Inference after sequence edition in CodonCode Aligner v. 3.7.1.1 (www.codoncode.com) and alignment using MUSCLE (Edgar, 2004) as implemented in MEGA v. 6 (Tamura *et al.*, 2013) under default settings. The best-fit model of evolution for each aligned marker was identified using jModelTest v. 2.1.3 (Darriba *et al.*, 2012) and the Akaike Information Criterion (AIC), as follows: GTR+G+I for all mitochondrial markers, HKY for *bdnf* and *c-mos*, GTR for *prlr*, GTR+I for *rag1*, and HKY+I for *mkl1*. Phylogenetic analyses were performed using MrBayes v. 3.2 (Ronquist *et al.*, 2012) and the *BEAST tool (a coalescent-based method) in BEAST 1.8.3 (Drummond *et al.*, 2012) through the Cipres Science Gateway (Miller *et al.*, 2010). For MrBayes analyses, two independent Bayesian runs were performed with four Markov chains each, starting from a random tree; each run consisted of 10,000,000 generations, with trees sampled every 1,000 generations. For *BEAST analyses, two independent runs of 100 million generations each were used, with a sampling frequency of 10,000

generations. For both MrBayes and BEAST runs, we discarded the first 25% of trees as burn-in; convergence of independent runs and stationarity of model parameters (effective sampling sizes > 200) were verified using Tracer v. 1.6 (Rambaut and Drummond, 2009). A 50% majority consensus tree with clade frequencies was summarized for MrBayes analyses, while a maximum clade credibility tree was summarized for *BEAST runs using TreeAnnotator (Drummond *et al.*, 2012). Resulting phylogenies were visualized using FigTree 1.4.0 (http://tree.bio.ed.ac.uk/). The haplotypic phase of heterozygotes was determined using PHASE 2.1.1 (Stephens & Donnelly, 2003) under default settings implemented in DNAsp v5.1 (Librado & Rozas, 2009).

Alternative scenarios of demographic history

Phylogenetic analyses recovered a number of clades within *Bothrops bilineatus* that are restricted geographically (see Results). To investigate the history of range expansions in *B. bilineatus*, we tested alternative historical demographic scenarios based on these inferred lineages using coalescent simulations and Approximate Bayesian Computation (ABC). Our approach compares the observed (i.e., sampled) genetic data to data simulated under each competing scenario, based on informative summary statistics that capture patterns of genetic variation (Beaumont, 2010; Csilléry *et al.*, 2010). We were particularly interested in testing which spatially-restricted Amazonian lineage (western Amazonia, central Amazonia, or Guiana Shield; see Results) most likely acted as the colonization source of the Atlantic Forest group. Moreover, to shed light on the magnitude of former forest connections, we aimed to test whether colonization of the Atlantic Forest was accompanied by a population bottleneck, as expected as a result of spatially or temporally restricted connections

across domains, as opposed to a scenario of vicariance of a widely-distributed ancestor, which did not involve pronounced shifts in population sizes following divergence.

We used the historical relationships inferred by phylogenetic analyses to inform the population tree topology in alternative models of *B. bilineatus* demographic history. However, because phylogenetic analyses based on both MrBayes and *BEAST found little support for which Amazonian clade (central, western or central/western) is sister to a clade containing the Atlantic Forest plus Guiana Shield samples (see Results), we included all possible tree topologies at that node (i.e., switching the relationship among major clades) in our competing historical models. These alternative topologies include (see Results): a central Amazonian clade as the sister of the common ancestor of the remaining *B. bilineatus* samples, with a western Amazonian lineage as sister to the ancestor of Atlantic Forest + Guiana Shield (used in scenarios 1-4; see below); a western Amazonian clade as the sister of the ancestor of the remaining clades, with a central Amazonian clade as sister to the ancestor of Atlantic Forest + Guiana Shield (scenarios 5-8); and a central and western Amazonian clades forming a clade that is sister to Atlantic Forest + Guiana Shield (scenarios 9-12; see Fig. S1.1 for a visual representation of these scenarios).

Based on those three alternative topologies, 12 historical scenarios were simulated and confronted with each other (Fig. S1.1). In scenario 1, an ancestral population occurring in western Amazonia (central Amazonia in scenario 5) and the Atlantic Forest was split following expansion of open and dry domains in South America (a vicariant split); a second vicariant event subsequently separated Guiana Shield and Atlantic Forest clades (Fig. S1.1). In scenarios 2 and 6, a founder population colonized the Atlantic Forest from the Guiana Shield region in northern

Amazonia through a forest corridor in the presently dry belt of northeastern South America, with subsequent population expansion within the Atlantic Forest (Fig. S1.1). In scenario 3, a vicariant split separated an ancestral population occurring in western Amazonia (central Amazonia in scenario 7) and the Atlantic Forest, with a subsequent event of forest expansion leading to colonization of the Guiana Shield from the Atlantic Forest, which would then be split following expansion of open and dry domains in northeastern South America (Fig. S1.1). In Scenario 4, a founding population colonized the Atlantic Forest from western Amazonia (central Amazonia in scenario 8), with subsequent population expansion within the Atlantic Forest; in this same scenario, a second founder event led to colonization of the Guiana Shield from the Atlantic Forest (Fig. S1.1).

In scenarios 9-12, western and central Amazonia were treated as sister lineages whose ancestor is sister to a clade formed by Atlantic Forest and Guiana Shield clades. In scenario 9, an ancestral population occurring in central and western Amazonia and the Atlantic Forest was split following expansion of open and dry domains in South America (a vicariant split); a second vicariant event separated the Guiana Shield and Atlantic Forest clades (Fig. S1.1). In scenario 10, a founder population colonized the Atlantic Forest by an Amazonian ancestor from the Guiana Shield region through a forest corridor in northeastern South America, with subsequent population expansion within the Atlantic Forest (Fig. S1.1). In scenario 11, a vicariant split separated an ancestral population occurring in central and western Amazonia and the Atlantic Forest; a subsequent event of forest expansion led to colonization of the Guiana Shield from the Atlantic Forest (Fig. S1.1). In scenario 12, a founding population colonized the Atlantic Forest from a central and western Amazonian ancestor, with subsequent population expansion within the Atlantic Forest; in this same scenario, another founder

event led to colonization of the Guiana Shield from the Atlantic Forest (Fig. 4 and Fig. S1.1). In summary, scenarios 1, 5 and 9 similarly test for vicariant events in the demographic history of *B. bilineatus*; scenarios 2, 6 and 10 similarly test for a colonization event of the Atlantic Forest from a Guiana Shield ancestor; scenarios 3, 7 and 11 similarly test for a colonization event of Amazonia (Guiana Shield region) from a Atlantic Forest ancestor; and lastly, scenarios 4, 8 and 12 similarly test for a colonization event of the Atlantic Forest from a central and western Amazonia ancestor, with a second colonization event of the Guiana Shield from an Atlantic Forest ancestor.

We performed historical demographic analyses based on six loci: one mitochondrial locus (unphased data) comprising four genes (*12s*, *16s*, *cytb* and *nd4*), and five independent nuclear loci (*mkl1*, *rag1*, *prlr*, *c-mos* and *bdnf*; phased data). We follow Prates et al. (Prates *et al.*, 2016a) and performed ten million coalescent simulations (evenly distributed among all demographic scenarios) followed by ABC inference with *DIYABC* 2.1 (Cornuet *et al.*, 2014). We chose summary statistics based on preliminary runs that confirmed that Tajima's D (Tajima, 1989), number of private segregating sites, and pairwise Fst (Hundon *et al.*, 1992) were appropriate for discrimination among simulated scenarios. We also confirmed if the observed genetic data were contained within the space of simulated data by performing a principal component analysis (PCA) based on the chosen summary statistics (Cornuet et al., 2014).

Prior distributions of model parameters were set as follows: (1) divergence times between independently-evolving lineages with a uniform distribution ranging between 0.1-5 million years; (2) effective population size in each independently-evolving lineages with a uniform distribution ranging between 0.01-5 millions of

individuals; (3) effective population size during a founder event with a uniform distribution ranging from 0.001-0.1 millions of individuals; and (4) effective population size prior to vicariant events with a uniform distribution ranging from 0.01-5 millions of individuals. We set a mutation rate based on the number of substitutions among sequences of Crotalinae species (Wuster *et al.*, 2008), with a uniform prior distribution ranging from 10^{-9} to 10^{-8} substitutions per site per generation for the mitochondrial locus and 10^{-10} to 10^{-8} substitutions per site per generation for the nuclear loci. A *Tamura-Nei* (Tamura & Nei, 1993) substitution model of evolution was implemented for each locus. To convert the number of generations in our models to years, we assumed a generation time (T) of three years in *Bothrops*. We established this number based on the equation $T = a + [s/1(1- s)]$ (Lande et al., 2003), where a is the age of maturity and s is the annual survival rate. Almeida-Santos & Salomão (2002) and Almeida-Santos *et al.* (2004) suggested that the maturity age of *Bothrops* species is around 1.5-3 years. While it is hard to estimate the annual survival rate of these snakes over time, which probably vary with climatic conditions and food supply, some data for populations of crotalinae species suggest annual survival rates around 0.39 to 0.77 (Brown *et al.*, 2007; Maritz & Alexander, 2012). Based on these data, we estimate generation times in *Bothrops bilineatus* as being around 2-3 years.

To assess how data simulated under each of the 12 scenarios compare to the empirical genetic data, we estimated the statistical support of each model based on Euclidean distances between the simulated data and the observed data. For this, we selected the 1.000 simulated datasets (0.01% of the total simulations) that were closest to the observed data. We then estimated posterior distributions of population parameters under the best-fit scenario, using the 500 simulated datasets that were closest to the observed data.

Lastly, we tested the accuracy of the model selection procedure by examining whether the different simulated scenarios can be discriminated based on the summary statistics used. For this, we simulated 100 pseudo-observed datasets under each scenario, such that the true scenario was known for each pseudo-observed dataset. We then performed ABC inference on each pseudo-simulated dataset, thus calculating the proportion of pseudo-observed datasets that were correctly assigned to its generating model.

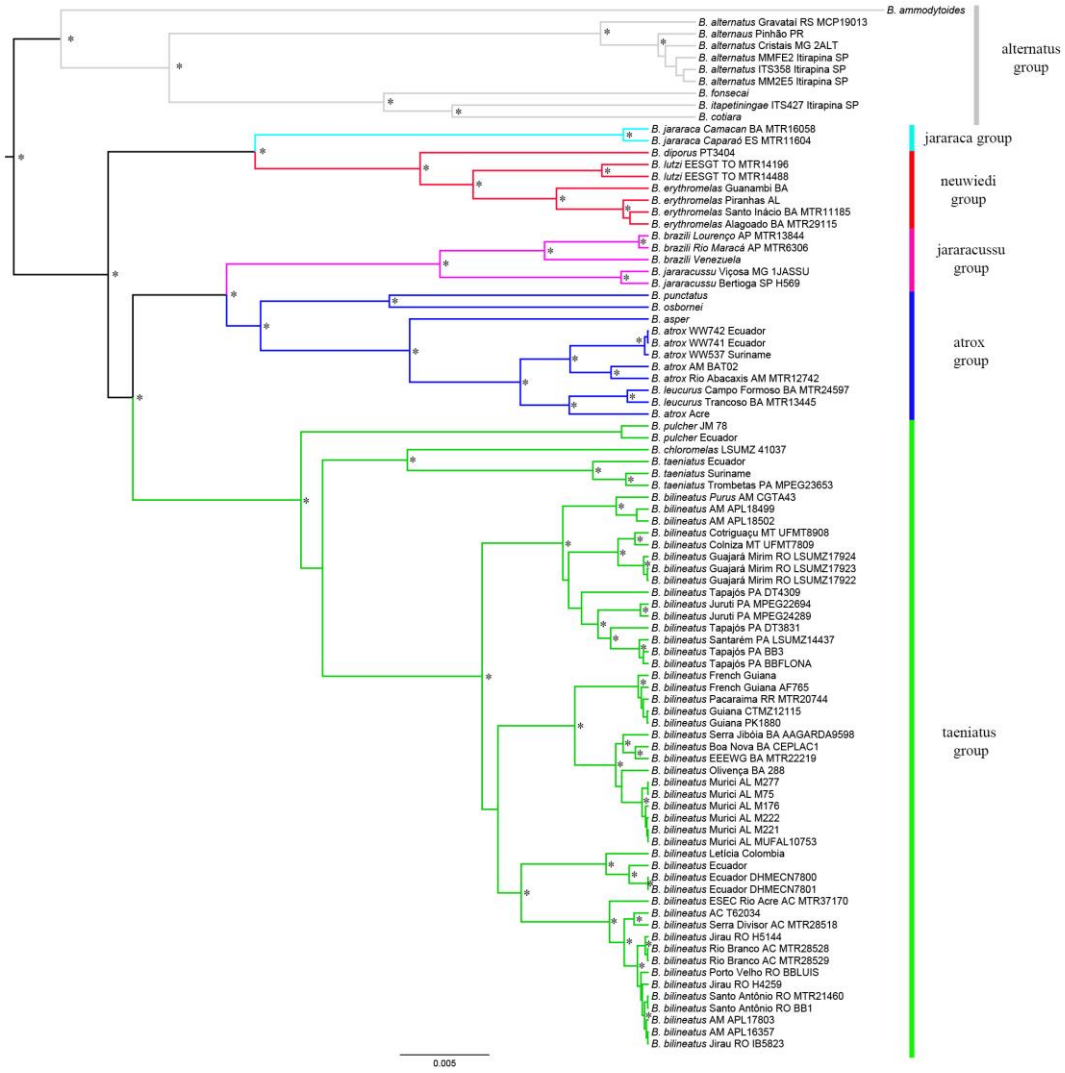

Figure 1. Phylogenetic relationships within *Bothrops* performed on MrBayes based on a concatenated dataset with nine genes, showing its six species groups: *alternatus* in

grey, *jararaca* in light blue, *neuwiedi* in red, *jararacussu* in purple, *atrox* in dark blue and *taeniatus* in green. Asterisks on nodes indicate posterior probabilities higher than 0.95. Scale bar indicates substitutions per site.

RESULTS

Phylogenetic relationship and species delimitation

Phylogenetic relationships within *Bothrops* based on a concatenated dataset (using MrBayes) indicate that the *taeniatus* species group is monophyletic (PP=1), and sister to the *atrox* + *jararacussu* species groups (Fig. 1). The species tree (using *BEAST) recovered a different relationship among these species groups, with a clade formed by the *atrox*, *jararacussu*, *jararaca* and *neuwiedi*, groups as sister to the *taeniatus* group, albeit with low statistical support (Fig. 2). In all phylogenetic analyses, *B. bilineatus* is nested within the *taeniatus* species group (Fig. 2 and Fig. 3). *Bothrops bilineatus* is constituted by four highly supported major clades (PP=1), all of which restricted in geographic space, as follows (Fig. 3): 1) an Atlantic Forest clade; 2) a Guiana Shield clade occurring north of the Amazon river and east of the Orinoco and Rio Negro rivers; 3) a western Amazonian clade occurring west of the Orinoco and Rio Negro rivers (in Colombia and Ecuador) and western Brazil (states of Acre and Rondônia, possibly also occurring in Peru and Bolivia); and 4) a central Amazonian clade distributed to the south of the Amazon river, in the Brazilian states of Amazonas, Rondônia, Mato Grosso and Pará (Fig. 3).

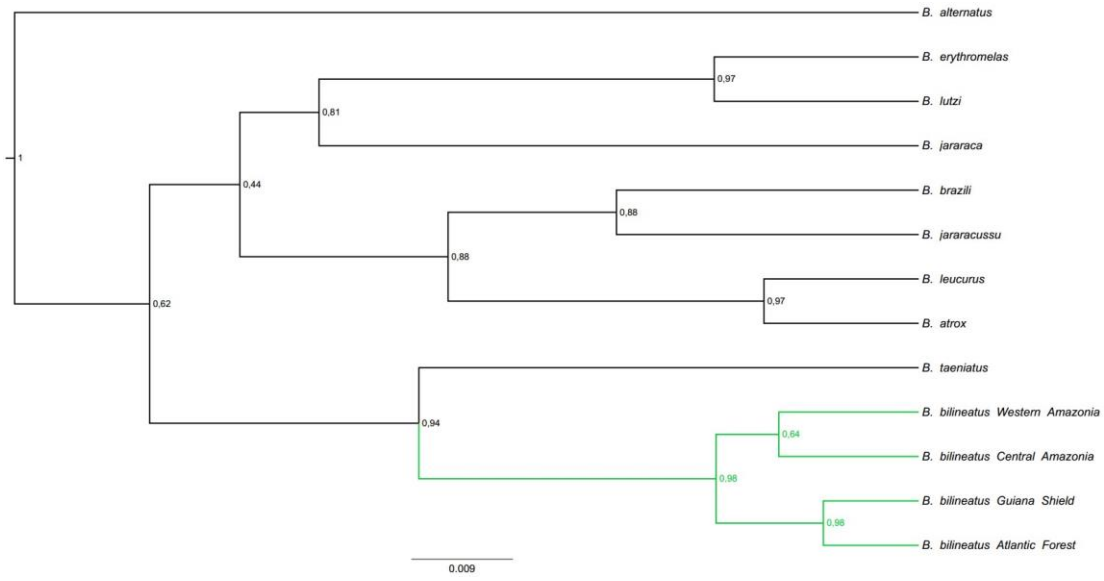

Figure 2. Species tree within *Bothrops* based on coalescent analysis performed in *BEAST implemented in BEAST. The green branch represents *B. bilineatus* (corresponding to four geographically restricted populations; see text). Numbers in nodes represent posterior probability values. Scale bar indicates substitutions per site.

Within *Bothrops bilineatus*, phylogenetic results using MrBayes found the Atlantic Forest and Guiana Shield clades to be sister to each other with high support (PP=1). This clade is the sister group of a clade formed by western Amazonian samples with moderate support (PP=0.73). The clade formed by these three geographically restricted clades is the sister-group of the more broadly distributed central Amazonian clade (PP=1) (Fig. 3). On the other hand, the species tree analysis based on *BEAST recovered a sister relationship between the western and central Amazonian clades (PP=0.64), whose ancestor is the sister of the Atlantic Forest + Guiana Shield clade (PP=0.98) (Fig. 2).

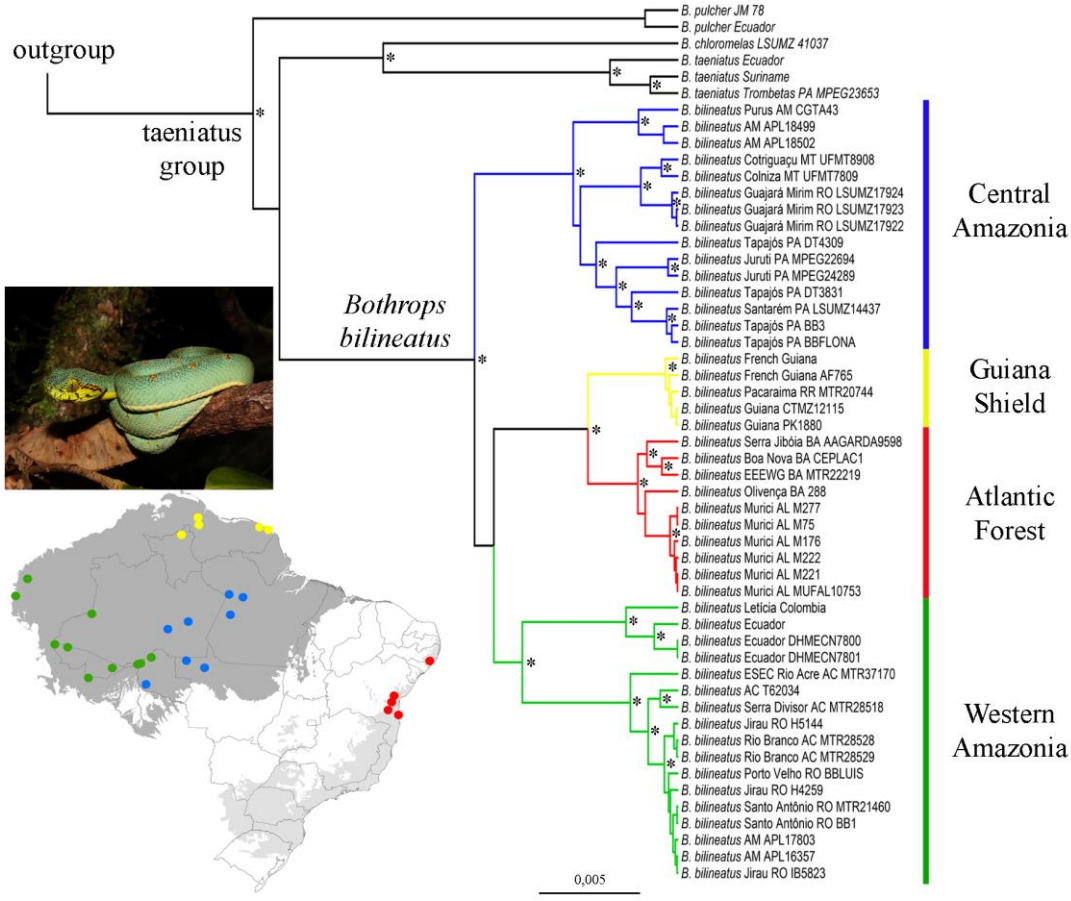

Figure 3. Phylogenetic relationships within the *taeniatus* group and phylogeography of *Bothrops bilineatus* based on nine molecular markers (five nuclear and four mitochondrial genes) performed on MrBayes. Colors in map correspond to major clades in historical demographic analyses. Asterisks on nodes indicate posterior probabilities higher than 0.95. Scale bar indicates substitutions per site. Photo: Renato Recoder.

