

UNIVERSIDADE FEDERAL DE ALAGOAS
INSTITUTO DE CIÊNCIAS BIOLÓGICAS E DA SAÚDE
Programa de Pós-Graduação em Diversidade Biológica e Conservação nos
Trópicos

JOÃO PAULO FELIX AUGUSTO DE ALMEIDA

**O PAPEL RELATIVO DOS REFÚGIOS GLACIAIS E DAS BARREIRAS
GEOGRÁFICAS ATUAIS NA DIVERSIFICAÇÃO DE UM SQUAMATA FOSSORIAL NA
MATA ATLÂNTICA BRASILEIRA**

MACEIÓ - ALAGOAS
Fevereiro/2017

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Dissertação apresentada ao Programa de Pós-Graduação em Diversidade Biológica e Conservação nos Trópicos, Instituto de Ciências Biológicas e da Saúde. Universidade Federal de Alagoas, como requisito para obtenção do título de Mestre em CIÊNCIAS BIOLÓGICAS, área de concentração em Conservação da Biodiversidade Tropical.

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MACEIÓ - ALAGOAS
Fevereiro/2017

Catálogo na fonte
Universidade Federal de Alagoas
Biblioteca Central

Bibliotecário Responsável: Valter dos Santos Andrade

- A447p Almeida, João Paulo Felix Augusto de.
O papel relativo dos refúgios glaciais e das barreiras geográficas atuais na diversificação de um Squamata fossorial na Mata Atlântica brasileira/ João Paulo Felix Augusto de Almeida. – 2017.
57 f. : il.
- Orientadora: Tamí Mott.
Coorientador: Marcelo José Sturaro.
Coorientador: Ricardo José Pereira.
- Dissertação (Mestrado em Diversidade Biológica e Conservação nos Trópicos) – Universidade Federal de Alagoas. Instituto de Ciências Biológicas e da Saúde. Maceió, 2017.
- Inclui bibliografias.
1. Diversidade genética. 2. Squamata. 3. *Amphisbaena pretei*.
4. Nicho ecológico - Modelagem. I. Título.
- CDU: 598.113.5

Folha de aprovação

João Paulo Felix Augusto de Almeida

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BARREIRAS GEOGRÁFICAS ATUAIS NA DIVERSIFICAÇÃO DE
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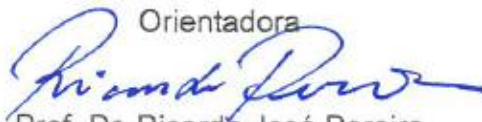
Dissertação apresentada ao Programa de Pós-Graduação em Diversidade Biológica e Conservação nos Trópicos, Instituto de Ciências Biológicas e da Saúde, Universidade Federal de Alagoas, como requisito para obtenção do título de Mestre em CIÊNCIAS BIOLÓGICAS, área de concentração em Conservação da Biodiversidade Tropical.

Dissertação aprovada em 21 de fevereiro de 2017.



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MACEIÓ - AL

Fevereiro / 2017

AGRADECIMENTOS

A toda minha família, especialmente minha mãe, e meus amigos pelo apoio constante em todos os momentos.

A todos os amigos da minha turma de graduação, especialmente Thamires, Larissa, Sarah, Nanda, Nanda Alves, Ana, Jacque, Evys, Elidiane, Lara, Lucas, Renato, Jamille, Isabela, Isabela, Lauro, Dani, Jadson e Thiago; que direta ou indiretamente contribuíram para meu crescimento pessoal e profissional. Pelo compartilhamento de tantos momentos bons e aqueles que não foram tão bons, mas que certamente nos ajudaram a crescer um pouco mais. Pelas conversas e momentos incríveis e inspiradores. Aprendi muito com vocês durante o tempo que compartilhamos, agradeço profundamente.

A todos meus amigos e companheiros de laboratório; Larissa, Luana, Marcos, Jessika, André e Adel; por toda experiência compartilhada no laboratório e fora dele. Por todas as conversas e suporte nos momentos de desespero acadêmico (ok, um pouco de exagero aqui.... ou não), pela ajuda nos procedimentos de laboratório, pela companhia nos intervalos para o café... ahh café, o que seria de nós sem você? Desde que você chegou no lab você também desempenhou um papel importante no nosso desenvolvimento. Você nos reuniu e permitiu que nós compartilhássemos histórias de vida e muitas vezes descobríssemos aquela solução improvável para os problemas acadêmicos que nos apareciam, ahhh... Enfim, de volta aos amigos de laboratório (será que já estou considerando o café um companheiro de laboratório?... é possível), obrigado por tudo! Aprendi bastante com vocês também. Vocês certamente fizeram essa jornada mais divertida!