Best-fit historical scenarios

Based on coalescent simulations and Approximate Bayesian Computation, we found that the observed genetic data are consistent with the hypothesis that *Bothrops bilineatus* colonized the Atlantic Forest from an Amazonian ancestor (which presumably occurred in central + western Amazonia) and subsequently colonized the

Guiana Shield region through a back colonization from the Atlantic Forest (scenario 12; PP=0.99) (Fig. 4). These results support the idea of two independent colonization events in opposite directions across currently disjunct rainforest blocks in South America.

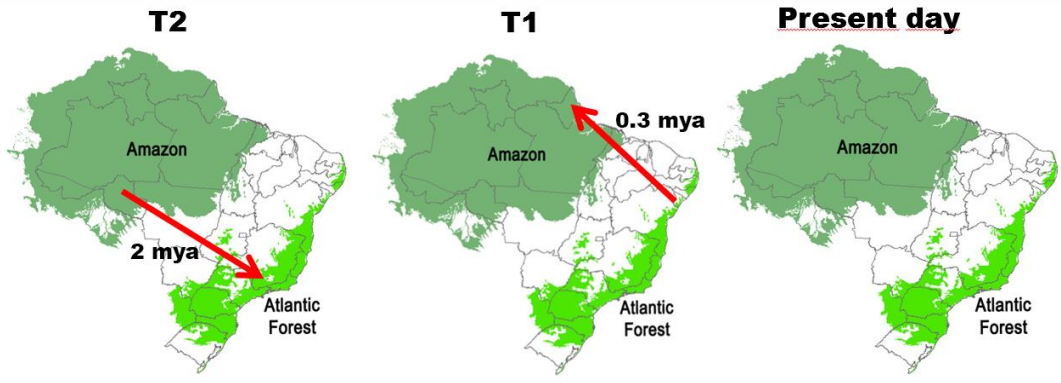

Figure 4. Best-fit scenario supported by historical demography analyses using DIYABC based on nine genes (four mitochondrial and five nuclear) from *Bothrops bilineatus*. The model includes a colonization event by an Amazonian ancestor (which presumably occurred in central + western Amazonia) into the Atlantic Forest at T2 (2 Mya), with another colonization event into the Guiana Shield by an Atlantic Forest ancestor at T1 (0.3 Mya). Arrows represent colonization events; T2 and T1 represent times in the past (T2 older than T1; T1 older than today). For details, see text.

Posterior parameter estimates based on the best-fit scenario support a late-Pliocene divergence between the ancestor of the central and western Amazonian clade and the ancestor of the Atlantic Forest and Guiana Shield clades, dating back to 1.95 mya (median value; 95% credibility interval (C.I.) = 1.5-4.5 mya); and a late-Pleistocene divergence between the Atlantic Forest and Guiana Shield clades, dating

back to 0.3 mya (median value; 95% credibility interval (C.I.) = 0.19-0.8 mya). Effective population size estimates suggest that the initial pool of Atlantic Forest colonizers was composed of about 53,000 individuals (ca. 1.2% of the ancestral Amazonian population), while the Guiana Shield was colonized by about 98,000 individuals originated from the Atlantic Forest (ca. 4.6% of the ancestral Atlantic population), supporting a pronounced population bottleneck following dispersal into both the Atlantic Forest and Guiana Shield. Median substitution rates were estimated as 5×10^{-9} substitutions per site/year for the mitochondrial locus (median value; 95% credibility interval (C.I.) = $3.4–8.6 \times 10^{-9}$ substitutions per site/year) and $0.4–0.7 \times 10^{-9}$ substitutions per site/year for the nuclear loci (median value; 95% credibility interval (C.I.) = $0.3 – 2.1 \times 10^{-9}$ substitutions per site/year)

Model validation based on principal component analyses of the summary statistics confirmed that the observed data were contained within the space of simulated data. Posterior error rates based on pseudo-observed datasets were estimated as 0.69. However, most incorrect inferences (53% from the total of pseudo-observed datasets) happened between scenarios that depict the same history of Atlantic Forest and Guiana Shield colonization but have topological differences regarding which *Bothrops bilineatus* clade that is sister to the remaining ones. This is the case of confusion between scenarios 2, 6 and 10, or 4, 8 and 12 (see Methods). As a result, the error rate between scenarios that depict distinct histories of colonization (e.g. colonization of the Atlantic Forest from a Guiana Shield ancestor versus colonization of the Guiana Shield from an Atlantic Forest ancestor, or a vicariant event between these two regions) is effectively much lower: 0.16.

DISCUSSION

The idea of past biogeographic connections between Amazonia and the Atlantic Forest has received increasing attention in the last few years, with phylogeographic and phylogenetic studies pointing to connection events between these two domains (Zamudio & Green, 1997; Costa, 2003; Batalha-Filho *et al.*, 2013 Prates *et al.*, 2016a, 2017). Speleothem (Auler *et al.*, 2004; Cheng *et al.*, 2013), and paleopalynological records (Oliveira *et al.*, 1999) have also supported the view of past dynamism in South American rainforest habitats, with pulses of forest expansion and fragmentation. In agreement with these studies, our results for *Bothrops bilineatus* support the idea that changing climates have led to shifts in South American rainforest distribution and, ultimately, in biotic interchange between regions, allowing lineages to colonize new areas, where pressures from competitors and predators could potentially be relaxed. These events seem to have had major consequences for the composition of local species pools, and therefore for large-scale biogeographic patterns in both Amazonia and the Atlantic Forest.

Our tests of alternative historical demographic scenarios based on multi-locus genetic data from *Bothrops bilineatus* point to a history of two consecutive events of forest colonization, as well as of back invasion of Amazonia – to our knowledge, a pattern that has not been yet identified in studies of other organisms. Specifically, we found support for a scenario of colonization of the Atlantic Forest from Amazonia (presumably by an ancestor occurring in both central and western Amazonia), followed by colonization of the Guiana Shield from the Atlantic Forest. A history of connections between western Amazonia and the Atlantic Forest, supported by our historical demographic study of *B. bilineatus*, was recovered by previous studies based on genetic data of birds and lizards (Batalha-Filho *et al.*, 2013; Prates *et al.*, 2017). Phylogeographic studies have also found evidence of northern colonizations between

the Atlantic Forest and northeastern Amazonia (Costa, 2003; Batalha-Filho *et al.*, 2013; Prates *et al.*, 2016a,b;). In spite of these two distinct proposed connection routes, previous studies have by and large found that Amazonia acted as the source of colonizations into the Atlantic Forest. Our study is one of the first (as that of Rodrigues *et al.*, 2014), however, to find evidence of the Atlantic Forest serving as a source of colonizations into Amazonia, particularly into northern Amazonia in the Guiana Shield region. It is currently unclear why the Atlantic Forest seems to have more often received than provided biodiversity to Amazonia over the course of these forests' histories, a pattern that may be related to their significant differences in total size.

While phylogenetic patterns have provided clues about the localization of former rainforest corridors in South America, we have a limited understanding of how often Amazonia and Atlantic Forest have been connected in the past, and for how long. Batalha-Filho *et al.* (2013) suggested multiples contact events based on divergence times between species and clades of birds restricted to each of these two forest systems, suggesting that the proposed forest bridge in present-day northeastern Brazil would be Pleistocenic in age. This timing is consistent with studies of co-distributed arboreal lizards and of the south American bush master *Lachesis muta*, which proposed colonization of the Atlantic Forest from eastern Amazonia at around 300-950 kya (Zamudio & Green, 1997; Prates *et al.*, 2016a). Similarly, our estimates for *Bothrops bilineatus* also suggest a Pleistocenic contact at around 0.3 mya between the Atlantic Forest and northern Amazonia (at the Guiana Shield) through northeastern South America. It is worth noting, however, that the directionality of range expansions in *B. bilineatus* and other studied organisms is fundamentally different, as our study suggests that this snake seems to have colonized Amazonia from an Atlantic Forest

ancestral population, while organisms such as lowland anole lizards were found to have expanded their ranges in the opposite direction (Prates *et al.*, 2016a).

Besides a colonization event in present-day northeastern Brazil, the complex history of *Bothrops bilineatus* seems to include an event of range expansion into the Atlantic Forest from an Amazonian ancestor, as supported by the results of phylogenetic and historical demographic analyses. Costa (2003) suggested a similar, seemingly southern route for small mammals, but without detailed information regarding its timing. The available divergence time estimates suggest that recurrent forest connection events occurred during the Pliocene, dating back to 2 mya in the case of *Bothrops bilineatus*, 11 mya for *Anolis* lizards (Prates *et al.*, 2017), and 3 to 13 mya for a range of small mammal and bird taxa (Batalha-Filho *et al.*, 2013; Ledo & Coli, 2017).

Taken together, biogeographic studies focusing on species and clades whose distribution span distinct South American rainforest blocks suggest that Amazonia and the Atlantic Forest have an intricate history of physical connections over time, which led to taxonomic similarity across presently disjunct regions. Despite the numerous examples of taxa that seem to have undergone range expansions across these domains, there is a limited number of studies that have investigated the routes, timing and especially the demographic processes involved, an information that is crucial to improve our understanding of the origin of these extraordinarily diverse ecosystems. By involving consecutive colonization events across Amazonia and the Atlantic Forest in a single taxon, the population history of *Bothrops bilineatus* is unique. As such, this study provides further insight, and adds complexity, to the dynamic history of South American rainforests during the last millions of years, seemingly as a result of pronounced climate shifts. We hope that the results presented here inspire further

studies aiming to deepen our knowledge about former biogeographic connections and biotic interchange among South American regions.

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BIOSKETCH

Francisco Dal Vechio is PhD student in the Herpetology Lab in the Biosciences Institute of the University of Sao Paulo. His main research interests are the systematics and biogeographic history of Neotropical pit vipers.

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Appendix

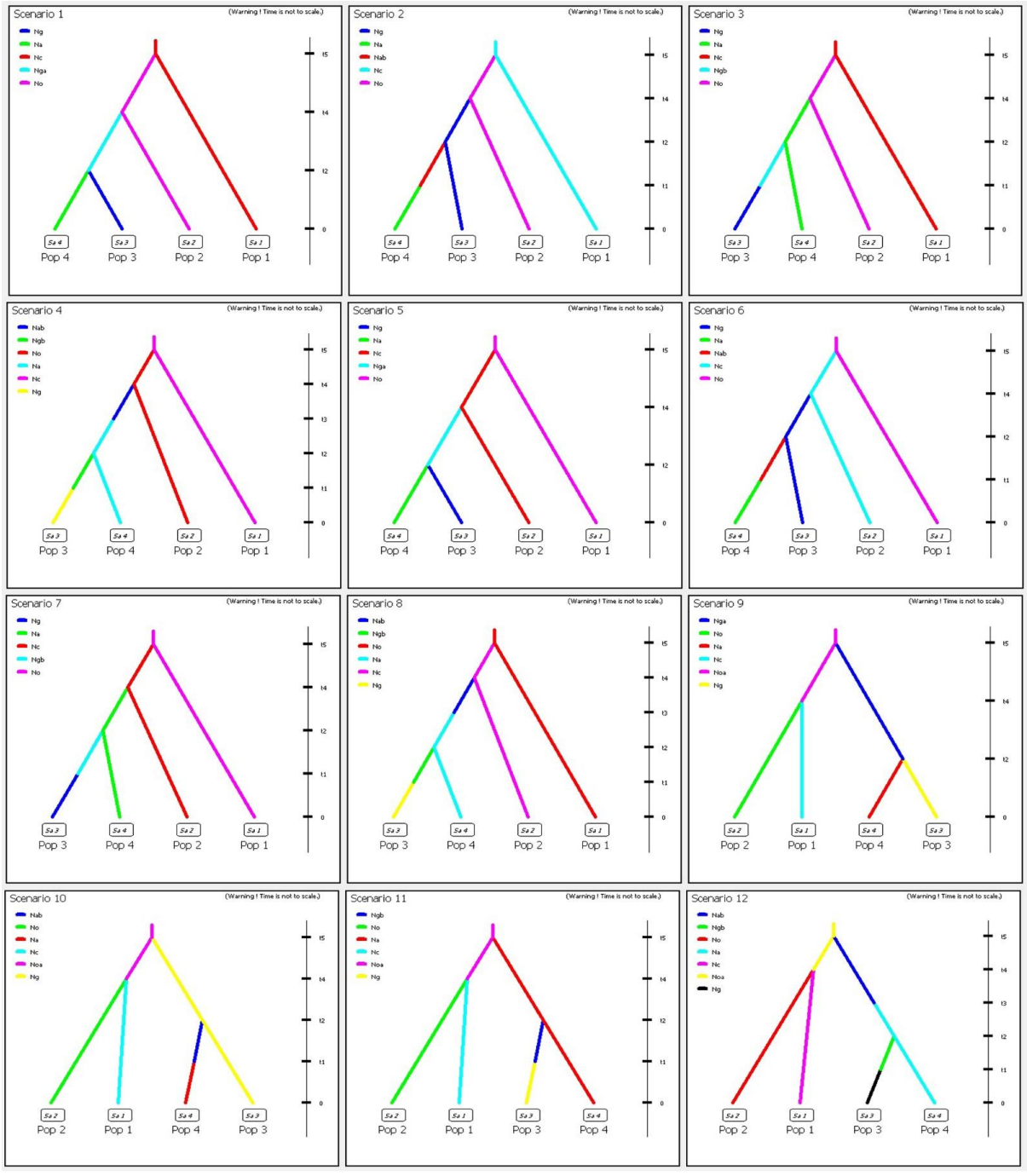

Figure 1. Twelve historical models used to test the evolutionary history of *Bothrops bilineatus* based on a dataset of nine molecular genes (four mitochondrial and five nuclear) using DIYABC. Scenarios 1-4 have the same tree topology with central Amazonia as the sister of remaining samples and western Amazonia as sister to an Atlantic Forest + Guiana Shield clade. Scenarios 5-8 have share a tree with western

Amazonia as the sister of remaining samples and central Amazonia as sister to Atlantic Forest + Guiana Shield. Scenarios 9-12 have the same tree topology with central Amazonia as sister to western Amazonia and their ancestor as sister to Atlantic Forest + Guiana Shield. Scenarios 1, 5, 9 test for vicariant processes. Scenarios 2, 6, 10 test for colonization of the Atlantic Forest by an ancestor from the Guiana Shield. Scenarios 3, 7, 11 test for a colonization of the Amazon (Guiana Shield region), by an ancestor from the Atlantic Forest. Scenarios 4, 8, 12 test for two colonization events in opposite directions: First, a colonization of the Atlantic Forest by an Amazonian ancestor from central Amazonia (Scenario 4), western Amazonia (Scenario 8), or an ancestor distributed in both central and western Amazonia (Scenario 12). Each colonization event is followed by a population expansion. Na: present population in the Atlantic Forest; Nc: present population in central Amazonia; Ng: present population in the Guiana Shield; No: present population in western Amazonia; Nab: hypothetical historical population that colonized the Atlantic Forest; Nga: hypothetical historical population ancestor of the Atlantic Forest + Guiana Shield; Ngb: hypothetical historical population that colonized the Guiana Shield; Noa: hypothetical historical population ancestor of central + western Amazonia. T1-T5 are time in the past, with T5 older than T4, T3, T2 and T1; T4 older than T3, T2 and T1; T3 older than T2 and T1; T2 older than T1; and T1 older than the present day.

Capítulo 4

Demografia populacional dos Crotalíneos Neotropicais sugere sincronia espacial e temporal para o dinamismo histórico das paisagens da América do Sul (Serpentes: Viperidae)

Biogeografia de *Bothrops* do grupo *atrox* e *Crotalus durissus*

Francisco Dal Vechio[1*], Miguel Trefaut Rodrigues[1]

[1]Departamento de Zoologia, Instituto de Biociências, Universidade de São Paulo, Rua do Matão, Trav. 14, nº 321, Cidade Universitária, CP 11461, CEP 05508-090, São Paulo, SP, Brasil.

[*]Autor de correspondência. E-mail: francisco.dalvechio@gmail.com

Palavras chave: Amazônia, biogeografia, *Bothrops atrox*, *B. leucurus*, Caatinga, Cerrado, *Crotalus durissus*, filogeografia, Mata Atlântica, refugio florestal.

Resumo

Esse capítulo apresenta-se de forma preliminar em comparação com os anteriores, porém não menos importante e relevante. Aqui sigo a linha biogeográfica da tese, ao investigar o dinamismo histórico das paisagens da América do Sul, utilizando outras espécies de Crotalíneos como modelos. As análises abordaram as relações filogenéticas e dados de demografia populacional do complexo *B. atrox-B. leucurus*, linhagens exclusivas de áreas florestadas, com ocorrência na Amazônia e Mata Atlântica. Como teste de hipótese alternativo à fragmentação florestal Amazônia-Mata Atlântica, foi investigada a história demográfica populacional de *Crotalus durissus*, como representante da diagonal de áreas abertas da América do Sul, composta pela Caatinga, Cerrado e Chaco. Os resultados filogenéticos suportam para/polifia de *B. atrox*, *B. moojeni* e *Crotalus durissus*, revelando a necessidade de aprofundamento no estudo sistemático para essas linhagens. O melhor cenário histórico para as espécies de *B.* do grupo *atrox* suporta conexão florestal Amazônia-Mata Atlântica durante o Plioceno, com colonização da Mata Atlantica por ancestrais do oeste da Amazônia, possivelmente pela ponte sul. Já o melhor cenário histórico para *Crotalus durissus* sugere colonização das áreas abertas ao sul do Rio Amazonas durante o médio Plesitoceno, com expansão populacional pela diagonal de áreas abertas, nos últimos 500 mil anos. Resultado esse que sugere sincronia temporal e espacial para perda da ponte florestada Amazônia-Mata Atlântica pelo nordeste brasileiro, devido à expansão de áreas abertas da Caatinga e Cerrado norte.