A todos os amigos do mestrado; Gustavo, Lays, Waltyane, Bruno, Norah e Edinir; pela parceria e incentivos durante esses dois anos.

A minha orientadora, profa. Dra. Tamí Mott, pelas orientações e todos os ensinamentos não só neste estudo, mas desde que me juntei ao laboratório há seis anos. Agradeço também a meus co-orientadores, Dr. Ricardo Pereira e Dr. Marcelo Sturaro, pelas orientações e todo o auxílio no desenvolvimento deste estudo.

Ao PPG DIBICT e a todos os professores do programa, pelos ensinamentos e pela oportunidade de continuar meu crescimento acadêmico.

A Julliene, aquela pessoa que sempre nos salva nos momentos de necessidade e burocracias institucionais.

Por fim, a CAPES, pelo financiamento durante esses dois anos de mestrado e consequentemente pela oportunidade de aprimoramento acadêmico.

“Everyone you will ever meet knows
something you don’t”

Bill Nye

RESUMO

A região tropical abriga uma biodiversidade extraordinária, porém os processos evolutivos responsáveis por esse padrão permanecem obscuros. Diferentes fatores foram elencados como responsáveis pela diversificação das espécies nessa região. Aqui, testamos o papel relativo de dois desses fatores, as oscilações climáticas do Pleistoceno e a presença do rio São Francisco na estruturação morfológica e genética de *Amphisbaena pretrei*, um anfisbenídeo largamente distribuído na Mata Atlântica que possui um estilo de vida fossorial, morfologia conservada e baixa vagilidade. Os principais objetivos deste estudo foram (1) identificar áreas climaticamente estáveis adequadas para a ocorrência de *A. pretrei* e (2) avaliar se os padrões de diversidade morfológica e genética da espécie estão associados com essas áreas de adequabilidade ambiental ou com a posição do rio São Francisco. O mapa de estabilidade climática mostra que desde 21 mil anos atrás há uma grande área de adequabilidade ambiental para a espécie que se inicia ao sul do rio São Francisco e se estende até o extremo norte do nordeste do Brasil. Por sua vez, os dados morfológicos mostram um grupo distinto no extremo sul da distribuição da espécie, sugerindo que nesta localidade uma diversificação morfológica já ocorreu. Análises filogenéticas sugerem que a diversificação na espécie começou há pelo menos 1,31 milhões de anos atrás e uma forte diferenciação genética relacionada à distância geográfica e a presença do rio São Francisco. Esses resultados sugerem que o processo de diversificação em *A. pretrei* iniciou durante o Pleistoceno, provavelmente devido às oscilações climáticas, e continua no presente devido às barreiras atuais.

Palavras-chave: Diversidade genética. América do Sul. *Amphisbaena*. Morfologia. modelagem de nicho ecológico.

ABSTRACT

The tropical region harbors an extraordinary biodiversity, but the evolutionary processes responsible for this pattern remain obscure. Several factors were suggested to play a role in the species diversification in this region. Herein, we test the relative role of the Pleistocene climate oscillations as well as the presence of the São Francisco River on the morphological and genetic structure of *Amphisbaena pretrei*, an amphisbaenid widely distributed in the Atlantic Forest with a fossorial lifestyle, conserved morphology and low vagility. The goals of this study were (1) to identify climatically stable areas suitable for the occurrence of *A. pretrei* and (2) to assess whether patterns of morphological and genetic diversity of the species are associated with these areas of environmental suitability or with the São Francisco River. The climatic stability map shows that since 21 thousand years ago there is a large area of environmental suitability for the species that extends from the south of the São Francisco River until the northernmost portion of northeastern Brazil. The morphology data show a distinct group in the southernmost part of the species distribution, suggesting that in this locality morphological diversification has already occurred. Phylogenetic analyses suggest that diversification within this species started at least 1.31 million years ago. Furthermore, there is a strong genetic differentiation related to geographic distance and the presence of the São Francisco River. These results suggest that the process of diversification in *A. pretrei* began during the Pleistocene, probably due to the climatic oscillations, and continues in the present due to current barriers.

Keywords: Genetic diversity. South America. Amphisbaenia. Morphology. ecological niche modeling.