Abstract

This chapter is preliminary in comparison with previous ones, but not less important and relevant. Here I follow the biogeographic line of the thesis, investigating the

historical dynamism of the landscapes within South America, using other pit vipers species as models. The analyzes addressed the phylogenetic relationships and historical demography of the *B. atrox-B. leucurus* species complex, lineages restricted to forested areas ofAmazon and Atlantic Forest. As an alternative test for Amazon-Atlantic Forest fragmentation hypothesis we was investigate the population demography of *Crotalus durissus*, a representative of the South American diagonal of open areas, compounded by Caatinga, Cerrado and Chaco. The results of historical demography for the *B. atrox-B. leucurus* complex support an Amazonian- Atlantic Forest connection during the Pliocene, with colonization of the Atlantic Forest by ancestors of western Amazonia, possibly by the southern bridge. The best-fit historical scenario for *Crotalus durissus* suggests colonization of the open areas south of the Amazon River during the middle Plesitocene, with population expansion throughout the diagonal of open areas, in the last 500 thousand years. This result suggests a temporal and spatial synchrony for the loss of northern bridge between Amazon-Atlantic Forest due to the expansion of open areas through the Caatinga and Cerrado North.

Introdução

Desde o Século XIX, a conformação dos Rios da Bacia Amazônica tem aquecido debates e formulação de hipóteses biogeográficas para diversificação de fauna da Amazônia. De fato, naturalistas como Alfred Russel Wallace já teorizavam esses rios como barreira, ao observar espécies distintas de macacos em margens opostas, concebendo umas das primeiras teorias biogeográficas para a Amazônia (Wallace, 1852).

Depois das ideias de Wallace, outras tantas surgiram para tentar explicar a diversidade de espécies na Amazônia. Haffer (1969) e Vanzolini & Williams (1970) publicaram quase que simultaneamente seus trabalhos associando a diversificação da fauna amazônica com as mudanças climáticas durante o Pleistoceno (últimos 1.8 milhão de anos). Suas publicações encantaram e entusiasmaram o meio acadêmico, mobilizando a comunidade científica a testar e debater os resultados obtidos sob essa nova óptica, criticando ou mesmo acatando essa nova teoria.

Apesar de rejeitada como o motivo central para geração de diversidade de espécies na Amazônia (Colinveaux *et al*., 1996; Bush *et al*., 2004) a Teoria dos Refúgios Florestais (Haffer, 1969) é ainda intensamente citada e extremamente influente nos dias atuais. Isso porquê, hipotetiza fragmentação de área florestal ancestral e consequentemente de populações associadas a esses ambientes, possibilitando o rastreamento genético desses eventos, inferindo sobre a história evolutiva das linhagens e das paisagens.

Assim, a Teoria dos Refúgios sugere que durante períodos passados frios e secos, as florestas se retrairiam, originando diversos refúgios climáticos de áreas florestadas no meio da dominância de paisagens abertas; ao passo que nos períodos quentes e úmidos as florestas se expandiriam, possibilitando o contato entre as áreas anteriormente fragmentadas e o intercâmbio faunístico entre elas; ao mesmo tempo, originaria refúgios de áreas abertas no domínio de áreas florestadas.

Do modo como foi hipotetizada a Teoria dos Refúgios, áreas fragmentadas poderiam ser outrora tão abrangentes como domínios morfoclimáticos, atualmente tratadas pelos biogeógrafos como “mega-refúgios”. Nessa nova interpretação, ambientes como Amazônia e Mata Atlântica atuariam como áreas relictuais que se conectaram fisicamente ao longo do tempo, levando ao intercâmbio de fauna em

ambas direções, seguido de especiação ou isolamento geográfico das populações depois da separação das matas (Prates *et al*., 2016a, 2017; 2018; Dal Vechio *et al*., 2018).

Os estudos vêm mostrando diversidade temporal e espacial quanto as possíveis conexões florestais entre a Amazônia e a Mata Atlântica. Alguns estudos sugeremo um contato mais antigo, durante o Mioceno (23 – 5 milhões de anos), pela parte sudoeste da Amazônia (Rondônia, norte do atual Chaco no estado do Mato Grosso, Mato Grosso do Sul, leste da Bolívia e norte-leste do Paraguai) com a região sul da Mata Atlântica (Prates *et al*., 2017; Batalha-Filho *et al*., 2013, Ledo & Colli, 2017).

Outro contato, mais recente (Plio/Pleistoceno – últimos 5 milhões de anos), teria ocorrido através da expansão de áreas florestadas pela Caatinga e pelo norte do Cerrado, ou mesmo pela costa nordeste do Brasil (Maranhão, Piauí, Ceará, Rio Grande do Norte, Pernambuco) (Costa, 2003, Batalha-Filho *et al*., 2013; Prates *et al*., 2016a; Dal Vechio *et al*., 2018, 2019 *aceito*).

Os crotalíneos representam um interessante grupo para estudos biogeográficos por apresentarem espécies fiéis a seu tipo de ambientes, linhagens com ampla distribuição, altas taxas de mutação molecular a nível citoplasmático, e muitas espécies de fácil amostragem em campo. Assim, crotalíneos do novo mundo exclusivos de ambientes florestados e que ocorram em disjunção distribucional Amazônia/Mata Atlântica, como as jararacas do grupo atrox (*Bothrops leucurus* e *B. atrox*), juntamente com aquelas de ambiente aberto, como *Crotalus durissus*, podem ser interessantes modelos no teste de hipótese de “mega-refúgios”, envolvendo expansão, retração e fragmentação de ambientes, além de ajudar no entendimento do dinamismo das paisagens sul-americanas ao longo do tempo.

Materiais e Métodos

1. Inferências filogenéticas e filogeográficas

Dados moleculares foram utilizados para investigar as relações filogenéticas em *Bothrops e Crotalus*. Para isso, amostras de tecido (fígado, escama ou muda de pele) preservados em etanol 100% foram utilizadas para a extração de DNA (Fetzner, 1999). Foram utilizados nove marcadores gênicos (seis locus) para as análises moleculares, sendo quatros mitocondriais – os ribossomais *16s* e *12s* (Palumbi, 1996) e os codificadores de proteína *nd4* (Arévalo *et al*., 1994) e *cytb* (Pook *et al*., 2000); e cinco marcadores nucleares *c-mos* (Godinho *et al*., 2006), *rag1* (Wiens *et al*., 2008) *prlr*, *bdnf*, *mkl1* (Townsend *et al*., 2008). Todos os fragmentos foram amplificados seguindo protocolo padrão de PCR, com ciclo inicial a 94°C por 5 min, seguido de 35 ciclos (94°C por 40 segundos; **X**°C por 40 segundos; 72°C por 40 segundos) e um ciclo final de extensão por 7 min a 72°C; em que **X** representa a temperatura de anelamento de 49°C para *nd4*; 51°C para *16s*, *cytb*, *prlr* e *rag1*; 52°C para *c-mos*; 54°C para *12s* e *mkl1*; 55°C para *bdnf*.

As sequências resultantes foram manualmente editadas utilizando o programa CodonCode Aligner v. 3.7.1.1. (http://www.codoncode.com) e alinhadas sob algoritmo MUSCLE implementado no programa MEGA v. 6, com parâmetros padrão (Tamura *et al*., 2013). Os fragmentos gênicos codificadores de proteína, *nd4*, *cytb*, *c-mos*, *prlr*, *rag1*, *bdnf*, *mkl1*, foram transformados em sequências de aminoácidos para conferir o alinhamento.

Foi obtido o melhor modelo de evolução para cada marcador usando o programa jModelTest v. 2.1.3 sob o critério de seleção AIC (Akaike Information Criterion), resultando o modelo GTR+I+G para todos os marcadores mitocondriais;

HKY para o marcador nuclear *c-mos*, *rag1* e BDNF; HKY+I para *mkl1*; e GTR+I para *prlr*.

O alinhamento concatenado dos nove marcadores genéticos resultou numa matriz com 6006 pb. Essa matriz foi utilizada para reconstrução das relações filogenéticas sob a perspectiva probabilística de Inferencia Bayesiana (IB), rodadas no programa MrBayes v. 3.2 (Ronquist *et al.*, 2012) e *Beast* v. 1.8.3 (Drummond *et al*., 2012) disponíveis *on line* no *Site Cipres Science Gateway* - http://www.phylo.org/ (Miller *et al.*, 2010). Foram geradas duas corridas Bayesianas independentes com quatro cadeias de Markov cada, iniciando com uma árvore aleatória. Cada corrida consistiu de 10.000.000 gerações, com árvores amostradas a cada 1.000 gerações, resultando em 10.000 árvores; com 25% das árvores iniciais descartadas como *burn in*; a convergência das corridas independentes e escores para ESS foram observados utilizando o programa Tracer v. 1.6 (Rambaut & Drummond, 2009). A topologia final, assim como as Probabilidades Posteriores (PP) para os nós foram visualizadas no programa FigTree 1.4.0 (http://tree.bio.ed.ac.uk/), sendo considerados significantes valores superiores ou iguais a 0.95 para PP.

2. Teste de cenários demográficos

Foram utilizadas simulações coalescentes e ABC (*Approximate Bayesian Computation*) para testar cenários históricos de vicariância, colonização e expansão de distribuição para as diferentes linhagens. Em casos de vicariância assume-se que uma população ancestral em panmixia seja quebrada em duas menores. Em cenários de colonização, uma população ancestral atua como fonte, onde um número "*n*" de indivíduos se dispersa e coloniza uma outra região, gerando uma rota de colonização num dado tempo "*t*". Nesses casos há o pressuposto de perda inicial de variabilidade

genética "*bottleneck*" da população colonizadora, seguida de expansão e aumento de população. Esse tipo de cenário foi testado para as linhagens que apresentaram relação de irmandade entre clados ocorrentes na Amazônia e na Mata Atlântica (*B. atrox-B. leucurus*). Adicionalmente esse cenário foi utilizado para testar a dinâmica populacional de *Crotalus durissus* nas áreas abetas da América do Sul.

Primeiramente, as linhagens utilizadas nos testes de cenários foram aquelas recuperadas nas análises filogenéticas e filogeográficas performadas no MrBayes v. 3.2 (Ronquist *et al.*, 2012) e *Beast* v. 1.8.3 (Drummond *et al*., 2012). A topologia recuperada nas análises IB foi mantida para os testes de cenários, adicionado de parâmetros que testam os processos demográficos. Foi utilizada a matriz concatenada dos quatro marcadores mitocondriais (*16s*, *12s*, *cytb* e *nd4*) para realização dos testes.

Para *Bothrops atrox-B. leucurus* foram utilizadas três linhagens (ver resultados): Escudo das Guianas, Amazônia oeste ao sul do Rio Amazonas e Mata Atlântica, com topologia ((Mata Atlântica +Amazônia oeste) Escudo das Guianas) (Ver resultados). Cinco cenários alternativos foram testados: 1- vicariância entre Escudo das Guianas e população ancestral Amazônia oeste sul do Rio Amazonas+Mata Atlântica e vicariância entre Mata Atlântica e Amazônia oeste; 2- vicariância entre Escudo das Guianas e Amazônia oeste ao sul do Rio Amazonas e colonização da Mata Atlântica por indivíduos da Amazônia oeste; 3- vicariância entre Escudo das Guianas e Mata Atlântica e colonização da Amazônia oeste por indivíduos da Mata Atlântica; 4- colonização da Mata Atlântica por indivíduos do Escudo das Guianas e recolonização da Amazônia (oeste) por indivíduos da Mata Atlântica; 5- colonização da Amazônia oeste por indivíduos do Escudo das Guianas e colonização da Mata Atlântica por indivíduos da Amazônia oeste (Figura 1).

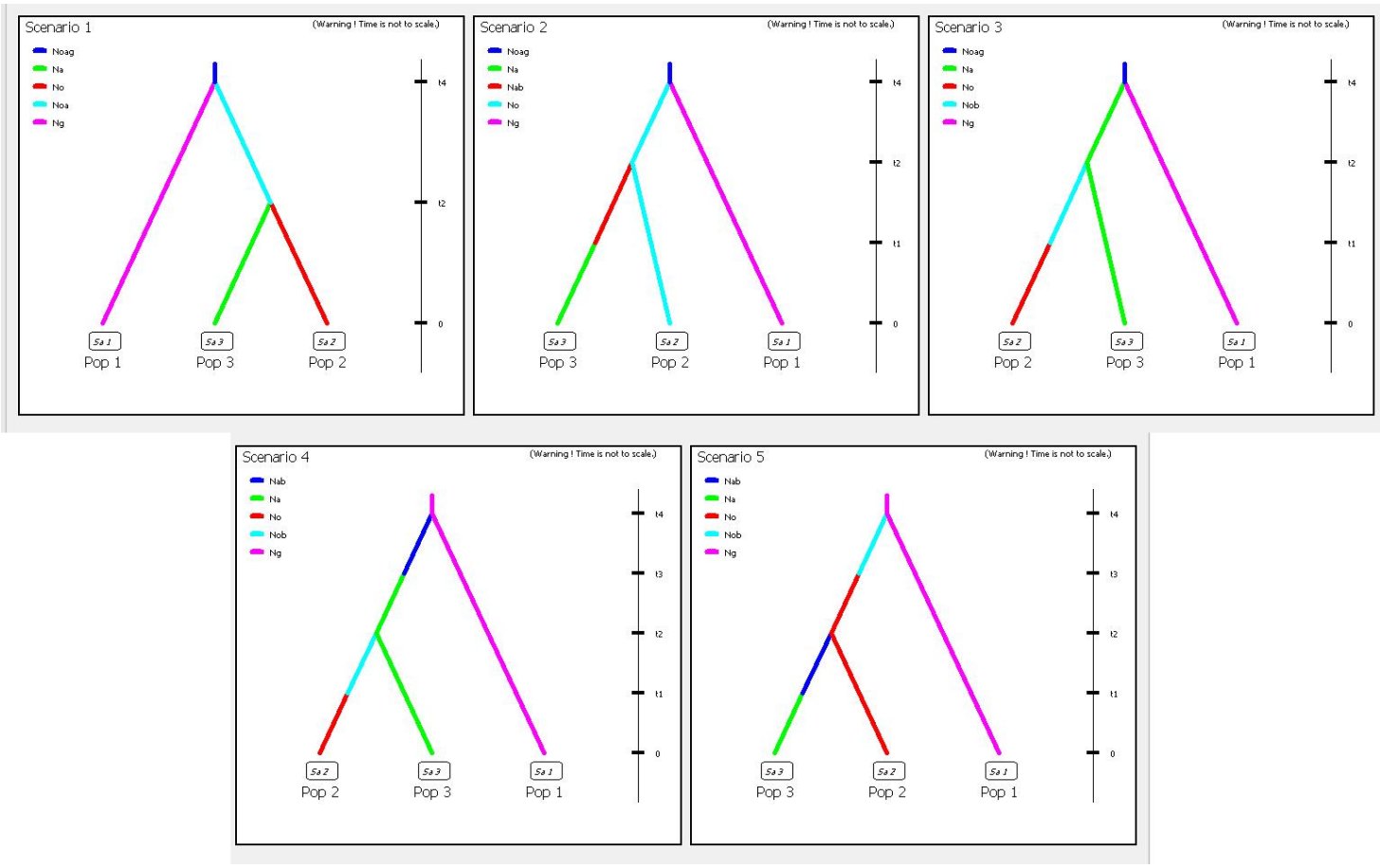

Figura 1. Cenários de demografia histórica propostos para testar o processo de diversificação das linhagens de *Bothrops atrox-B. leucurus*. As cores dos ramos não são correspondentes entre os cenários. Na: população atual na Mata Atlântica; Ng: população atual no Escudo das Guianas; No: população atual na Amazônia oeste, a sul do Rio Amazonas; Nab: população hipotética que colonizou a Mata Atlântica; Noa: população hipotética ancestral de Mata Atlântica+Amaônia oeste; Nob: população hipotética que colonizou a Amazônia oeste; Noag: população hipotética ancestral de Noa+Escudo das Guianas; T1-T4 são tempo no passado, com T4 mais antigo que T3, T2 e T1; T3 mais antigo que T2 e T1; T2 mais antigo que T1; e T1 mais antigo que os dias atuais.

Para *Crotalus durissus* foram utilizadas três linhagens (ver resultados): Venezuela, Escudo das Guianas e sul do Rio Amazonas, com topologia ((Escudo das Guianas+sul do Rio Amazonas) Venezuela). Quatro cenários foram testados: 1- vicariância entre população da Venezuela e população ancestral norte+sul do Rio Amazonas; 2- vicariância entre Venezuela e Norte do Rio Amazonas e colonização do sul do Rio Amazonas por indivíduos do Escudo das Guianas; 3- colonização do Escudo das Guianas por indivíduos da Venezuela e colonização do sul do Rio Amazonas por indivíduos do Escudo das Guianas; 5- colonização do norte+sul do Rio Amazonas por indivíduos da Venezuela e vicariância entre norte e sul do Rio Amazonas (Figura 2).

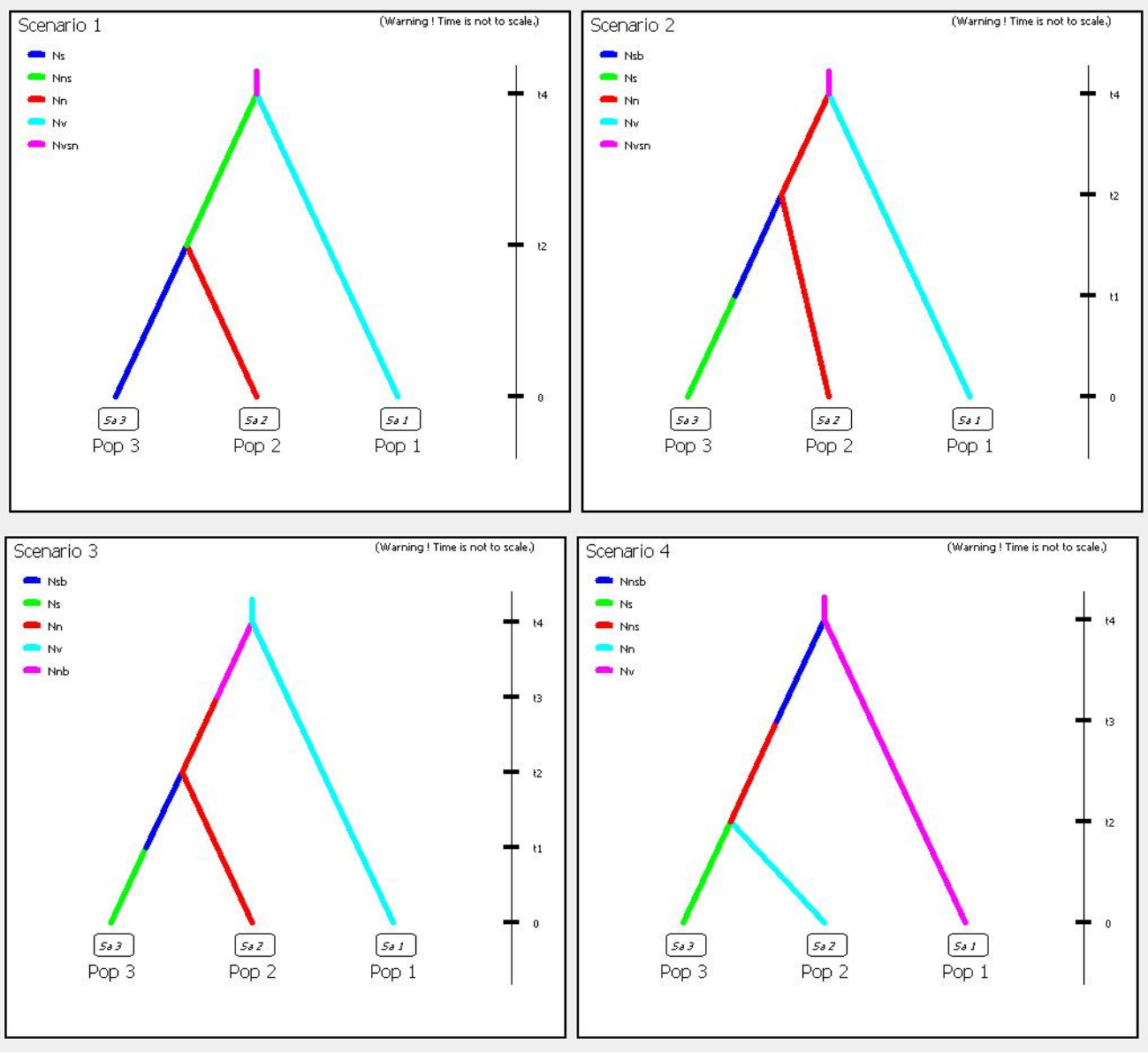

Figura 2. Cenários de demografia histórica propostos para testar o processo de diversificação das linhagens de *Crotalus durissus*. As cores dos ramos não são correspondentes entre os cenários. NS: população atual a sul do Rio Amazonas; Nn: população atual a norte do Rio Amazonas; Nv: população atual na Venezuela; Nns: população hipotética ancestral de sul+norte do Rio Amazonas; Nvns: população hipotética ancestral de Nns+Venezuela; Nsb: população hipotética que colonizou as áreas ao sul do Rio Amazonas; Nnb: população hipotética que colonizou o Escudo das Guianas; Nnsb: população hipotética que colonizou Escudo das Guianas+sul do Rio Amazonas.