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1 APRESENTAÇÃO

A região tropical abriga uma biodiversidade ímpar (HILLEBRAND, 2004) e vários mecanismos já foram propostos para explicar a origem e manutenção desta diversidade (GASTON, 2000; MORITZ et al., 2000). A hipótese dos refúgios pleistocênicos (HAFFER, 1969), por exemplo, é uma ideia frequentemente utilizada para explicar a extraordinária diversidade encontrada na América do Sul (TURCHETTO-ZOLET et al., 2013). Esta hipótese foi proposta com base em estudos na Amazônia (HAFFER, 1969), entretanto, estudos na Mata Atlântica têm encontrado que as oscilações climáticas do Pleistoceno também têm contribuído para a diversificação das espécies nesse bioma (CARNAVAL et al., 2009; CARNAVAL; MORITZ, 2008; RIBEIRO et al., 2011). A hipótese de que os rios podem atuar como barreira promovendo a diversificação das espécies também foi inicialmente proposta para a Amazônia (WALLACE, 1854), no entanto alguns estudos na Mata Atlântica apontam que o rio São Francisco pode exercer um papel importante no processo de diversificação das espécies dessa região (e.g. CAZÉ et al., 2016; PELLEGRINO et al., 2005).

Com apenas aproximadamente 11% de sua área original restante (RIBEIRO et al., 2009), a Mata Atlântica é uma ecorregião altamente diversa e sob forte influência antrópica, sendo considerado um dos *hotspots* mundiais de biodiversidade (MYERS et al., 2000). O crescente número de estudos filogeográficos com diversos grupos taxonômicos distribuídos na Mata Atlântica (CARNAVAL et al., 2009; CAZÉ et al., 2016; D'HORTA et al., 2011; MALDONADO-COELHO, 2012; PELLEGRINO et al., 2005; THOMÉ et al., 2010; VALDEZ; D'ELÍA, 2013) é extremamente importante, pois através da obtenção da distribuição espacial das linhagens genealógicas é possível inferir os processos evolutivos responsáveis pela diversificação das espécies (AVISE, 2000). Além disso, áreas de maior concentração da diversidade genética e de espécies vêm sendo identificadas e estas informações são extremamente importantes para guiar medidas de conservação apropriadas para esse bioma altamente ameaçado (CARNAVAL et al., 2014). No entanto, os fatores históricos sugeridos como responsáveis pelo isolamento das linhagens genealógicas nessa região (como oscilações climáticas do Pleistoceno e rios como barreira) não são mutuamente exclusivos e podem contribuir simultaneamente no processo de diversificação das espécies (MORITZ et al., 2000). Dessa maneira, estudos de

diferentes grupos animais com diferentes graus de diversidade e capacidade de dispersão são extremamente importantes para uma melhor compreensão de como esses processos afetam as espécies distribuídas no bioma.

Amphisbaena pretrei Duméril & Bibron, 1839 é um réptil Squamata de hábitos fossoriais com ampla distribuição na costa leste do Brasil, estando amplamente associada com áreas de Mata Atlântica (UETZ; FREED; HOSEK, 2017). Sua distribuição se estende do sudeste do Brasil no Estado de Minas Gerais cruzando o rio São Francisco até o nordeste brasileiro no Estado do Ceará através de regiões da Mata Atlântica propostas como historicamente estáveis (CARNAVAL; MORITZ, 2008). Sua baixa capacidade de dispersão ligada ao seu estilo de vida fossorial bem como sua distribuição associada à Mata Atlântica brasileira fazem de *A. pretrei* um excelente modelo para estudar os processos históricos de diversificação do bioma através de uma abordagem integrativa entre modelagem de nicho ecológico e análises morfológicas e moleculares.

Esta dissertação inicia-se com um capítulo de revisão de literatura, onde são discutidas as principais temáticas aqui abordadas. Segue-se um capítulo principal contendo o artigo formatado para o periódico *Zoological Journal of the Linnean Society*, que expõe as motivações e metodologias utilizadas no desenvolvimento deste estudo e os resultados obtidos a partir dessas análises bem como sua contextualização diante da literatura vigente. Por fim, são delineadas as conclusões gerais obtidas a partir deste estudo.

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2 REVISÃO DE LITERATURA

2.1 Diversificação de espécies nos neotrópicos

Entender como as espécies estão distribuídas e quais interações regem essa distribuição é um dos principais objetivos da ecologia (BEGON et al. 2007). Entretanto, entender os padrões de distribuição da biodiversidade não é uma tarefa simples. É de comum conhecimento que a biodiversidade não é distribuída de maneira homogênea na superfície da terra, sendo mais concentrada na região tropical (HILLEBRAND, 2004). Várias hipóteses já foram propostas para tentar explicar os processos que regem a formação e distribuição das espécies (GASTON, 2000; MORITZ et al., 2000), estando elas ligadas a fatores macroecológicos mais abrangentes, por exemplo, a distribuição de algumas espécies de répteis está altamente relacionada com a temperatura ambiental e com o nível de energia que é recebida pelo ambiente, enquanto a distribuição de várias espécies de anfíbios é afetada principalmente pelos níveis de precipitação (HAWKINS et al., 2003). Além disso, fatores locais e regionais também são invocados como explicação para a distribuição das espécies, por exemplo, a conservação de nicho tem papel essencial na determinação da tolerância ambiental de determinada espécie, da mesma maneira a capacidade de dispersão de uma espécie ou a competição interespecífica podem explicar porque uma espécie não está presente em uma região que tem condições adequadas para sustentá-la (WIENS; DONOGHUE, 2004).

A influência dos grandes rios na distribuição e diversificação das espécies foi inicialmente proposta por Wallace (1854) para explicar o padrão de distribuição e diversificação de primatas na Amazônia. Segundo essa teoria, os rios atuam como uma barreira à dispersão dos indivíduos e assim desempenham um papel importante no isolamento de populações e geração de biodiversidade por meio do processo de especiação alopátrica. Esse padrão de distribuição deixa sinais moleculares particulares, por exemplo, é esperado que exista uma maior divergência genética entre populações em margens opostas do rio, bem como que essas sejam reciprocamente monofiléticas. Por outro lado, não se espera encontrar sinais de expansão populacional ou mesmo diferenças na diversidade genética das populações isoladas (MORITZ et al., 2000). Porém, a influência do rio no isolamento das linhagens pode variar ao longo de seu curso devido a alterações nas dimensões do rio (MORITZ et al., 2000). Além disso, a dinâmica histórica de mudanças no

próprio curso do rio também deve ser levada em consideração, uma vez que a posição de um determinado curso fluvial pode variar ao longo do tempo (BERMINGHAM; MORITZ, 1998; MORITZ et al., 2000).

Vários estudos têm mostrado que os grandes rios da América do Sul têm influenciado o processo de diversificação das espécies nessa região (CAZÉ et al., 2016; CHEVIRON; HACKETT; CAPPARELLA, 2005; PELLEGRINO et al., 2005; RIBAS et al., 2012). Por outro lado, alguns estudos sugerem que os rios podem não exercer tamanha influência sobre algumas espécies (MALDONADO-COELHO, 2012; PATTON; SILVA; MALCOLM, 1994). Além dos fatores históricos relacionados a essas discrepâncias, como a própria dinâmica dos rios, essas diferentes respostas também devem ser avaliadas do ponto de vista dos organismos sob análise, uma vez que diferentes organismos podem responder de maneira diferente aos processos históricos estudados devido a suas características biológicas particulares (AVISE, 2000). Por exemplo, um rio pode funcionar como barreira ao fluxo gênico em uma espécie com relativamente baixa capacidade de dispersão, como uma perereca, mas não para uma espécie de com alta capacidade de dispersão como, por exemplo, certas espécies de aves. Dessa maneira, diferentes organismos co-distribuídos podem sugerir histórias evolutivas diferentes para determinada região se essas características não forem levadas em consideração.

Outro fator apontado como um dos principais responsáveis pela diversificação de espécies nos neotrópicos é a influência das oscilações climáticas durante o Pleistoceno (CARNAVAL; MORITZ, 2008; HAFFER, 1969; VANZOLINI; WILLIAMS, 1970). Segundo essa teoria, as oscilações climáticas durante esse período levaram à retração das florestas formando ilhas florestais isoladas por vegetação aberta (savana). Essas ilhas florestais funcionavam então como refúgio para espécies dependentes de floresta. Dessa maneira, o processo de retração e expansão das florestas, relacionado às oscilações climáticas desse período, pode ter atuado isolando populações e promovendo a quebra do fluxo gênico, conseqüentemente desencadeando eventos de especiação alopátrica. Na hipótese dos refúgios, é esperado um sinal de expansão populacional nas populações que atualmente ocupam áreas fora dos refúgios florestais, uma vez que estas novas áreas foram ocupadas provavelmente após o período de oscilações climáticas, quando as áreas florestadas voltaram a se expandir. É esperado ainda que, se os indivíduos de uma determinada espécie ficarem isolados nos refúgios florestais, após várias gerações,

essas populações subdivididas formem grupos monofiléticos congruentes com seu isolamento nesses refúgios, isto é, que correspondam espacialmente à localização dos refúgios. Ademais, uma maior diversidade genética é esperada nos grupos que ocupam áreas de refúgios com relação àquelas que ocupam áreas fora dos refúgios (MORITZ et al., 2000).