Um milhão de simulações coalescentes foram realizadas para cada cenário demográfico, seguidas de ABC, rodadas no programa DIYABC (Cornuet *et al.*, 2014). Para cada análise foi utilizada um conjunto específico de Estatísticas Sumárias (ES), a fim de maximizar a análise, sendo *Tajima's D* (Tajima, 1989), *number of private segregating sites* e *pairwise Fst* (Hundon *et al.*, 1992), para *B. atrox-B.leucurus* e *Crotalus durissus*.

A fim de verificar a presença dos dados empíricos entre a nuvem de pontos simulados, foi realizada Análise de Componente Principais (PCA) das ES dos pontos simulados. O suporte estatístico para cada cenário foi calculado através da distância Euclidiana entre os dados observados e os simulados, usando regressão logística das ES (Beaumount, 2010). Adicionalmente foi calculada a probabilidade posterior de cada cenário, baseado em 0.01 % (1.000 simulações) do total de pontos simulados que foram recuperados próximos aos dados observados. A distribuição posterior dos parâmetros populacionais do melhor cenário foi recuperada usando 500 pontos simulados que foram recuperados próximos dos dados empíricos.

Foi ainda avaliada a precisão do processo de seleção de modelos, isto é, a precisão em discriminar os modelos pelas ES selecionadas; assim, foram simulados 100 pseudos-pontos observados, em que o cenário verdadeiro era conhecido para cada ponto simulado. Foi então realizado ABC, calculando a proporção de pseudo-pontos observados corretamente identificados pelo processo de seleção de modelo.

Os *priors* utilizados para cada parâmetro foram: 1- Tempo de divergência entre linhagens, variando de 0.1-5 milhões de anos, assumindo 2-3 anos como o tempo de geração para *Bothrops* e *Crotalus* (Dal Vechio *et al*., 2018); 2- Tamanho efetivo populacional para cada região, variando de 0.01-5 milhões; 3- Tamanho efetivo

populacional durante o evento fundador (colonização), variando de 0.001-0.1 milhão de anos; 4- Tamanho efetivo populacional em eventos de vicariância, variando de 0.01-5 milhões. O modelo de substituição *Tamura nei* (Tamura & Nei, 1993) foi implementado para a matriz mitocondrial, com taxas de mutação variando de 10^{-8} a 10^{-9} por geração.

Resultados

Inferências filogenéticas: B. atrox, B. leucurus e B. moojeni

Bothrops leucurus foi recuperada como monofilética com alto suporte (PP = 1), as populações de Alagoas foram recuperadas como irmãs de todas outras amostras; as populações do sul da Bahia foram recuperadas como irmãs das populações da Chapada Diamantina, com alto suporte (PP> 0.95) (Figura 3).

Bothrops moojeni foi recuperada como polifilética com relação a *B. atrox*, apresentando uma linhagem monofilética (PP =1) com as populações de Mato Grosso, Minas Gerais, São Paulo, Goiás e Brasília. Já as populações das matas de galeria do Tocantins e Piauí foram recuperadas junto com as populações de *B. atrox* do sudesde da Amazônia (sul/sudeste do Pará, norte do Mato Grosso, e sudeste do Amazonas) (Figura 3).

Bothrops atrox foi recuperada como polifilética. No entanto, o clado contendo as populações do oeste amazônico ao sul do Rio Amazonas (Porto velho-RO, Acre e Rio Purus e Campo Tupana-AM) foi recuperado como monofilético e irmão de *B. leucurus* com alto suporte (PP = 1) e externamente a esse agrupamento foi recuperado um clado reunindo as amostras de *B. atrox* do escudo guianês (Roraima, Amapá e Silves-AM, norte do Rio Amazonas), também com alto valores de probabilidade posterior (PP = 1) (Figura 3). Desse modo, o agrupamento (*B. atrox* do Escudo das

Guianas (B. atrox do oeste amazônico+*B. leucurus*)) foi utilizado para as análises subsequentes dos testes de cenários históricos.

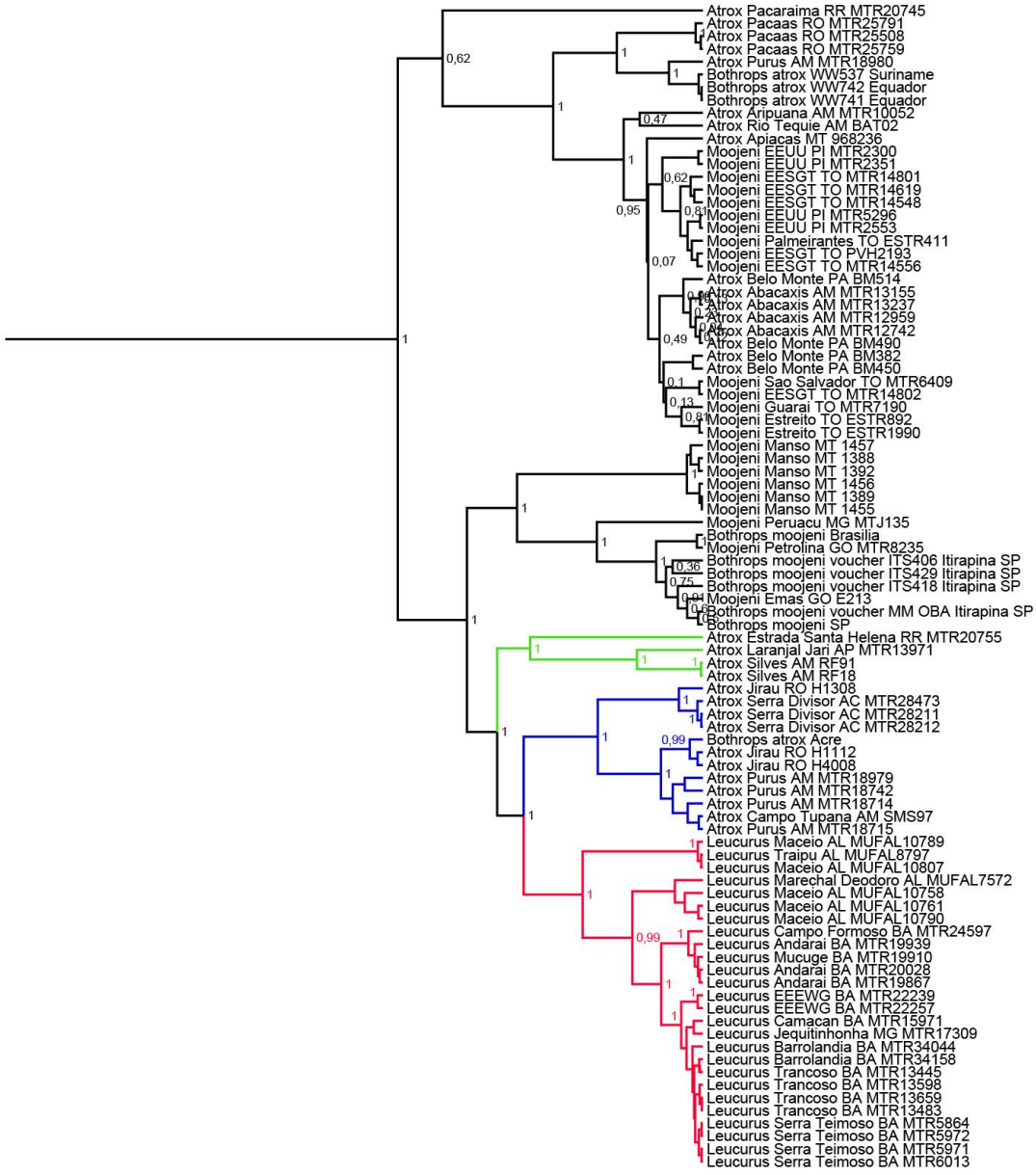

Figura 3. Árvore filogenética e filogeográfica baseada em IB com matriz concatenada para 9 marcadores moleculares (4 mitocondriais e 5 nucleares) para relações inter e intraespecíficas das espécies de *Bothrops atrox* (em azul e verde) e *B. leucurus* (vermelho).

Inferências filogenéticas: Crotalus dusissus

Crotalus durissus foi recuperado em polifilia apesar do baixo suporte para alguns nós da árvore filogenética (Figura 4). As amostras pertencentes ao Escudo Guianês + sul do Rio Amazonas formam um clado com alto suporte (PP=1). As amostras ao sul do Rio Amazonas se separam em três linhagens: uma com amostras de enclaves de áreas abertas na Amazônia (Santarém, PA) + extremo oeste do Cerrado (Pontes Lacerda, MT); e as outras duas linhagens ocupando toda porção do Cerrado e da Caatinga, com baixa divergência genética entre as amostras (Figura 4).

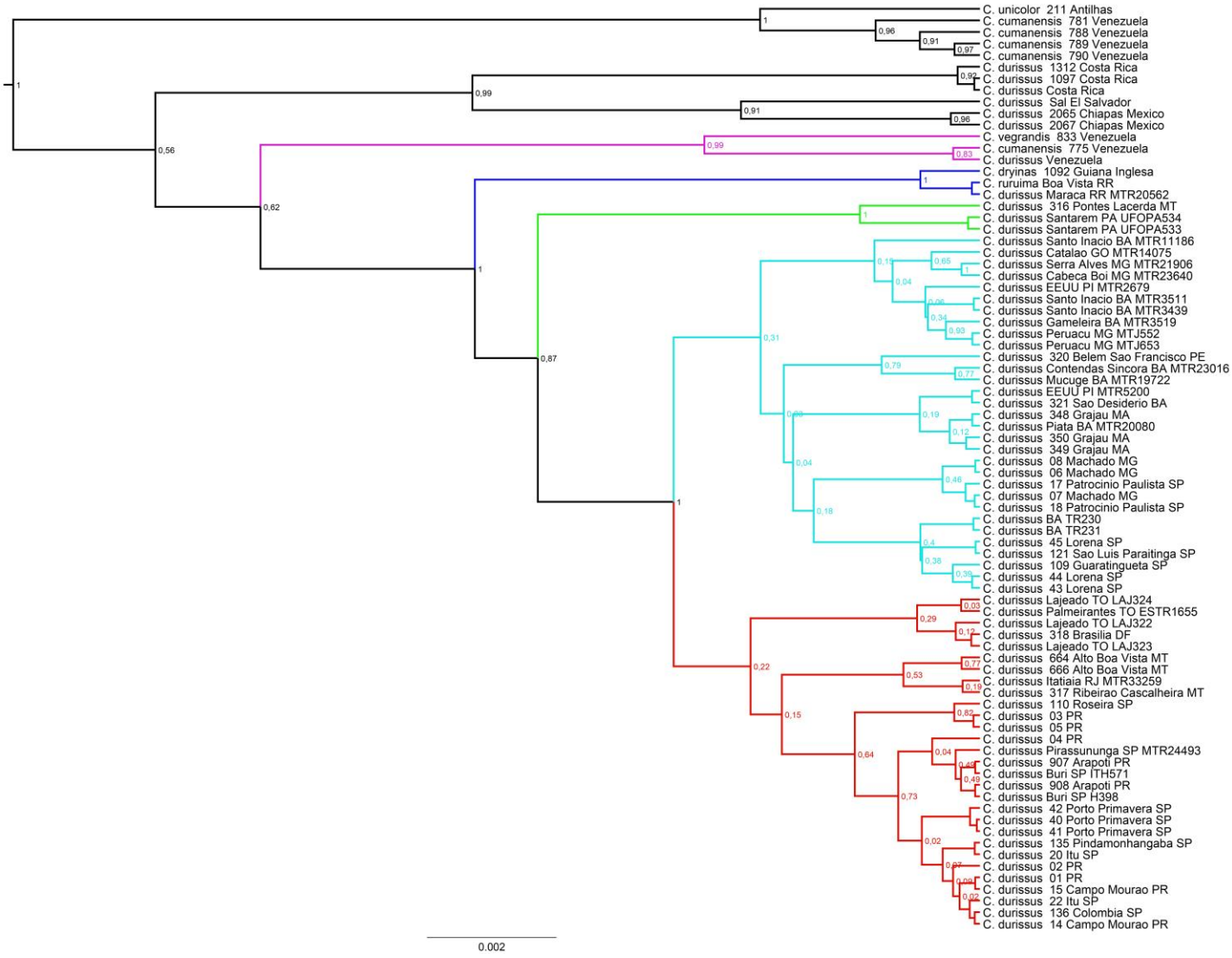

Figura 4. Árvore filogenética e filogeográfica baseada em IB para as relações interespecíficas em *Crotalus* com ênfase nas relações intraespecíficas de *C. durissus*. Ramos em azul claro, vermelho e verde representam as populações de *C. durissus* ao sul do Rio Amazonas; Ramos em azul escuro representa as populações de *C. durissus* no Escudo Guianês; Ramos em roxo representa as populações de *C. durissus* na Venezuela.

Testes de cenários históricos de demografia populacional

Para *Bothrops atrox-B. leucurus* o melhor cenário histórico baseado em simulações coalescentes e ABC (P = 0.45) sugere vicariância entre a população do escudo guianês e oeste da Amazônia a sul do Rio Amazonas, há aproximadamente 3.6 milhões de anos (Intervalo de Confiança [IC] 95% = 1.6 – 5 milhões de anos). Os resultados também sugerem colonização da Mata Atlântica por um pool de 55 mil indivíduos do oeste amazônico há aproximadamente 2.6 milhões de anos (IC = 1.1 – 4.4 milhões de anos), com pronunciada perda de variabilidade genética e expansão de população pelo bioma atlântico há aproximadamente 800 mil anos (Figuras 5 e 6). A média da taxa de mutação estimada foi de 2.38×10^{-9} substituições por sítio/ano para o locus mitocondrial. Adicionalmente, o erro posterior baseado nos pontos pseudo-observados foi estimado em 0.47.

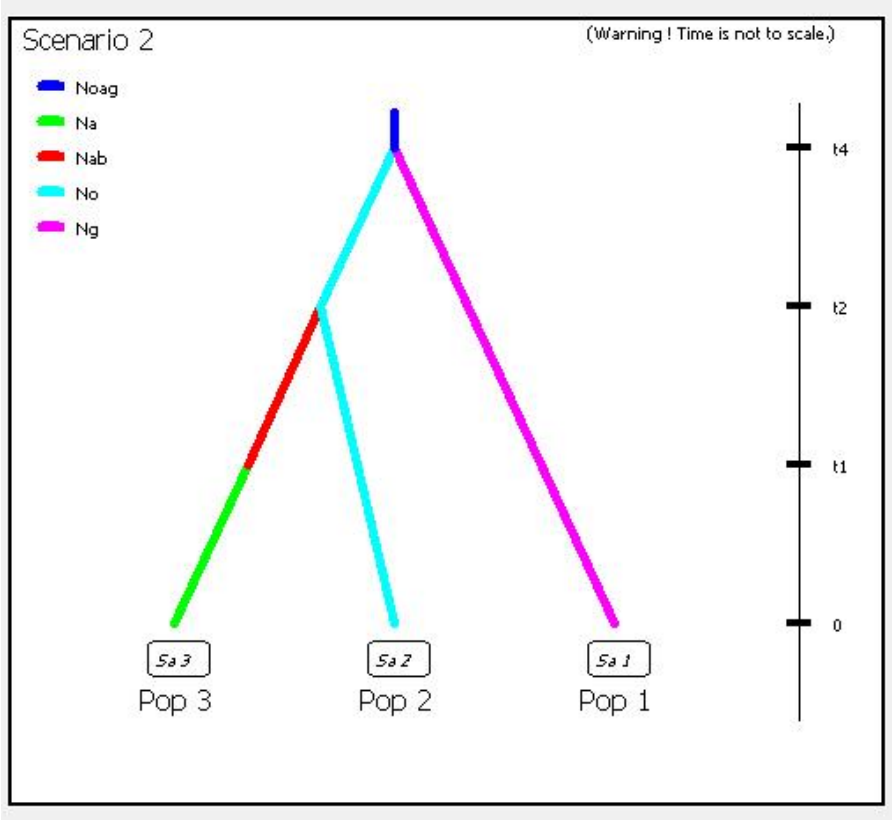

Figura 5. Melhor cenário histórico obtido para as linhagens de *Bothrops atrox* – *B. leucurus*. Na = Mata Atlântica; Ng = Escudo das Guianas; No = oeste amazônico e sul do Rio Amazonas; Nab = população ancestral hipotética de colonizadores de origem do oeste amazônico Noag: população hipotética ancestral de oeste amazônico+Escudo das Guianas.

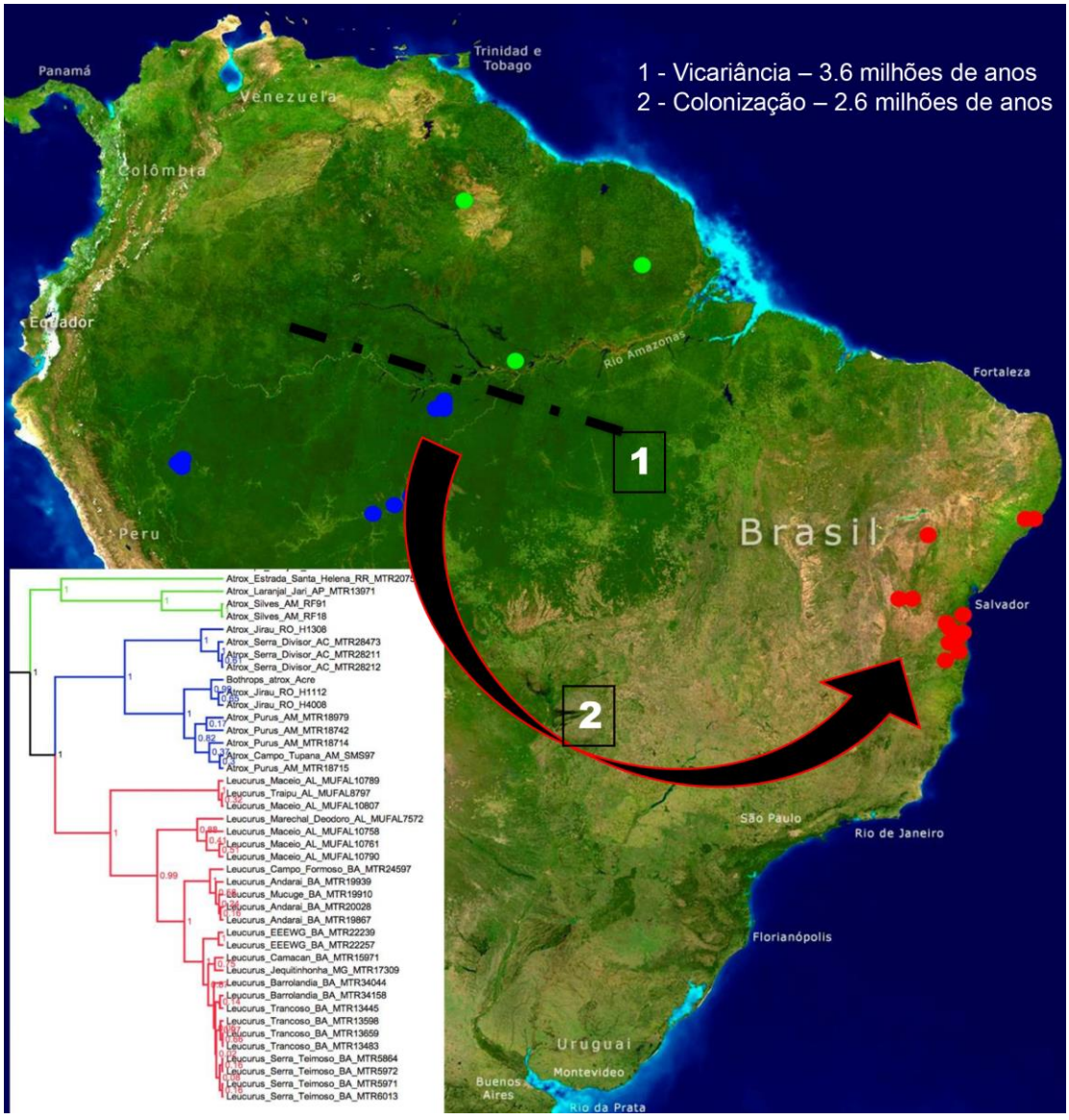

Figura 6. Relações filogenéticas para *Bothrops atrox – B. leucurus* com mapa de distribuição geográfica das linhagens recuperadas nas análises com base em IB. Seta representa evento de colonização e linha tracejada evento de vicariância.

Para *Crotalus durissus* o melhor cenário histórico baseado em simulações coalescentes e ABC (P = 0.62) sugere vicariância entre as populações da Venezuela com as do Escudo Guianês em torno de 1.9 milhões de anos (IC = 0.8 – 4.2 milhões de anos) e colonização das áreas ao sul do Rio Amazonas por um pool de 71 mil indivíduos da população norte do rio, datado de aproximadamente 1.26 milhões de

anos (IC = 0.5 – 3.81 milhões de anos). Subsequentemente, as populações ao sul do Rio Amazonas se expandiriam por todo Cerrado e Caatinga, há 0.5 milhões de anos (IC = 0.2 – 1.74 milhões de anos) (Figuras 6 e 7). A média da taxa de mutação estimada foi de 5.1 x 10^{-9} substituições por sítio/ano para o locus mitocondrial. Adicionalmente, o erro posterior baseado nos pontos pseudo-observados foi estimado em 0.39.

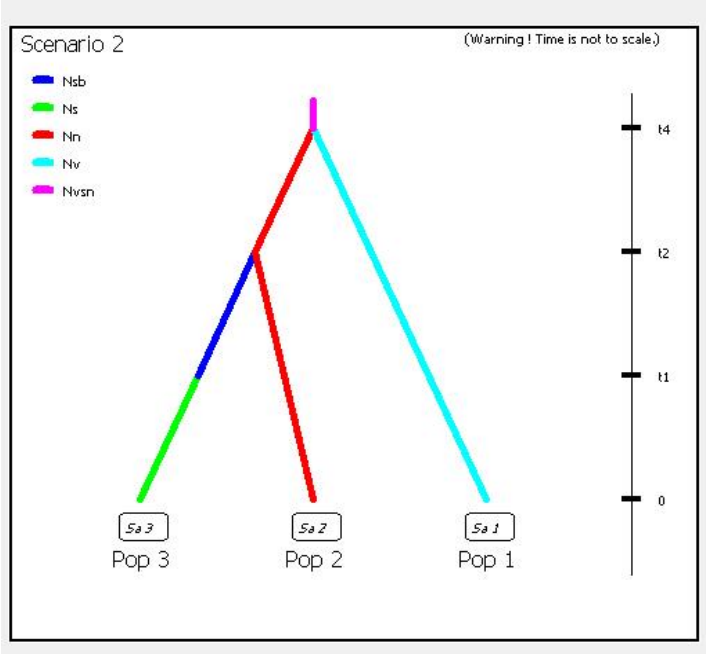

Figura 6. Melhor cenário histórico obtido para as linhagens de *Crotalus durissus*. Nn = Norte Rio Amazonas; Ns = Sul Rio Amazonas; Nv = Venezuela; Nsb = população ancestral hipotética de colonizadores de origem do Norte do Rio Amazonas, Nvsn: população hipotética ancestral de norte do Rio Amazonas+Venezuela.

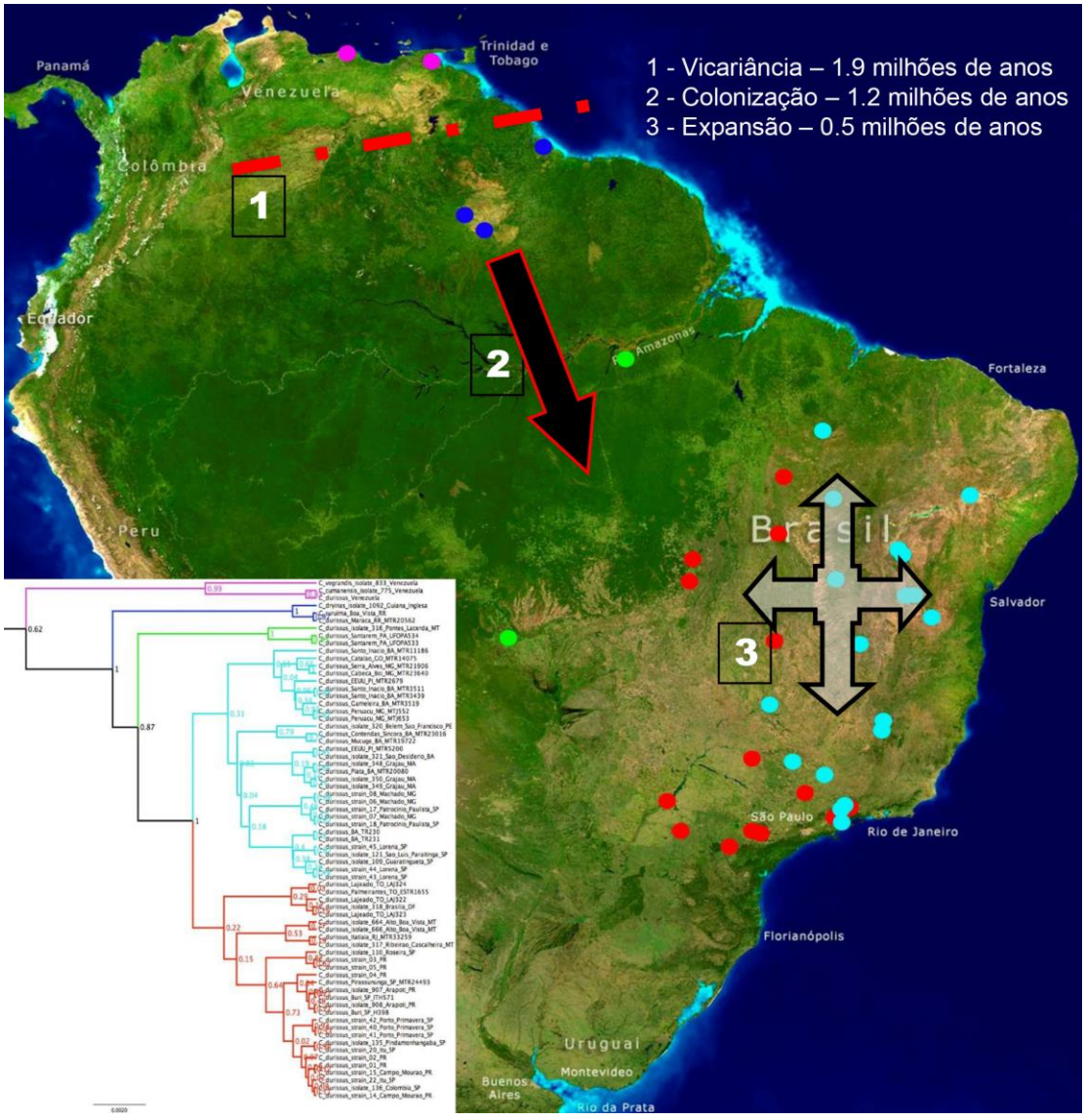

Figura 7. Relações filogeográficas para *Crotalus durissus* com mapa de distribuição geográfica das linhagens recuperadas nas análises sob IB. Seta única representa evento de colonização, linha tracejada evento de vicariância e setas em cruz evento de expansão populacional.

Discussão

Inferências filogenéticas e implicações taxonômicas

As análises filogenéticas baseadas nos dados concatenados recuperam o grupo *atrox* como uma linhagem monofilética em *Bothrops*, em concordância com as recentes investigações (Fenwick *et al*., 2009; Alencar *et al*., 2016, Dal Vechio *et al*., 2018). Os resultados genéticos mostram que *B. atrox* e *B. moojeni* apresentam instabilidade taxonômica e problemas no reconhecimento do limite das espécies. Trabalhos filogeográficos recentes têm encontrado os mesmos problemas taxonômicos ao estudar a estrutura populacional em outros grupos de espécies em *Bothrops* (Machado *et al*., 2014; Saldarriaga-Córdoba *et al*., 2017, Dal Vechio *et al*., 2018, aceito). Já *B. leucurus* foi recuperada como monofilética e irmã das amostras de *B. atrox* do oeste amazônico ao sul do Rio Amazonas.

As análises filogenéticas recuperam *Crotalus durissus* em polifilia, com nenhuma associação das linhagens moleculares com o nível taxonômico sub-específico proposto atualmente para as populações ao sul do Rio Amazonas (Campbell & Lamar, 2004). *Crotalus durissus ruruima* apresenta-se como uma linhagem monofilética, irmã das amostras ao sul do Rio Amazonas, sendo potencialmente uma espécie válida. O mesmo ocorre para as outras populações de *C. durissus* presentes no norte da América do Sul e América Central.

História demográfica e biogeografia

As análises de demografia histórica suportam um cenário de vicariância entre as populações de *Botrops atrox* do Escudo das Guianas e Amazônia oeste ao sul do Rio Amazonas, datado do Mioceno tardio. Com base nesse resultado é possível sugerir que a formação e estabilização do Rio Amazonas tenha atuado como uma barreira vicariante separando essas populações, e continue atuando no presente como uma barreira impedindo fluxo gênico. A formação do Rio Amazonas está relacionada com

o soerguimento final dos Andes, num período de intenso tectonismo no oeste da América do Sul por volta de 10-15 milhões de anos (Hoorn *et al*., 2010, 2017). Nesse processo haveria o escoamento do sistema lacustre *Pebas* em direção ao Oceano Atlântico, por volta de 7-9 milhões de anos, originando a calha do atual Rio Amazonas (Hoorn *et al*., 2010, 2017). No entanto, como *B. atrox* é um grande complexo com diversas linhagens e pouco sabemos sobre sua história evolutiva, acredito que se faz necessário um aprofundamento filogeográfico para se testar a influência dos rios amazônicos na sua diversificação.

Os resultados de demografia histórica ainda suportam colonização da Mata Atlântica por ancestral do oeste da Amazônia durante o Plioceno, provavelmente devido à expansão florestal com contato físico entre as partes sul/sudoeste desses ambientes florestados. A pretérita conexão sul entre Amazônia e Mata Atlântica tem sido invocada como rota de dispersão e intercâmbio faunístico para diversos grupos da fauna (Aves: Batalha-Filho *et al*., 2013; Mamíferos: Costa, 2003, Lagartos: Prates *et al*., 2017; Serpentes: Dal Vechio *et al*., 2018). Apesar da variação temporal entre os trabalhos para a presença da ponte sul Amazônia-Mata Atlântica, Ledo & Coli (2017) comentam que essa conexão teria sido mais antiga e mais duradoura que a conexão norte, a qual teria ocorrido pelo nordeste brasileiro.

É importante salientar que são ainda poucos os trabalhos que investigam a direcionalidade do fluxo de colonização e mesmo os processos biogeográficos envolvidos na diversificação da fauna. No entanto, os resultados de demografia histórica para *B. atrox-B. leucurus* estão em concordância em: rota geográfica, direcionalidade de fluxo de fauna e período geológico, com os encontrados por Dal Vechio *et al*. (2018) para a jararaca verde *B. bilineatus*, sugerindo conexão sul entre

Amazônia-Mata Atlântica, durante o Plioceno, e com um fluxo colonizador da Mata Atlântica por ancestral do oeste amazônico.

Já as análises de demografia histórica suportam um cenário de vicariância entre as populações de *Crotalus durissus* do norte da América do Sul presentes na Venezuela e as do Escudo Guianês, datado do Plio-Pleistoceno, provavelmente associado a um evento de expansão florestal no norte da Amérca do Sul. Durante o médio Pleistoceno *C. durissus* teria colonizado as áreas ao sul do Rio Amazonas com expansão populacional por toda diagonal aberta (Cerrado e Caatinga) nos últimos 500 mil anos. Esses resultados estão em concordância com os já publicados para a espécie (Wuster *et al*., 2005; Quijada-Mascareñas *et al*., 2007) e em sincronia temporal para expansão populacional de outras linhagens simpátricas pelo ambiente árido da Caatinga (Gehara *et al*., 2017).

Os dados da história demográfica de *Crotalus durissus* são de extrema importância no entendimento do dinamismo do cinturão de áreas abertas na América do Sul, e consequentemente, das mudanças na paisagem dos ambientes florestados. Isto porque ao projetarmos uma perda na conexão florestal pretérita Amazônia-Mata Atlântica pelas atuais áreas ocupadas por Caatinga e Cerrado, admitimos ao mesmo tempo expansão dessas áreas abertas e das linhagens associadas a elas.

Conclusões

Os resultados de demografia histórica para *Bothrops* do grupo *atrox* e *Crotalus durissus* suportam pulsos de expansão/retração florestal durante os últimos 2.5 milhões de anos, com contato físico entre a Amazônia e a Mata Atlântica e o intercâmbio faunístico entre esses ambientes. Além disso, os resultados revelam o dinamismo histórico das áreas abertas que separam esses blocos florestais mostrando

sincronia temporal para eventos de expansão/retração de paisagens. Por último, as análises filogenéticas realçam a necessidade de aprofundamento sistemático/taxonômico no grupo *atrox* e para *Crotalus durissus*.

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Discussão Geral e Conclusões

Padrões biogeográficos

Esta tese teve como objetivo investigar o dinamismo histórico das paisagens do Neotrópico, elucidando a história evolutiva dos crotalíneos sul-americanos. Apesar de terem sido pouco investigados os processos biogeográficos que influenciaram a diversificação da fauna neotropical, levando-se em consideração tempo, rota e direcionalidade, os resultados apresentados aqui estão em sincronia com a literatura e suportam conectividade e intercâmbio de fauna entre Amazônia-Mata Atlântica.

Com base em dados filogenéticos e em testes de cenários demográficos alternativos para as serpentes *Bothrops* dos grupos *jararacussu*, *atrox* e *taeniatus* e *Crotalus durissus* foi possível detectar ao menos três pulsos de expansão e retração de áreas florestadas e de áreas abertas na região Neotropical com sincronia temporal e espacial entre esses eventos biogeográficos.

O primeiro pulso teria ocorrido por volta de 2-2.5 milhões de anos (Plio-Pleistoceno), com expansão florestal generalizada pela América do Sul, possibilitando o contato físico e intercâmbio de fauna entre Amazônia e Mata Atlântica (*Bothrops bilineatus* [Dal Vechio *et al*., 2018]; *B. atrox-B. leucurus*); e concomitante retração das áreas abertas, separando as populações de *Crotalus durissus* da Venezuela e as do Escudo Guianês.

O segundo pulso teria ocorrido no médio Pleistoceno, por volta de 1.2 milhões de anos, com expansão das áreas abertas pelo cinturão seco da Amazônia, possibilitando a *Crotalus durissus* colonizar áreas ao sul do Rio Amazonas. Já o terceiro pulso teria ocorrido por volta de 500 mil anos, novamente com perda de conexão florestal Amazônia-Mata Atlântica (*Bothrops bilineatus* [Dal Vechio *et al*.,

2018]; *B.* do grupo *jararacussu* [Dal Vechio *et al*., 2019 aceito]) devido à expansão das áreas abertas no Cerrado e Caatinga, com expansão populacional de *C. durissus* por toda diagonal seca da América do Sul.

As pontes históricas entre as florestas da Amazônia e Mata Atlântica têm sido invocadas para explicar o intercâmbio faunístico entre esses dois blocos florestais. A ponte sul tem sido sugerida como mais antiga e duradoura (Ledo & Colli, 2017), com múltiplas estabilizações durante o Mioceno (Batalha-Filho *et al*., 2013; Ledo & Colli, 2017; Prates *et al*., 2017; Dal Vechio *et al*., 2018). Esse corredor florestal possivelmente favoreceu a colonização da Mata Atlântica por linhagens ancestrais vindas do oeste amazônico para *Bothrops bilineatus* (Dal Vechio *et al*., 2018) e *B. leucurus*, datado do Plioceno (Mioceno Tardio).

Já a ponte norte é sugerida como mais recente, com estabilizações durante o Pleistoceno (Batalha-Filho *et al*., 2013; Prates et al., 2016, Dal Vechio *et al*., 2018, 2019 aceito). A hipótese da ponte norte pelo nordeste brasileiro é suportada pela presença dos Brejos Nordestinos, que são áreas relictuais de mata em meio à Caatinga semi-árida, mantendo uma biota mista de elementos amazônicos e atlânticos (Pôrto *et al*., 2004). Além disso, estudos paleopolínicos, espeleológicos e paleontológicos têm suportado mudanças na paisagem da Caatinga no último milhão de anos, com alto grau de substituição do ambiente xérido por um florestado (Dever *et al*., 1987; Hartwing & Cartelle, 1996; Czaplewski & Cartelle, 1998; Oliveira *et al*., 1999; Auler & Smart, 2001; Auler *et al*., 2004, Wang *et al*., 2004).

A ponte norte teria favorecido o intercâmbio faunístico para *Bothrops bilineatus* e para as espécies do grupo *jararacussu*, apesar de direções opostas para o fluxo colonizador da fauna. Os resultados de demografia histórica têm suportado para ambos os grupos, a perda da conexão florestal Amazônia-Mata Atlântica durante o

Pleistoceno tardio, exatamente no mesmo período em que se recuperam sinais de expansão populacional para diversos répteis e anfíbios associados ao ambiente árido da Caatinga, (ex. *Crotalus durissus*, *Philodryas naterreri*, *Vanzosaura multiscutata*, *Micrablepharus maximiliani*, *Lygodactylus klugei*, *Phyllopezus pollicaris*, *Polychrus acutirostris*, *Dermatonotus muelleri*, *Phylomedusa nordestina*, *Rhinella jimi* e *Pleurodema diplolister* [Wuster *et al*., 2005; Quijada-Mascareñas *et al*., 2007; Gehara *et al*., 2017]).

Adicionalmente, as análises de demografia histórica revelam o papel do Rio Amazonas na diversificação da fauna Neotropical, sugerindo que sua formação e estabilização no Mioceno tardio (Hoorn *et al*., 2010, 2017) tenha atuado como uma barreira vicariante separando *Bothrops brazili* (sul do rio) e *B. oligobalius* **sp. nov.** (norte do rio) (Dal Vechio *et al*., 2019 aceito) e possivelmente populações de *B. atrox*.

No entanto, apesar de *B. bilineatus* apresentar o mesmo padrão filogeográfico que o encontrado para as espécies do grupo *jararacussu*, com a linhagem atlântica irmã do clado do Escudo das Guianas, os resultados de biogeografia histórica sugerem que o Rio Amazonas não tenha atuado como uma barreira vicariante para a espécie e sim como um limitador de distribuição e fluxo gênico (Dal Vechio *et al*., 2018).

Historicamente a formação e estabilização dos grandes rios amazônicos têm sido associada a eventos de especiação parapatrica, gerando diversidade (Wallace, 1852). Atualmente os grandes rios amazônicos são associados a limites de áreas de emdemismo (Cracraft, 1985; Silva *et al*., 2005; Ribas, *et al*., 2012), provovendo separação vicariante para diversos grupos, em particular aves e mamíferos (Aleixo, 2004; Heyes & Sewlal, 2004; Ribas, *et al*., 2012; Boubli *et al*., 2012; Pomara *et al*., 2014), e agora para serpentes também (Dal Vechio *et al*., 2019 aceito).