Essa ideia foi inicialmente fundamentada na análise da distribuição dos níveis de precipitação na Amazônia juntamente com a análise da distribuição espacial de espécies de aves amazônicas (HAFFER, 1969). Foi proposto que, durante o período frio e seco dos eventos glaciais, algumas áreas no bioma teriam mantido um nível de precipitação alto o suficiente para manter condições adequadas para a manutenção de áreas florestadas. Ao mesmo tempo, Haffer (1969) também observou que a distribuição de alguns grupos de aves na Amazônia não se sobrepõe, ou seja, a distribuição de um determinado grupo se expande apenas até certa região onde se inicia a distribuição de outro grupo. Baseado nessas evidências indiretas, Haffer (1969) propôs as localizações dos refúgios pleistocênicos na Amazônia.

Alguns estudos publicados após a proposição de Haffer (1969) encontraram suporte à sua hipótese (e.g. BROWN; SHEPPARD; TUNER, 1974; GARZÓN-ORDUÑA; BENETTI-LONGHINI; BROWER, 2014; MAYR; O'HARA, 1986). No entanto, muito tem se debatido sobre a validade dessa hipótese na Amazônia. Alguns estudos sugerem que na verdade eventos orogênicos em períodos pré-pleistocênicos, como, por exemplo, o soerguimento dos Andes, teriam sido responsáveis pela origem da diversidade encontrada no bioma (HOORN et al., 2010). Além disso, análises de dados palinológicos e de espeleotemas sugerem que, enquanto houve uma variação climática mais acentuada na porção leste da Amazônia, a porção oeste do bioma se manteve bem mais estável ao longo dos últimos 250 mil anos (CHENG et al., 2013; COLINVAUX; DE OLIVEIRA, 2000). Ao mesmo tempo, os maiores níveis de diversidade encontrados na porção oeste do bioma, sugerem que as oscilações climáticas tiveram influência reduzida no processo de diversificação das espécies (CHENG et al., 2013). Da mesma maneira, alguns estudos em genética de populações de aves e mamíferos com distribuição na Amazônia não têm encontrado suporte a hipótese dos refúgios (CHEVIRON; HACKETT; CAPPARELLA, 2005; LESSA; COOK; PATTON, 2003; MALDONADO-COELHO et al., 2013). Ao invés disso, a influência dos Andes bem como a presença

dos rios foram sugeridos como principais fatores explicando a diversidade genética das espécies.

Ambas as hipóteses abordadas aqui (rios e refúgios) sugerem um processo de especiação alopátrica atuando na diversificação da biodiversidade neotropical, entretanto, elas diferem quanto à assinatura molecular esperada. Embora esses processos não sejam mutuamente exclusivos, a contribuição relativa deles para o processo de diversificação das espécies ainda não é claro. A maior parte dos estudos tem como foco espécies com capacidade de dispersão relativamente alta, como aves e mamíferos ou com divergência morfológica marcante, como anfíbios. Estudos com espécies de ampla distribuição, mas com baixa capacidade de dispersão e morfologia conservada pode ajudar a entender como esses processos afetam o processo de diversificação das espécies.

2.2 Processos promotores de diversidade na Mata Atlântica

Carnaval e Moritz (2008) sugeriram, através de modelos paleoclimáticos e da comparação com padrões filogeográficos de espécies codistribuídas na Mata Atlântica brasileira, a existência de áreas que podem ter funcionado como refúgios para a biodiversidade durante as oscilações climáticas do Pleistoceno, gerando diversificação das linhagens e endemismo nessa região. Duas áreas foram sugeridas como regiões de estabilidade climática, ou seja, que mantiveram condições de abrigar áreas florestadas durante o Pleistoceno. Uma área ao norte do rio São Francisco, denominada refúgio de Pernambuco, que engloba o que hoje corresponde aos Estados de Alagoas e Pernambuco e uma área de maiores dimensões localizada ao sul do rio São Francisco, denominada refúgio da Bahia, ocupando boa parte da região costeira do Estado da Bahia (CARNAVAL; MORITZ, 2008). Subsequentemente, uma terceira área foi proposta no sudeste brasileiro (CARNAVAL et al., 2009).