Implicações Taxonômicas

Investigando a história evolutiva dos crotalíneos, com ênfase nas espécies dos grupos *jararacussu*, *atrox* e *taeniatus* em *Bothrops* e de *Crotalus durissus* ficou evidente a subestimação da diversidade a nível de espécies, reconhecida atualmente. As análises moleculares e testes de delimitação de espécies suportam parafilia ou polifilia para *B. brazili*, *B. jararacussu*, *B. atrox*, *B. moojeni* e *Crotalus durissus* e problemas no reconhecimento do limite específico para *B. bilineatus e C. durissus*.

A fim de se alcançar estabilidade taxonômica para o grupo *jararacussu*, proponho a descrição de *B. oligobalius* **sp. nov.** No entanto, faz-se necessário uma investigação sistemática do clado atlântico composto pelas espécies: *B. jararacussu*, *B. pirajai* e *B. muriciensis* para melhor delineamento de suas linhagens evolutivas.

O grupo atrox apresenta problemas taxonômicos históricos. Acredito que obteremos avanços taxonômicos somente após uma abordagem conjunta de *B. atrox*, *B. moojeni* e *B. leucurus* e possivelmente de outras espécies do grupo, sob uma perspectiva integrada de dados moleculares e morfológicos. O mesmo ocorre para *Crotalus durissus*, que atualmente apresenta uma série de subespécies reconhecidas na América do Sul, porém sem associação dos nomes as linhagens moleculares.

Principais Conclusões

Usando uma abordagem multi-locus, os dados filogenéticos e de demografia histórica suportam múltiplos pulsos de fragmentação e expansão florestal nos últimos 2.5 milhões de anos, com contato físico entre a Amazônia e a Mata Atlântica e intercâmbio de fauna em ambas as direções. As análises de cenários históricos ainda suportam pulsos de fragmentação e expansão de abertas na América do Sul em sincronia temporal e espacial com os eventos paelobiogeográficos encontrados para as

áreas florestadas. Adicionalmente, os resultados de demografia populacional elucidam o papel dos rios amazônicos na diversificação da fauna, sugerindo que a formação e estabilização do Rio Amazonas atuou como uma barreira vicariante na separação de populações de serpentes amazônicas e tem atuado nos dias atuais como uma barreira para fluxo gênico. Por último, as análises moleculares e delimitação de linhagens evolutivas independentes apontam para problemas taxonômicos no reconhecimento do limite específico e diversidade críptica em *Crotalus durissus* e nos diversos grupos de espécies em *Bothrops* aqui estudados, sendo imprecindíveis revisões sistemáticas integrando-se dados moleculares com os de morfologia externa.

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Resumo

O contato pretérito Amazônia-Mata Atlântica vem sendo recorrentemente citado na literatura para diversos grupos da fauna Neotropical e teria ocorrido por diferentes rotas geográficas e períodos temporais. Segundo esta hipótese, em períodos quentes e úmidos as florestas se expandiram provocando retração/fragmentação das áreas abertas, ao passo que em períodos frios e secos, o inverso ocorreria. Assim, utilizando-se de crotalíneos como modelo, esta tese pretende: (1) testar o pretérito contato Amazônia-Mata Atlântica, assim como o tempo de separação entre as linhagens relictuais nesses ambientes florestados, elucidando a história demográfica das espécies de *Bothrops* do grupo *jararacussu*, do grupo *atrox* e de *B. bilineatus*; (2) paralelamente *Crotalus durissus*, espécie associada à diagonal de áreas abertas da América do Sul (Caatinga, Cerrado e Chaco), foi utilizada para testar a ocorrência de expansão desses ambientes, o que levaria a quebra da hipotetizada ponte florestada Amazônia-Mata Atlântica. Através de *dataset* multi-locus sob Inferência Bayesiana (quatro genes mitocondriais e cinco nucleares) foram testadas as relações filogenéticas e filogeográficas entre as amostras em *Bothrops* e *Crotalus*. Cenários históricos alternativos foram testados com base em simulações coalescentes e ABC (approximate Bayesian computation). Adicionalmente, foi empregado teste de delimitação de linhagens evolutivas para o reconhecimento da diversidade, a nível de espécie, em cada grupo estudado, usando a implementação Bayesiana do algoritimo *Generalized Mixed Yule-Coalescent*. Os resultados filogenéticos e análises de delimitação de espécies indicam diversidade críptica para os grupos *jararacussu*, *atrox* e *taeniatus*. Os testes de cenários históricos sugerem (1) múltiplas conexões florestais Amazônia–Mata Atlântica nos últimos 2.5 milhões de anos, com intercâmbio faunístico em ambas

as direções e (2) expansão das áreas abertas do Cerrado e Caatinga em sincronia temporal com perda de conectividade florestal Amazônia–Mata Atlântica durante o Pleistoceno tardio (3) que a formação e estabilização do Rio Amazonas teve influência na diversificação do grupo *jararacussu*, gerando diversidade. Os resultados trazem informações sobre o dinamismo histórico das paisagens florestadas e abertas no Neotrópico ao longo do tempo, assim como o papel dos rios amazônicos na diversificação da fauna. Adicionalmente, os resultados apontam para instabilidade taxonômica e diversidade críptica em diversos grupos em *Bothrops* e em *Crotalus durissus*, revelando a necessidade do aprofundamento sistemático para essas serpentes venenosas de importância médica.

Abstract

The hypotetized historical contact Amazon-Atlantic Forest has been recurrently cited in the literature for several groups of Neotropical fauna and would have occurred by different geographic routes and time periods. According to this hypothesis, in hot and humid periods the forests expanded causing retraction / fragmentation of the open areas, while in cold and dry periods, the reverse would occur. Thus, by using Neotropical pit vipers as a model, this thesis aims to: (1) test the historical forest contact Amazon-Atlantic Forest, as well as the time of separation between relict lineages in these forested environments, elucidating the demographic history of *Bothrops* species from *jararacussu* group, *atrox* group and *B. bilineatus*; (2) *Crotalus durissus*, a species associated with the diagonal of open areas of South America (Caatinga, Cerrado and Chaco), was used in parallel to test the occurrence of expansion of these environments, which would lead to the breakage of the hypothetical Amazon-Atlantic Forest forested bridge. Phylogenetic and phylogeographic relationships between samples of *Bothrops* and *Crotalus* were tested through a multi-locus dataset (four mitochondrial genes and five nuclear) under Bayesian Inference. Alternative historical scenarios were tested based on coalescent simulations and ABC (approximate Bayesian computation). In addition, was inferred independently evolving lineages to recognized species diversity in each group studied, using Bayesian implementation of the molecular species delimitation algorithm *Generalized Mixed Yule-Coalescent*. Phylogenetic results and analyzes of species delimitation indicate cryptic diversity for the groups *jararacussu*, *atrox* and *taeniatus*. The tests of historical scenarios suggest (1) multiple Amazon-Atlantic Forest forest connections in the last 2.5 million years, with faunal exchange in both directions and

(2) expansion of the open areas of the Cerrado and Caatinga in temporal synchrony with loss of forest bridge Amazon-Atlantic Forest during late Pleistocene (3) that the formation and stabilization of the Amazon River influenced the diversification of the *jararacussu* group, generating diversity. The results provide information on the historical dynamism of forested and open landscapes in the Neotropics over time, as well as the role of Amazonian rivers in the diversification of fauna. Moreover, the results point to taxonomic instability and cryptic diversity in several groups within *Brothrops* and *Crotalus durissus*, revealing the need for systematic deepening for these medically important venemous snakes.

Biografia

Cursei bacharelado em Ciencias Biológicas oferecido pelo Instituto de Biociências da Universidade de São Paulo – USP entre 2006-2011. Realizei durante este período uma iniciação científica no Laboratório de Bioquímica e Biofísica do Instituto Butantan, supervisionada pela Dra. Marilene Demasi, entre 2007-2009 (Número Fapesp: 2007/05620-6); e outra no Laboratório de Herpetologia do Instituto de Biociências - USP com o Prof. Dr. Miguel Trefaut Rodrigues (2010-2012) (Número Fapesp: 2010/15339-5). Dei continuidade aos estudos com a herpetofauna, realizando meu mestrado entre 2013-2014 (Número Fapesp: 2012/21213-0), no Departamento de Zoologia, IB-USP sob a orientação do Prof. Dr. Miguel, onde iniciei minha tese de doutorado no ano de 2015 (Número Fapesp: 2014/24431-3). Publiquei 24 trabalhos em revistas científicas como primeiro autor e como co-autor; e participei de congressos nacionais, Latino-Americanos e mundial na área de Herpetologia.

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Anexos

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RESEARCH PAPER

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Phylogeography and historical demography of the arboreal pit viper *Bothrops bilineatus* (Serpentes, Crotalinae) reveal multiple connections between Amazonian and Atlantic rain forests

Francisco Dal Vechio[1] | Ivan Prates[2] | Felipe G. Grazziotin[3] | Hussam Zaher[4] | Miguel Trefaut Rodrigues[1]

[1]Departamento de Zoologia, Instituto de Biociências, Universidade de São Paulo, São Paulo, SP, Brazil

[2]Department of Vertebrate Zoology, National Museum of Natural History, Smithsonian Institution, Washington, DC, 20560

[3]Laboratório de Coleções Zoológicas, Instituto Butantan, São Paulo, SP, Brazil

[4]Museu de Zoologia, Universidade de São Paulo, São Paulo, SP, Brazil

Correspondence
Francisco Dal Vechio, Departamento de Zoologia, Instituto de Biociências, Universidade de São Paulo, Rua do Matão, Trav. 14, n° 321, Cidade Universitária, CP 11461, CEP 05508-090, São Paulo, SP, Brazil.
Email: francisco.dalvechio@gmail.com

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Abstract

Aim: To examine historical connections between rain forest habitats in the Neotropics based on the evolutionary history of the arboreal pit viper *Bothrops bilineatus*.

Location: Amazonia and Atlantic Forest in South America.

Methods: We inferred phylogenetic relationships within *Bothrops* and phylogeographic relationships within *B. bilineatus* using both concatenated and species tree approaches under a Bayesian framework based on four mitochondrial and five nuclear genes. We inferred demographic history within *B. bilineatus* based on tests of alternative historical scenarios using coalescent simulations and Approximate Bayesian Computation.

Results: Phylogenetic analyses recovered four major clades within *Bothrops bilineatus*. The best-fit historical scenario involved colonization of the Atlantic Forest by an Amazonian ancestor around 2 Mya, followed by range expansion in the Atlantic Forest. Subsequently, there was a colonization event into the Guiana Shield from an Atlantic Forest ancestor, dating back to around 0.3 Mya.

Main conclusion: The complex demographic history of *Bothrops bilineatus* shows multiple connection events between Amazonia and the Atlantic Forest in the last 2 million years. Two previously hypothesized forest bridges, southern and northeastern, may have been involved in range expansions in this species. These results shed light on the historical processes involved in the diversification of this venomous South American snake, providing information about the historical dynamism of forested habitats in the Neotropics.

KEYWORDS

Bothriopsis, DIYABC, forest refuge, historical biogeography, molecular dating, Neotropics

1 | INTRODUCTION

Several biogeographic hypotheses have been proposed to explain the patterns of species richness and distribution in Amazonia, one of the most species-rich ecosystems on the planet. Restricted species ranges and composition turnover across this rain forest domain have been associated with contemporary and former landscape features (Bass et al., 2010), such as the present-day Amazon Basin rivers as dispersal barriers (Wallace, 1852), vicariance related to the formation of these major rivers, and tectonism in western South America linked to final uplift of the Andean chain during the Miocene (Hoorn et al., 2010). Another influential hypothesis is that of Pleistocene forest

refugia, which proposes cycles of forest fragmentation and expansion as a result of former climatic shifts in South America (Haffer, 1969). More recently, divergence time estimates between Amazonian taxa based on molecular data (Marques-Souza et al., 2018; Moritz, Patton, Schneider, & Smith, 2000; Prates, Rodrigues, Melo-Sampaio, & Carnaval, 2015) have suggested pre-Pleistocene diversification in Amazonia, which, together with palaeopalynological and speleothem data, contested the temporal framework implied by the refugia hypothesis (Bush, 2017; Bush & Oliveira, 2006; Colinvaux, Irion, Räsänen, Bush, & Nunes De Mello, 2001). Nevertheless, the idea that pulses of forest expansion and contraction during the Pleistocene have affected the demographic history of Amazonian species has found support in studies based on genetic data (Prates, Rivera, Rodrigues, & Carnaval, 2016). For instance, the possibility of the past forest bridges connecting Amazonia and the coastal Atlantic Forest has received attention in the last few years. These connections may explain the present-day ranges of a multitude of species whose ranges span both forest regions, but not the open areas of the dry diagonal of South America that separate them (the Caatinga, Chaco, and Cerrado domains).

The advance of bioinformatic techniques such as divergence time estimation, reconstruction of ancestral areas, and historical demographic inference has enabled new interpretations about spatial routes and the timing of former biogeographic connections between Amazonia and the Atlantic Forest. Studies with bird taxa proposed a Pleistocenic forest bridge between Amazonia and the Atlantic Forest along the northern coast of northeastern South America (Batalha-Filho, Fjeldsa, Fabre, & Miyaki, 2013). Such connection is also supported based on genetic data from small mammals (Costa, 2003), lizards (Prates, Rivera, et al., 2016; Prates, Xue, et al., 2016), and snakes (Zamudio & Greene, 1997). Pellegrino, Rodrigues, Harris, Yonenaga-Yassuda, and Sites (2011) and Rodrigues et al. (2014) suggested a similar but much older (Miocene) route based on lizard taxa. The idea of a northeastern forest connection along the northern Brazilian coast is consistent with the current presence of the "Brejos Nordestinos" in this region: relictual forest patches, surrounded by the semi-arid Caatinga, harbouring a mixture of Amazonian and Atlantic Forest animals and plants. Palaeopalynological (Oliveira, Barreto, & Suguio, 1999), speleothem (Auler & Smart, 2001; Auler et al., 2004; Dever, Fontes, & Richè, 1987; Wang et al., 2004), and palaeontological data (Czaplewski & Cartelle, 1998; Hartwig & Cartelle, 1996; Camolez & Zaher, 2010) point to a wetter climate in the semi-arid Caatinga during the Pleistocene, suggesting the possibility of preterit forest corridors extending through the central Caatinga, as opposed to along the northeastern coast. Finally, studies have also proposed a southern forest corridor between southwestern Amazonia and the southern Atlantic Forest. Batalha-Filho et al. (2013) found evidence of a southern Miocene bridge for birds through this route, while Costa (2003) found it to be consistent with phylogenetic patterns among small mammal taxa. Recently, Prates et al. (2017) found close relationships between southern Atlantic Forest anole lizards and species from western Amazonia and the Andes, suggesting a southwestern forest corridor that dates back to about 11 million years ago. As studies, to date, have found evidence of distinct times, magnitudes, and routes of presumed forest corridors, a general picture of biotic interchange between South American rain forests is yet to emerge. Since species responses to former habitat shifts may be affected by the biological attributes of organisms (Paz, Ibáñez, Lips, & Crawford, 2015; Prates, Rivera, et al., 2016; Prates, Xue, et al., 2016; Zamudio, Bell, & Mason, 2016), studies that target organisms with distinct ecologies and life histories can contribute to a better understanding of species interchange across regions over time.

In South America, snakes of the subfamily Crotalinae are a promising model to investigate historical changes in the distribution of rain forests, including the possibility of former connections between Amazonia (including the Guiana Shield region) and the Atlantic Forest, because several Crotalinae species show strong associations with dense closed-canopy wet forests. This is the case of *Bothrops bilineatus*, an arboreal pit viper of the *taeniatus* group of *Bothrops* that occurs in both Amazonia and the Atlantic Forest, but not in the intervening open and dry habitats. In this study, based on genetic data sampled throughout the distribution of this species, we infer the routes, directionality, and timing of former range expansions. Our approach combines phylogenetic reconstruction and historical demographic inference based on genetic coalescent simulations and Approximate Bayesian Computation. Beyond shedding light on the history of biotic interchange among highly diverse Neotropical rain forests, our study also has implications for lineage diversity and taxonomy in these medically important arboreal venomous snakes.

2 | MATERIALS AND METHODS

2.1 | Sampling and molecular protocols

We generated genetic data from 47 specimens of *Bothrops bilineatus* representing the entire range of this species as well as of one *B. taeniatus*. We also used sequences available on GenBank of two *B. taeniatus*, two *B. pulcher*, and one *B. chloromelas*, all of which members of the *taeniatus* species group within *Bothrops* (Figures 1–3 and Table 1). For broader phylogenetic inference, we also included 41 samples (22 from GenBank and 19 unpublished) of other *Bothrops* species as outgroups (Table 1). All samples used in this work were deposited in Genbank (accession numbers MH697889–MH698321).

Genomic DNA was extracted from tissue samples (liver, muscle, scale, or shed skin) and submitted to standard PCR protocols (see below). Nine DNA markers were amplified and sequenced (totaling 6,009 bp): the mitochondrial genes (matching available data sets for *Bothrops*) *16S ribosomal RNA* (*16s*, 534 bp; Palumbi, 1996), *12s ribosomal RNA* (*12S*, 394 bp; Palumbi, 1996), *cytochrome b* (*cytb*, 737 bp; Pook, Wüster, & Thorpe, 2000), and *NADH dehydrogenase subunit 4* (*nd4*, 797 bp; Arévalo, Davis, & Sites, 1994) as well as the nuclear genes *megakaryoblastic leukemia 1* (*mkl1*, 787 bp; Townsend, Alegre, Kelley, Wiens, & Reeder, 2008), *recombination-activating gene 1* (*rag1*, 995 bp; Wiens et al., 2008), *prolactin receptor* (*prlr*, 551 bp;

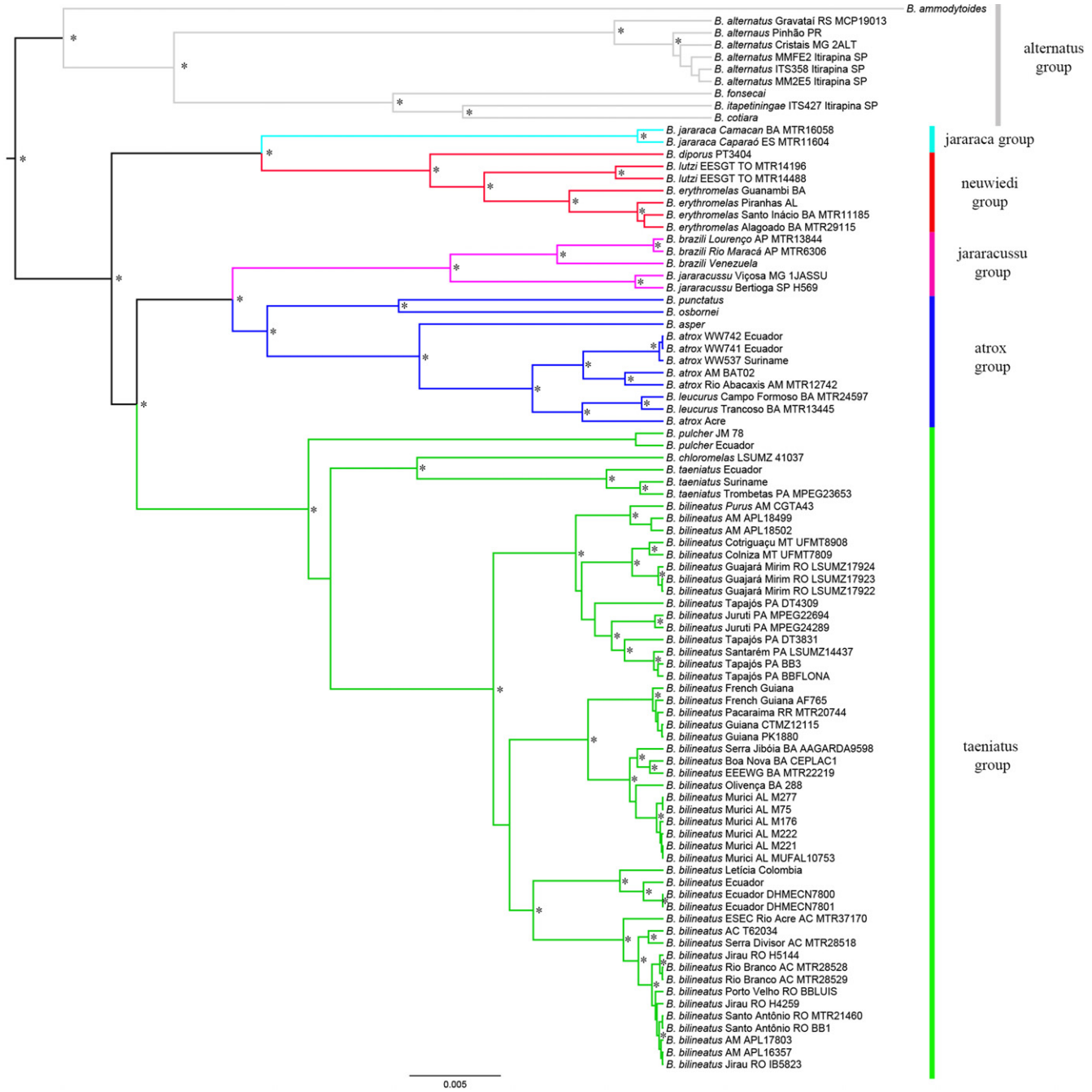

FIGURE 1 Phylogenetic relationships within *Bothrops* performed on MrBayes based on a concatenated data set with nine genes, showing its six species groups: *alternatus* in grey, *jararaca* in light blue, *neuwiedi* in red, *jararacussu* in purple, *atrox* in dark blue, and *taeniatus* in green. Asterisks on nodes indicate posterior probabilities higher than 0.95. Scale bar indicates substitutions per site

Townsend et al., 2008), *oocyte maturation factor* (*c-mos*, 575 bp; Godinho, Crespo, Ferrand, & Harris, 2006), and *brain-derived neurotrophic factor* (*bdnf*, 639 bp; Townsend et al., 2008). PCR reaction conditions were an initial denaturation step at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 40 s, annealing at X°C for 40 s, and extension at 72°C for 40 s, and a terminal extension cycle at 72°C for 7 min, in which X is the temperature for each set of primers, as follows: 51°C for *16s*, *12s*, *cytb*, *nd4*, *prlr* and *rag1*; 52°C for *c-mos*; 54°C for *mkl1*; and 55°C for *bdnf*.

2.2 | Phylogenetic inference

To reconstruct the historical relationships among samples, we used Bayesian Inference after sequence edition in CodonCode Aligner 3.7.1.1 (www.codoncode.com) and alignment using MUSCLE (Edgar, 2004) as implemented in MEGA 6 (Tamura, Stecher, Peterson, Filipski, & Kumar, 2013) under default settings. The best-fit model of evolution for each aligned marker was identified using jModelTest 2.1.3 (Darriba, Taboada, Doallo, & Posada, 2012) and the Akaike

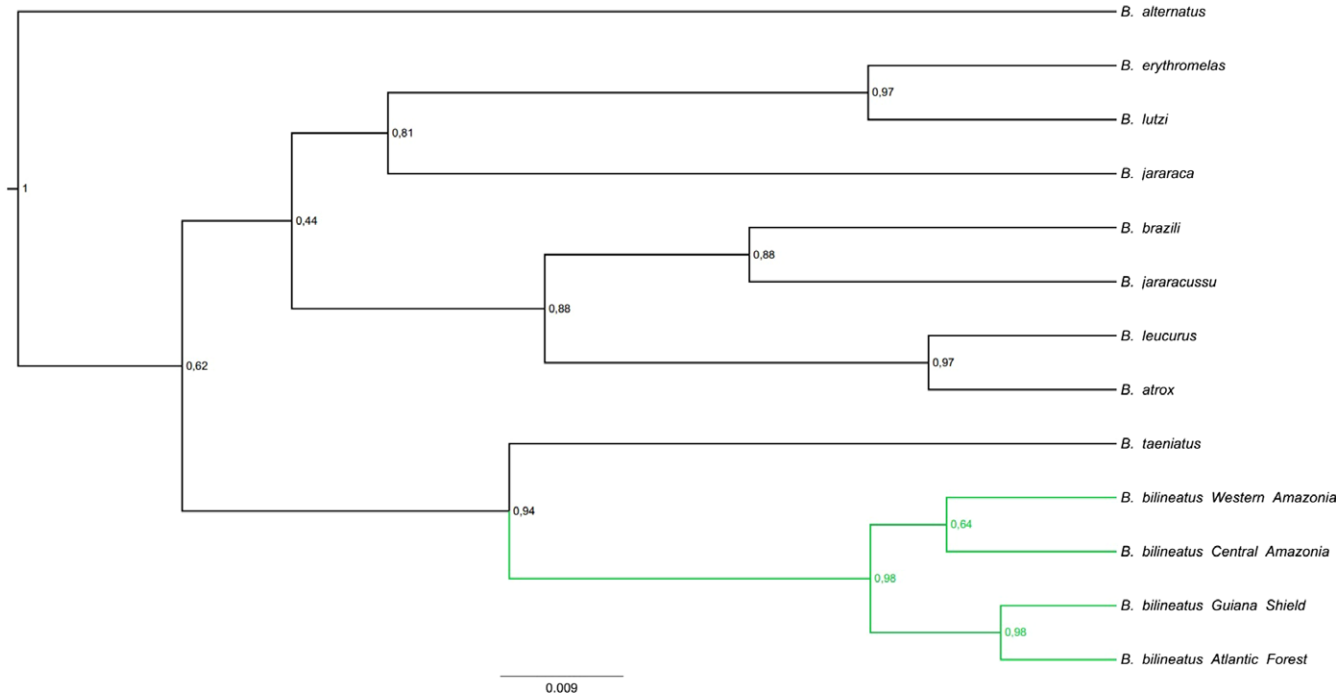

FIGURE 2 Species tree within *Bothrops* based on coalescent analysis performed in *BEAST implemented in BEAST. The green branch represents *B. bilineatus* (corresponding to four geographically restricted populations; see text). Numbers in nodes represent posterior probability values. Scale bar indicates substitutions per site

Information Criterion (AIC), as follows: GTR+G+I for all mitochondrial markers, HKY for *bdnf* and *c-mos*, GTR for *prlr*, GTR+I for *rag1*, and HKY+I for *mkl1*. Phylogenetic analyses were performed using MrBayes 3.2 (Ronquist et al., 2012) and the **BEAST* tool (a coalescent-based method) in BEAST 1.8.3 (Drummond, Suchard, Xie, & Rambaut, 2012) through the Cipres Science Gateway (Miller, Pfeiffer, & Schwartz, 2010). For MrBayes analyses, two independent Bayesian runs were performed with four Markov chains each, starting from a random tree; each run consisted of 10,000,000 generations, with trees sampled every 1,000 generations. For *BEAST analyses, two independent runs of 100 million generations each were used, with a sampling frequency of 10,000 generations. For both MrBayes and BEAST runs, we discarded the first 25% of trees as burn-in; convergence of independent runs and stationarity of model parameters (effective sampling sizes >200) were verified using Tracer 1.6 (Rambaut & Drummond, 2009). A 50% majority consensus tree with clade frequencies was summarized for MrBayes analyses, while a maximum clade credibility tree was summarized for *BEAST runs using TreeAnnotator (Drummond et al., 2012). Resulting phylogenies were visualized using FigTree 1.4.0 (http://tree.bio.ed.ac.uk/). The haplotypic phase of heterozygotes was determined using PHASE 2.1.1 (Stephens & Donnelly, 2003) under default settings implemented in DnaSP 5.1 (Librado & Rozas, 2009).

2.3 | Alternative scenarios of demographic history

Phylogenetic analyses recovered a number of clades within *Bothrops bilineatus* that are restricted geographically (see Results). To investigate the history of range expansions in *B. bilineatus*, we tested alternative historical demographic scenarios based on these inferred lineages using coalescent simulations and Approximate Bayesian Computation (ABC). Our approach compares the observed (i.e., sampled) genetic data to data simulated under each competing scenario, based on informative summary statistics that capture the patterns of genetic variation (Beaumont, 2010; Csilléry, Blum, Gaggiotti, & François, 2010). We were particularly interested in testing which spatially restricted Amazonian lineage (western Amazonia, central Amazonia, or Guiana Shield; see Results) most likely acted as the colonization source of the Atlantic Forest group. Moreover, to shed light on the magnitude of former forest connections, we aimed to test whether the colonization of the Atlantic Forest was accompanied by a population bottleneck, as expected as a result of spatially or temporally restricted connections across domains, as opposed to a scenario of vicariance of a widely distributed ancestor, which did not involve pronounced shifts in population sizes following divergence.

We used the historical relationships inferred by phylogenetic analyses to inform the population tree topology in alternative models of *B. bilineatus* demographic history. However, as phylogenetic analyses based on both MrBayes and *BEAST found little support for which Amazonian clade (central, western, or central/western) is sister to a clade containing the Atlantic Forest plus Guiana Shield samples (see Results), we included all possible tree topologies at that node (i.e., switching the relationship among major clades) in our competing historical models. These alternative topologies include (see Results): a central Amazonian clade as the sister of the common ancestor of the remaining *B. bilineatus* samples, with a western Amazonian

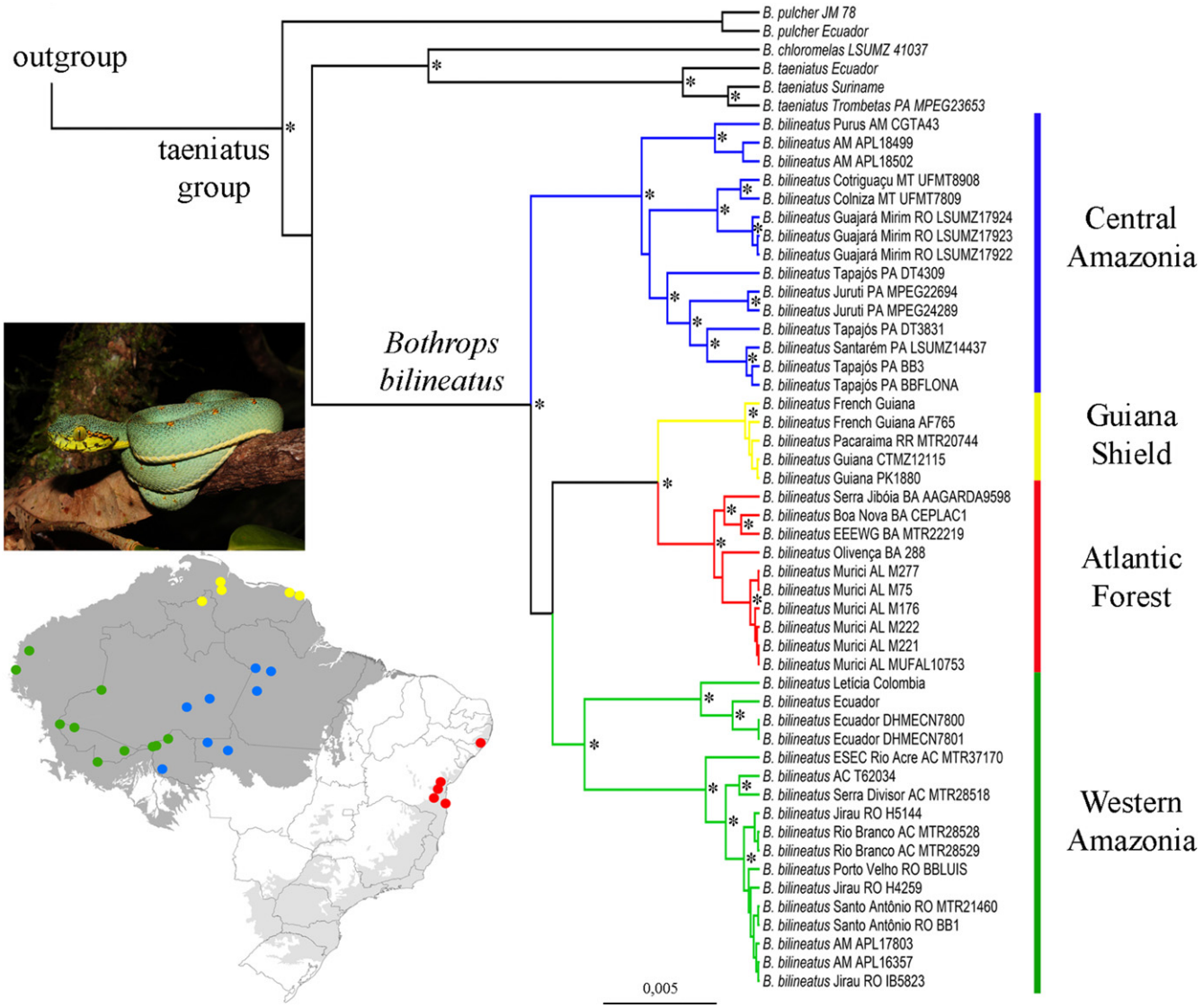

FIGURE 3 Phylogenetic relationship within the *taeniatus* group and phylogeography of *Bothrops bilineatus* based on nine molecular markers (five nuclear and four mitochondrial genes) performed on MrBayes. Colours in map correspond to major clades in historical demographic analyses. Asterisks on nodes indicate posterior probabilities higher than 0.95. Scale bar indicates substitutions per site. Photo: Renato Recoder

lineage as sister to the ancestor of Atlantic Forest + Guiana Shield (used in scenarios 1–4; see below); a western Amazonian clade as the sister of the ancestor of the remaining clades, with a central Amazonian clade as sister to the ancestor of Atlantic Forest + Guiana Shield (scenarios 5–8); and a central and western Amazonian clades forming a clade that is sister to Atlantic Forest + Guiana Shield (scenarios 9–12; see Supporting Information Figure S1.1 for a visual representation of these scenarios).

Based on those three alternative topologies, 12 historical scenarios were simulated and confronted with each other (Supporting Information Figure S1.1). In scenario 1, an ancestral population occurring in western Amazonia (central Amazonia in scenario 5) and the Atlantic Forest was split following expansion of open and dry domains in South America (a vicariant split); a second vicariant event subsequently separated Guiana Shield and Atlantic Forest clades (Supporting Information Figure S1.1). In scenarios 2 and 6, a founder population colonized the Atlantic Forest from the Guiana Shield region in northern Amazonia through a forest corridor in the presently dry belt of northeastern South America, with a subsequent population expansion within the Atlantic Forest (Supporting Information Figure S1.1). In scenario 3, a vicariant split separated an ancestral population occurring in western Amazonia (central Amazonia in scenario 7) and the Atlantic Forest, with a subsequent event of forest expansion leading to colonization of the Guiana Shield from the Atlantic Forest, which would then be split following expansion of open and dry domains in northeastern South America (Supporting Information Figure S1.1). In Scenario 4, a founding population colonized the Atlantic Forest from western Amazonia (central Amazonia in scenario 8), with subsequent population expansion within the Atlantic Forest; in this same scenario, a second founder event led to colonization of the Guiana Shield from the Atlantic Forest (Supporting Information Figure S1.1).

TABLE 1 Tissues samples used on phylogenetic and phylogeographic inferences

Voucher	Species	Locality
288	*Bothrops bilineatus*	Olivença, Bahia, Brazil
MTR22219	*Bothrops bilineatus*	EEEWG, Bahia, Brazil
MUFAL10753	*Bothrops bilineatus*	Murici, Alagoas, Brazil
CEPLAC1	*Bothrops bilineatus*	Boa Nova, Bahia, Brazil
M176	*Bothrops bilineatus*	Murici, Alagoas, Brazil
M75	*Bothrops bilineatus*	Murici, Alagoas, Brazil
M277	*Bothrops bilineatus*	Murici, Alagoas, Brazil
M221	*Bothrops bilineatus*	Murici, Alagoas, Brazil
M222	*Bothrops bilineatus*	Murici, Alagoas, Brazil
AAGarda9598	*Bothrops bilineatus*	Serra da Jibóia, Bahia, Brazil
AF765	*Bothrops bilineatus*	French Guiana
MTR20744	*Bothrops bilineatus*	Pacaraima, Roraima, Brazil
PK1880	*Bothrops bilineatus*	Lower slope of Maringma-tepui, Guiana
CTMZ12115	*Bothrops bilineatus*	Potaro-Siparuni, Guiana
–	*Bothrops bilineatus*	Petit Saut, French Guiana
MPEG22694	*Bothrops bilineatus*	Juruti, Pará, Brazil
MPEG24289	*Bothrops bilineatus*	Juruti, Pará, Brazil
UFMT8908	*Bothrops bilineatus*	Cotriguaçu, Mato Grosso, Brazil
UFMT-R7809	*Bothrops bilineatus*	Colniza, Mato Grosso, Brazil
DT3831	*Bothrops bilineatus*	Tapajós, Pará, Brazil
CGTA-C0043	*Bothrops bilineatus*	Médio Rio Purus, Amazonas, Brazil
APL18499	*Bothrops bilineatus*	Br-319, Amazonas, Brazil
APL18502	*Bothrops bilineatus*	Br-319, Amazonas, Brazil
BBFLONA	*Bothrops bilineatus*	Tapajós, Pará, Brazil
BB3	*Bothrops bilineatus*	Tapajós, Pará, Brazil
DT4309	*Bothrops bilineatus*	Tapajós, Pará, Brazil
LSUMZ14437	*Bothrops bilineatus*	Santarém, Pará, Brazil
LSUMZ17922	*Bothrops bilineatus*	Parque estadual Guajará-Mirim, Rondônia, Brazil
LSUMZ17923	*Bothrops bilineatus*	Parque estadual Guajará-Mirim, Rondônia, Brazil
LSUMZ17924	*Bothrops bilineatus*	Parque estadual Guajará-Mirim, Rondônia, Brazil
BB1	*Bothrops bilineatus*	UHE Santo Antônio, Rondônia, Brazil
T62034	*Bothrops bilineatus*	Cruzeiro do Sul, Acre, Brazil
MTR21460	*Bothrops bilineatus*	UHE Santo Antônio, Rondônia, Brazil
MTR28518	*Bothrops bilineatus*	Serra do Divisor, Acre, Brazil

(Continues)

TABLE 1 (Continued)

Voucher	Species	Locality
MTR28528	*Bothrops bilineatus*	Rio Branco, Acre, Brazil
MTR28529	*Bothrops bilineatus*	Rio Branco, Acre, Brazil
APL16357	*Bothrops bilineatus*	Rondônia, Brazil
APL17803	*Bothrops bilineatus*	Rondônia, Brazil
BBLUIS	*Bothrops bilineatus*	Porto Velho, Rondônia, Brazil
H4259	*Bothrops bilineatus*	UHE Jirau, Rondônia, Brazil
H5144	*Bothrops bilineatus*	UHE Jirau, Rondônia, Brazil
IB5823	*Bothrops bilineatus*	UHE Jirau, Rondônia, Brazil
MTR37170	*Bothrops bilineatus*	ESEC Rio Acre, Acre, Brazil
DHMECN7800	*Bothrops bilineatus*	Sucumbíos, Ecuador
DHMECN7801	*Bothrops bilineatus*	Sucumbíos, Ecuador
FHGO 983	*Bothrops bilineatus*	Morona Santiago, Macuma, Ecuador
–	*Bothrops bilineatus*	Letícia, Colombia
MVZ 223514	*Bothrops ammodytoides*	Neuquen, Argentina
IB 55543	*Bothrops fonsecai*	Campos do Jordão, São Paulo, Brazil
ITS 427	*Bothrops itapetiningae*	Itirapina, São Paulo, Brazil
WWW	*Bothrops cotiara*	Brazil
MCP19013	*Bothrops alternatus*	Gravataí, Rio Grande do Sul, Brazil
2ALT	*Bothrops alternatus*	Cristais, Minas Gerais, Brazil
MMFE2	*Bothrops alternatus*	Itirapina, São Paulo, Brazil
ITS358	*Bothrops alternatus*	Itirapina, São Paulo, Brazil
MM2E5	*Bothrops alternatus*	Itirapina, São Paulo, Brazil
IB 55314	*Bothrops alternatus*	Pinhão, Paraná, Brazil
MTR16058	*Bothrops jararaca*	Serra Bonita, Camacan, Bahia, Brazil
MTR11604	*Bothrops jararaca*	Caparaó, Espírito Santo, Brazil
PT3404	*Bothrops diporus*	Argentina
MTR14196	*Bothrops lutzi*	EESGT, Tocantins, Brazil
MTR14488	*Bothrops lutzi*	EESGT, Tocantins, Brazil
MTR11185	*Bothrops erythromelas*	Santo Inácio, Bahia, Brazil
MTR29115	*Bothrops erythromelas*	Alagoado, Bahia, Brazil
IB55541	*Bothrops erythromelas*	Guanambi, Bahia, Brazil
RG829	*Bothrops erythromelas*	Piranhas, Alagoas, Brazil
H569	*Bothrops jararacussu*	Bertioga, São Paulo, Brazil
1JASSU	*Bothrops jararacussu*	Viçosa, Minas Gerais, Brazil
MTR6306	*Bothrops brazili*	Rio Maracá, Amapá, Brazil