Estudos sobre a diversidade genética de plantas e de algumas espécies animais vêm demonstrando que a estruturação genética bem como o tempo de divergência entre as linhagens recuperadas sugerem que elas provavelmente divergiram em algum momento durante o Pleistoceno (aproximadamente, 2,5-0,2 milhões de anos) (AMARAL et al., 2013; CABANNE et al., 2008; CARNAVAL et al., 2009; D'HORTA et al., 2011; FITZPATRICK et al., 2009; MORAES-BARROS et al., 2006; RIBEIRO et al., 2011). Além disso, algumas dessas linhagens têm exibido sinais de expansão

populacional, sugerindo a expansão da distribuição dessas espécies, possivelmente como resultado da colonização de novas áreas a partir de áreas de refúgio, o que é esperado em espécies que ficaram isoladas nesses refúgios pleistocênicos. No entanto, outros estudos sugerem que os rios que cruzam a Mata Atlântica, por exemplo, o rio São Francisco e rio Doce, atuam como barreira ao fluxo gênico entre populações, sendo fatores importantes para a diversificação das espécies nessa região (e.g. CAZÉ et al., 2016; PELLEGRINO et al., 2005).

Alguns estudos sugerem que o rio São Francisco já passou por diversas mudanças durante sua história evolutiva, desde um curso inicial que desembocava no Atlântico equatorial (NASCIMENTO et al., 2013; POTTER, 1997), passando por uma fase endorreica que gerou o acúmulo de areia que possibilitou a formação das chamadas dunas do São Francisco (RODRIGUES, 1993, 1996). Subseqüentemente, adotando seu curso atual e durante esse processo atuou isolando populações de espécies nessas áreas de dunas, bem como em outras áreas dessa região, atuando dessa maneira como barreira ao fluxo gênico e gerando especiação alopátrica (PASSONI; BENOZZATI; RODRIGUES, 2008; RODRIGUES, 1996; SIEDCHLAG et al., 2010; WERNECK et al., 2012, 2015).

Na região norte da Mata Atlântica, as áreas de estabilidade climática propostas por Carnaval e Moritz (2008) ficam em margens opostas do rio São Francisco, fazendo com que a quebra filogeográfica esperada seja a mesma tanto para a hipótese dos refúgios quanto para a hipótese do rio como barreira. Nesse sentido, a análise e comparação de diferentes grupos taxonômicos codistribuídos bem como uma adequada análise de dados, incluindo estimativas de tempo de divergência, análises populacionais e de coalescência, são extremamente importantes para verificar como esses eventos evolutivos podem ter influenciado a biota dessa região.

2.3 Diversidade genética em *Amphisbaenia* Gray, 1844

As anfisbênias formam um clado de aproximadamente 198 espécies (UETZ; FREED; HOSEK, 2017) de répteis Squamata fossoriais e ápodas (com poucas exceções) alocadas em seis famílias atualmente reconhecidas: Amphisbaenidae, Bipedidae, Blanidae, Cadeidae, Rhineuridae e Trogonophidae (VIDAL; HEDGES, 2009; UETZ; FREED; HOSEK, 2017). Possuem uma distribuição predominantemente tropical e subtropical, ocorrendo no Oriente Médio, Europa e África (Amphisbaenidae, Blanidae e Trogonophidae), além de Américas do Norte,

Central e do Sul (Amphisbaenidae, Bipedidae, Cadeidae e Rhineuridae) (GANS, 2005; UETZ; FREED; HOSEK, 2017). Amphisbaenidae é a família mais especiosa englobando aproximadamente 90% (178 espécies) da diversidade do grupo, possuindo distribuição nos continentes africano e sul-americano (UETZ; FREED; HOSEK, 2017).

As espécies da subordem Amphisbaenia possuem uma morfologia altamente adaptada ao seu estilo de vida fossorial, com corpo alongado e ausência de membros (exceto nas três espécies da família Bipedidae), bem como uma estrutura cranial adaptada à escavação (GANS, 1978). Existem quatro morfotipos craniais em Amphisbaenia: arredondado, presente em Amphisbaenidae e Blanidae; em forma de pá (achatado dorsoventralmente), presente em Amphisbaenidae e Rhineuridae; em ângulo concavo, presente em Trogonophidae; e em forma de quilha, presente em Amphisbaenidae (KEARNEY, 2003). Cada morfotipo cranial está relacionado com um hábito de escavação particular (GANS, 1968) e evidências moleculares sugerem que o mesmo morfotipo evoluiu independentemente em várias linhagens de Amphisbaenia (KEARNEY; STUART, 2004; MOTT; VIEITES, 2009).

Eventos de evolução homoplástica são muito comuns em Amphisbaenia (KEARNEY, 2003; KEARNEY; STUART, 2004; MACEY et al., 2004; MOTT; VIEITES, 2009), e a utilização de caracteres morfológicos homoplásticos na taxonomia do grupo (formato da cabeça, por exemplo) resultou em agrupamentos artificiais que mascaravam suas relações filogenéticas (Kearney, 2003; Kearney e Stuart, 2004; Mott e Vieites, 2009). Dessa maneira, abordagens moleculares têm sido extremamente importantes no estudo da diversidade de Amphisbaenia (KEARNEY; STUART, 2004; MOTT; VIEITES, 2009; VIDAL et al., 2008).

O hábito fossorial desse clado dificulta o seu encontro na natureza e aspectos básicos de sua biologia ainda são desconhecidos (ANDRADE; NASCIMENTO; ABE, 2006). Da mesma maneira, a coleta e obtenção de amostras de tecidos desses animais é um desafio, dificultando a implementação de uma abordagem filogeográfica que, por sua vez, demanda uma amostragem ampla e espacialmente bem distribuída. Assim, poucos estudos filogeográficos foram realizados com anfisbênias. A maior parte deles se concentra na região do norte da África e sul da Europa e tem como foco as espécies alocadas no gênero *Blanus* (Blanidae) (ALBERT; ZARDOYA; GARCÍA-PARÍS, 2007; SAMPAIO et al., 2014; VACONCELOS; HARRIS; CARRETERO, 2006). A exceção é o trabalho de

Mulvaney e colaboradores (2005), que estudaram a espécie *Rhineura floridana*, única espécie atual do gênero *Rhineura* (Rhineuridae) e que têm ocorrência restrita aos Estados Unidos da América.

A diversidade genética dentro e entre essas espécies foi avaliada através da implementação de marcadores mitocondriais e nucleares e da avaliação de um n amostral bastante variável, desde de apenas 18 indivíduos em *R. floridana* (MULVANEY et al., 2005), até 72 indivíduos em *Blanus* (ALBERT; ZARDOYA; GARCÍA-PARÍS, 2007). No entanto, apesar da limitação amostral imposta pelo hábito de vida desses animais, esses estudos foram capazes de revelar uma alta estruturação genética nas espécies estudadas, sendo esse efeito atribuído a processos orogênicos e climáticos. É interessante ressaltar que apesar das diferentes escalas e amostragens utilizadas nesses estudos, todos eles encontraram estruturação genética relacionada à distribuição espacial das espécies estudadas. Alguns desses resultados revelaram a existência de diversidade críptica e algumas linhagens foram descritas como novas espécies (ALBERT; ZARDOYA; GARCÍA-PARÍS, 2007; SAMPAIO et al., 2014).

Até este momento, não existem publicações sobre filogeografia com espécies da família Amphisbaenidae, a família mais especiosa de Amphisbaenia (90% da diversidade atual do clado) e mais amplamente distribuída (América do Sul, Central e África, GANS, 2005). Entretanto, alguns estudos já chamaram a atenção para a variabilidade morfológica e genética presente em algumas espécies com ampla distribuição na América do Sul, como *Amphisbaena vermicularis* (GANS, 1965), *A. alba* e *A. microcephala* (MOTT; VIEITES, 2009). Isso sugere que essas espécies amplamente distribuídas podem apresentar diversas linhagens geograficamente estruturadas ou mesmo se tratarem de complexos de espécies.

2.4 *Amphisbaena pretrei* Duméril & Bibron, 1839

Amphisbaena pretrei é um anfisbenídeo com ampla distribuição na porção leste do Brasil, podendo ser encontrada desde o norte do estado de Minas Gerais estendendo-se aproximadamente 1.500 km ao norte até ao estado do Ceará (Figura 1; UETZ; FREED; HOSEK, 2017). A maioria dos estudos que citam *A. pretrei* são inventários de espécies locais: Santo Inácio, Bahia (RODRIGUES, 1996); Chapada do Araripe, Ceará (RIBEIRO et al., 2008); Planalto da Ibiapaba, Ceará (Loebmann &

Haddad, 2010); Abaeté, Bahia (DIAS; ROCHA, 2014); Camaçari, Lauro de Freitas, Mata de São João e Salvador, todos na Bahia (FREITAS, 2014).