(Continues)

TABLE 1 (Continued)

Voucher	Species	Locality
MTR13844	*Bothrops brazili*	Lourenço, Amapá, Brazil
FHGO live 2452	*Botrops punctatus*	Ecuador
FHGO live 2166	*Bothrops osbornei*	Ecuador
MZUCR 11152	*Bothrops asper*	Costa Rica
-	*Bothrops atrox*	Acre, Brazil
MTR13445	*Bothrops leucurus*	Trancoso, Bahia, Brazil
MTR24597	*Bothrops leucurus*	Campo Formoso, Bahia Brazil
MTR12742	*Bothrops atrox*	Rio Abacaxis, Amazonas, Brazil
BAT02	*Bothrops atrox*	Amazonas, Brazil
-	*Bothrops atrox*	Suriname
WW741	*Bothrops atrox*	Ecuador
WW742	*Bothrops atrox*	Ecuador
JM78	*Bothrops pulcher*	Peru
FHGO live 2142	*Bothrops pulcher*	Zamora Chinchipe, Ecuador
LSUMZ41037	*Bothrops chloromelas*	Pasco, Peru
MPEG23653	*Bothrops taeniatus*	FLONA Trombetas, Pará, Brazil
FHGO live 1407	*Bothrops taeniatus*	Morona Santiago, Macuma, Ecuador
-	*Bothrops taeniatus*	Suriname

In scenarios 9–12, western and central Amazonia were treated as sister lineages whose ancestor is sister to a clade formed by Atlantic Forest and Guiana Shield clades. In scenario 9, an ancestral population occurring in central and western Amazonia and the Atlantic Forest was split following expansion of open and dry domains in South America (a vicariant split); a second vicariant event separated the Guiana Shield and Atlantic Forest clades (Supporting Information Figure S1.1). In scenario 10, a founder population colonized the Atlantic Forest by an Amazonian ancestor from the Guiana Shield region through a forest corridor in northeastern South America, with subsequent population expansion within the Atlantic Forest (Supporting Information Figure S1.1). In scenario 11, a vicariant split separated an ancestral population occurring in central and western Amazonia and the Atlantic Forest; a subsequent event of forest expansion led to colonization of the Guiana Shield from the Atlantic Forest (Supporting Information Figure S1.1). In scenario 12, a founding population colonized the Atlantic Forest from a central and western Amazonian ancestor, with subsequent population expansion within the Atlantic Forest; in this same scenario, another founder event led to colonization of the Guiana Shield from the Atlantic Forest (Figure 4 and Figure S1.1). In summary, scenarios 1, 5, and 9 similarly test for vicariant events in the demographic history of *B. bilineatus*; scenarios 2, 6, and 10 similarly test for a colonization event of the Atlantic Forest from a Guiana Shield ancestor; scenarios 3, 7, and 11 similarly test for a colonization event of Amazonia (Guiana Shield region) from a Atlantic Forest ancestor; and finally, scenarios 4, 8, and 12 similarly test for a colonization event of the Atlantic Forest from a central and western Amazonia ancestor, with a second colonization event of the Guiana Shield from an Atlantic Forest ancestor.

We performed historical demographic analyses based on six loci: one mitochondrial locus (unphased data) comprising four genes (12S, 16S, cytb, and ND4), and five independent nuclear loci (MKL1, RAG1, PRLR, C-mos, and BDNF; phased data). We follow Prates, Rivera, et al. (2016) and performed 10 million coalescent simulations (evenly distributed among all demographic scenarios) followed by ABC inference with *DIYABC* 2.1 (Cornuet et al., 2014). We chose summary statistics based on preliminary runs that confirmed that Tajima's *D* (Tajima, 1989), number of private segregating sites, and pairwise F_{st} (Hudson, Slatkin, & Maddison, 1992) were appropriate for discrimination among simulated scenarios. We also confirmed whether the observed genetic data were contained within the space of simulated data by performing a principal component analysis (PCA) based on the chosen summary statistics (Cornuet et al., 2014).

Prior distributions of model parameters were set as follows: (a) divergence times between independently evolving lineages with a uniform distribution ranging between 0.1 and 5 million years; (b) effective population size in each independently evolving lineages with a uniform distribution ranging between 0.01 and 5 millions of individuals; (c) effective population size during a founder event with a uniform distribution ranging from 0.001 to 0.1 millions of individuals; and (d) effective population size prior to vicariant events with a uniform distribution ranging from 0.01 to 5 millions of individuals. We set a mutation rate based on the number of substitutions among sequences of Crotalinae species (Wüster, Peppin, Pook, & Walker, 2008), with a uniform prior distribution ranging from 10^{-9} to 10^{-8} substitutions per site per generation for the mitochondrial locus and 10^{-10} to 10^{-8} substitutions per site per generation for the nuclear loci. A *Tamura-Nei* (Tamura & Nei, 1993) substitution model of evolution was implemented for each locus. To convert the number of generations in our models to years, we assumed a generation time (*T*) of 3 years in *Bothrops*. We established this number based on the equation $T = a + [s/1(1-s)]$ (Lande, Engen, & Saether, 2003), where *a* is the age of maturity and *s* is the annual survival rate. Almeida-Santos and Salomão (2002) and Almeida-Santos et al. (2004) suggested that the maturity age of *Bothrops* species is around 1.5–3 years. While it is hard to estimate the annual survival rate of these snakes over time, which probably vary with climatic conditions and food supply, some data for populations of Crotalinae species suggest annual survival rates around 0.39 to 0.77 (Brown, Kéry, & Hines, 2007; Maritz & Alexander, 2012). Based on these data, we estimate generation times in *Bothrops bilineatus* as being around 2–3 years.

To assess how data simulated under each of the 12 scenarios compare to the empirical genetic data, we estimated the statistical support of each model based on Euclidean distances between the simulated data and the observed data. For this, we selected the 1.000 simulated data sets (0.01% of the total simulations) that were closest to the observed data. We then estimated posterior

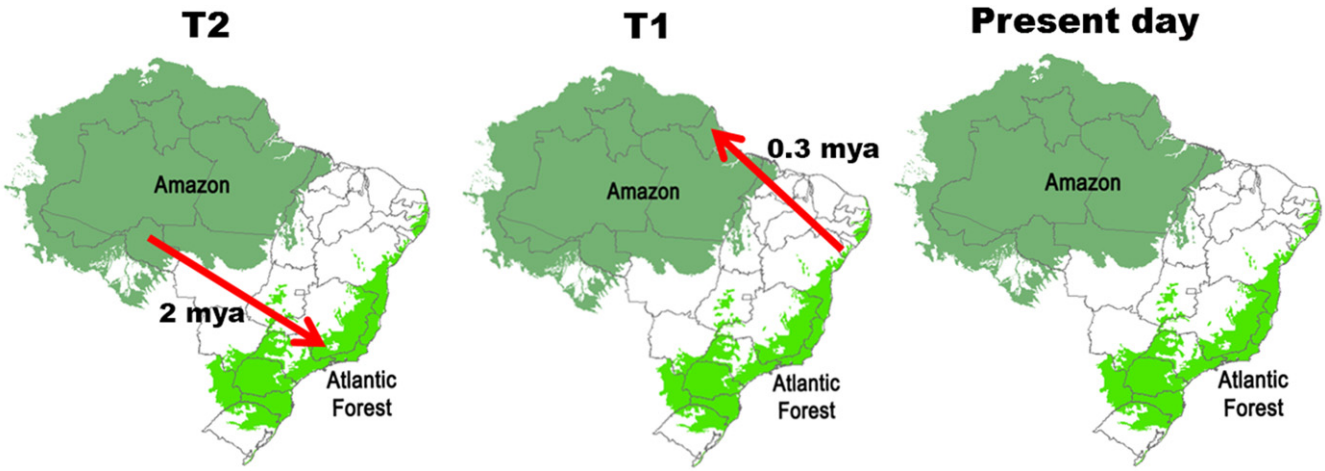

FIGURE 4 Best-fit scenario supported by historical demography analyses using DIYABC based on nine genes (four mitochondrial and five nuclear) from *Bothrops bilineatus*. The model includes a colonization event by an Amazonian ancestor (which presumably occurred in central + western Amazonia) into the Atlantic Forest at T2 (2 Mya), with another colonization event into the Guiana Shield by an Atlantic Forest ancestor at T1 (0.3 Mya). Arrows represent colonization events; T2 and T1 represent times in the past (T2 older than T1; T1 older than today). For details, see text

distributions of population parameters under the best-fit scenario, using the 500 simulated data sets that were closest to the observed data.

Finally, we tested the accuracy of the model selection procedure by examining whether the different simulated scenarios can be discriminated based on the summary statistics used. For this, we simulated 100 pseudo-observed data sets under each scenario, such that the true scenario was known for each pseudo-observed data set. We then performed ABC inference on each pseudosimulated data set, thus calculating the proportion of pseudo-observed data sets that were correctly assigned to its generating model.

3 | RESULTS

3.1 | Phylogenetic relationship and species delimitation

Phylogenetic relationships within *Bothrops* based on a concatenated data set (using MrBayes) indicate that the *taeniatus* species group is monophyletic (PP = 1) and sister to the *atrox* + *jararacussu* species groups (Figure 1). The species tree (using *BEAST) recovered a different relationship among these species groups, with a clade formed by the *atrox*, *jararacussu*, *jararaca*, and *neuwiedi*, groups as sister to the *taeniatus* group, albeit with low statistical support (Figure 2). In all phylogenetic analyses, *B. bilineatus* is nested within the *taeniatus* species group (Figures 2 and 3). *Bothrops bilineatus* is constituted by four highly supported major clades (PP = 1), all of which restricted in geographic space, as follows (Figure 3): (a) an Atlantic Forest clade; (b) a Guiana Shield clade occurring north of the Amazon river and east of the Orinoco and Rio Negro rivers; (c) a western Amazonian clade occurring west of the Orinoco and Rio Negro rivers (in Colombia and Ecuador) and western Brazil (states of Acre and Rondônia, possibly also occurring in Peru and Bolivia); and (d) a central Amazonian clade distributed to the south of the Amazon river, in the Brazilian states of Amazonas, Rondônia, Mato Grosso, and Pará (Figure 3).

Within *Bothrops bilineatus*, phylogenetic results using MrBayes found the Atlantic Forest and Guiana Shield clades to be sister to each other with high support (PP = 1). This clade is the sister group of a clade formed by western Amazonian samples with moderate support (PP = 0.73). The clade formed by these three geographically restricted clades is the sister group of the more broadly distributed central Amazonian clade (PP = 1) (Figure 3). The species tree analysis based on *BEAST recovered a sister relationship between the western and central Amazonian clades (PP = 0.64), whose ancestor is the sister of the Atlantic Forest + Guiana Shield clade (PP = 0.98) (Figure 2).

3.2 | Best-fit historical scenarios

Based on coalescent simulations and Approximate Bayesian Computation, we found that the observed genetic data are consistent with the hypothesis that *Bothrops bilineatus* colonized the Atlantic Forest from an Amazonian ancestor (which presumably occurred in central + western Amazonia) and subsequently colonized the Guiana Shield region through a back colonization from the Atlantic Forest (scenario 12; PP = 0.99) (Figure 4). These results support the idea of two independent colonization events in opposite directions across currently disjunct rain forest blocks in South America.

Posterior parameter estimates based on the best-fit scenario support a late-Pliocene divergence between the ancestor of the central and western Amazonian clade and the ancestor of the Atlantic

Forest and Guiana Shield clades, dating back to 1.95 Mya (median value; 95% credibility interval (C.I.) = 1.5–4.5 Mya); and a late-Pleistocene divergence between the Atlantic Forest and Guiana Shield clades, dating back to 0.3 Mya (median value; 95% credibility interval (C.I.) = 0.19–0.8 Mya). Effective population size estimates suggest that the initial pool of Atlantic Forest colonizers was composed of about 53,000 individuals (ca. 1.2% of the ancestral Amazonian population), while the Guiana Shield was colonized by about 98,000 individuals originated from the Atlantic Forest (ca. 4.6% of the ancestral Atlantic population), supporting a pronounced population bottleneck following dispersal into both the Atlantic Forest and Guiana Shield. Median substitution rates were estimated as 5×10^{-9} substitutions per site/year for the mitochondrial locus (median value; 95% credibility interval (C.I.) = $3.4–8.6 \times 10^{-9}$ substitutions per site/year) and $0.4–0.7 \times 10^{-9}$ substitutions per site/year for the nuclear loci (median value; 95% credibility interval (C.I.) = $0.3–2.1 \times 10^{-9}$ substitutions per site/year).

Model validation based on principal component analyses of the summary statistics confirmed that the observed data were contained within the space of simulated data. Posterior error rates based on pseudo-observed data sets were estimated as 0.69. However, most incorrect inferences (53% from the total of pseudo-observed data sets) happened between scenarios that depict the same history of Atlantic Forest and Guiana Shield colonization but have topological differences regarding which *Bothrops bilineatus* clade that is sister to the remaining ones. This is the case of confusion between scenarios 2, 6, and 10, or 4, 8, and 12 (see Methods). As a result, the error rate between scenarios that depict distinct histories of colonization (e.g., colonization of the Atlantic Forest from a Guiana Shield ancestor versus colonization of the Guiana Shield from an Atlantic Forest ancestor or a vicariant event between these two regions) is effectively much lower: 0.16.

4 | DISCUSSION

The idea of the past biogeographic connections between Amazonia and the Atlantic Forest has received increasing attention in the last few years, with phylogeographic and phylogenetic studies pointing to connection events between these two domains (Batalha-Filho et al., 2013; Costa, 2003; Prates, Rivera, et al., 2016; Prates et al., 2017; Zamudio & Greene, 1997). Speleothem (Auler et al., 2004; Cheng et al., 2013) and palaeopalynological records (Oliveira et al., 1999) have also supported the view of the past dynamism in South American rain forest habitats, with pulses of forest expansion and fragmentation. In agreement with these studies, our results for *Bothrops bilineatus* support the idea that changing climates have led to shifts in South American rain forest distribution and, ultimately, in biotic interchange between regions, allowing lineages to colonize new areas, where pressures from competitors and predators could potentially be relaxed. These events seem to have had major consequences for the composition of local species pools, and therefore for large-scale biogeographic patterns in both Amazonia and the Atlantic Forest.

Our tests of alternative historical demographic scenarios based on multilocus genetic data from *Bothrops bilineatus* point to a history of two consecutive events of forest colonization as well as of back invasion of Amazonia—to our knowledge, a pattern that has not been yet identified in studies of other organisms. Specifically, we found support for a scenario of colonization of the Atlantic Forest from Amazonia (presumably by an ancestor occurring in both central and western Amazonia), followed by colonization of the Guiana Shield from the Atlantic Forest. A history of connections between western Amazonia and the Atlantic Forest, supported by our historical demographic study of *B. bilineatus*, was recovered by previous studies based on genetic data of birds and lizards (Batalha-Filho et al., 2013; Prates et al., 2017). Phylogeographic studies have also found evidence of northern colonizations between the Atlantic Forest and northeastern Amazonia (Batalha-Filho et al., 2013; Costa, 2003; Prates, Rivera, et al., 2016; Prates, Xue, et al., 2016). In spite of these two distinct proposed connection routes, previous studies have, by and large, found that Amazonia acted as the source of colonizations into the Atlantic Forest. Our study is one of the first (as that of Rodrigues et al., 2014), however, to find evidence of the Atlantic Forest serving as a source of colonizations into Amazonia, particularly into northern Amazonia in the Guiana Shield region. It is currently unclear why the Atlantic Forest seems to have more often received than provided biodiversity to Amazonia over the course of these forests' histories, a pattern that may be related to their significant differences in total size.

While phylogenetic patterns have provided clues about the localization of former rain forest corridors in South America, we have a limited understanding of how often Amazonia and Atlantic Forest domains have been connected in the past and for how long. Batalha-Filho et al. (2013) suggested multiples contact events based on divergence times between species and clades of birds restricted to each of these two forest systems, suggesting that the proposed forest bridge in the present-day northeastern Brazil would be Pleistocenic in age. This timing is consistent with studies of codistributed arboreal lizards and of the south American bush master *Lachesis muta*, which proposed colonization of the Atlantic Forest from eastern Amazonia at around 300–950 kya (Prates, Rivera, et al., 2016; Prates, Xue, et al., 2016; Zamudio & Greene, 1997). Similarly, our estimates for *Bothrops bilineatus* also suggest a Pleistocenic contact at around 0.3 mya between the Atlantic Forest and northern Amazonia (at the Guiana Shield) through northeastern South America. It is worth noting, however, that the directionality of range expansions in *B. bilineatus* and other studied organisms is fundamentally different, as our study suggests that this snake seems to have colonized Amazonia from an Atlantic Forest ancestral population, while organisms such as lowland anole lizards were found to have expanded their ranges in the opposite direction (Prates, Rivera, et al., 2016).

Besides a colonization event in present-day northeastern Brazil, the complex history of *Bothrops bilineatus* seems to include an event of range expansion into the Atlantic Forest from an Amazonian ancestor, as supported by the results of phylogenetic and historical demographic analyses. Costa (2003) suggested a similar, seemingly

southern route for small mammals, but without detailed information regarding its timing. The available divergence time estimates suggest that recurrent forest connection events occurred during the Pliocene, dating back to 2 Mya in the case of *Bothrops bilineatus*, 11 Mya for *Anolis* lizards (Prates et al., 2017), and 3–13 Mya for a range of small mammal and bird taxa (Batalha-Filho et al., 2013; Ledo & Colli, 2017).

Taken together, biogeographic studies focusing on species and clades whose distribution span distinct South American rain forest blocks suggest that Amazonia and the Atlantic Forest have an intricate history of physical connections over time, which led to taxonomic similarity across presently disjunct regions. Despite the numerous examples of taxa that seem to have undergone range expansions across these domains, there is a limited number of studies that have investigated the routes, timing and especially the demographic processes involved, an information that is crucial to improve our understanding of the origin of these extraordinarily diverse ecosystems. By involving consecutive colonization events across Amazonia and the Atlantic Forest in a single taxon, the population history of *Bothrops bilineatus* is unique. As such, this study provides further insight, and adds complexity, to the dynamic history of South American rain forests during the last millions of years, seemingly as a result of pronounced climate shifts. We hope that the results presented here inspire further studies aiming to our knowledge about former biogeographic connections and biotic interchange among South American regions.

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ORCID

Francisco Dal Vechio http://orcid.org/0000-0003-1328-0243

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BIOSKETCH

Francisco Dal Vechio is PhD student in the Herpetology Laboratory in the Biosciences Institute of the University of Sao Paulo. His main research interests are the systematics and biogeographic history of Neotropical pit vipers.

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SUPPORTING INFORMATION

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