Desde sua descrição, o único estudo mais abrangente realizado com *A. pretrei* foi a redescritção da mesma feita por Gans (1965). Nesse estudo, o autor avaliou principalmente a variabilidade morfológica da espécie e sua relação com as três espécies que se encontravam em sua sinonímia: *A. brachyura* (Amaral, 1932), *A. subocularis* (Peters, 1878) e *A. leucocephala* Peters, 1878. Gans (1965) notou que *A. leucocephala* era distinta das outras formas de *A. pretrei* por ter uma coloração predominantemente amarela na região cefálica, pela disposição das escamas parietais, pelo baixo número de anéis caudais e segmentos em um anel no meio do corpo, além do alto número de poros pré-cloacais. A espécie foi então elevada ao status de “*species iquirenda*” (espécie que requer uma melhor análise para confirmação) e caracterizada principalmente por ter 233-240 anéis corporais, 26-27 anéis caudais, 18-20 segmentos dorsais e 21-22 segmentos ventrais em um anel no meio do corpo, 12 poros pré-cloacais e sítio autotômico presente no sexto anel caudal (Gans, 1965). A validade taxonômica de *A. leucocephala* foi corroborada posteriormente por meio de uma comparação com espécimes de *A. pretrei* através de evidências morfológicas, osteológicas e moleculares (DAL VECHIO et al., 2016).

Amphisbaena pretrei, por sua vez, foi caracterizada principalmente por ter 231-255 anéis corporais, 22-26 anéis caudais, 20-27 segmentos dorsais e 22-28 segmentos ventrais em um anel no meio do corpo, cinco a nove poros pré-cloacais sequenciais e sítio autotômico presente no quinto ou sexto anel caudal (Gans, 1965). Além de redescrever a espécie, Gans (1965) sugeriu que existem variações morfológicas relacionadas à distribuição geográfica em *A. pretrei*. Por exemplo, espécimes provenientes da porção sul da distribuição da espécie (Bahia e Minas Gerais) apresentaram um número de anéis corporais no limite inferior da amplitude de variação dessa característica, porém alguns espécimes ao norte da distribuição também apresentaram baixo número de anéis corporais. Dessa maneira, o autor ressaltou que essas variações eram fracas e que poderiam ser resultado de vieses de coleta e, por isso, sua amostragem no momento (40 espécimes) era insuficiente para estabelecer qualquer padrão de estruturação espacial.

Figura 1 – Distribuição conhecida de *Amphisbaena pretrei*. Registros baseados em dados de literatura e coleções científicas. AL – Alagoas, BA – Bahia, CE – Ceará, MG – Minas Gerais, PB – Paraíba, PE – Pernambuco, SE – Sergipe. Gans, 1965: 1 – Maceió, 2 – Queimadas, 3 – Campina Grande, 4 – João Pessoa, 5 – Igarassu, 6 – Pesqueira, 7 – Poção, 8 – Recife, 9 – Lagoa Papari, Nísia Floresta, 10 – Natal, 11 – Maruim; Vanzolini, 1974: 12 – Agrestina, 13 – Ponta de Pedras, Goiana; Vanzolini, 1981: 14 – Arajara, Chapada do Araripe; Rodrigues, 1996: 15 – Santo Inácio; Ribeiro et al., 2008: 16 – Chapada do Araripe; Loebmann & Haddad, 2010: 17 – Planalto de Ibiapaba; Dias & Rocha, 2014: 18 – Abaeté, Salvador; Freitas, 2014: 19 – Camaçari, 20 – Lauro de Freitas, Salvador, 21 – Mata de São João; Roberto et al., 2014: 22 – Barbalha, 23 – Crato, 24 – Lagoa dos Gatos, 25 – Paracuru, 26 – Quebrangulo; MCNR: 27 – Itambé, 28 – Usina Hidrelétrica Irapé, Grão Mogol; MTR: 29 – Morro do Chapéu, 30 – Mucugê, 31 – Wenceslau Guimarães; MUFAL: 32 – Coruripe, 33 – Flexeiras, 34 – Murici, 35 – Paripueira, 36 – Rio Largo, 37 – Traipu, 38 – União dos Palmares; UFSE: 39 – Aracaju, 40 – Brejo Grande, 41 – Capela, 42 – Indiaroba, 43 – Malhador, 44 – Santa Luzia do Intahi, 45 – São Cristóvão, 46 – Simão Dias.

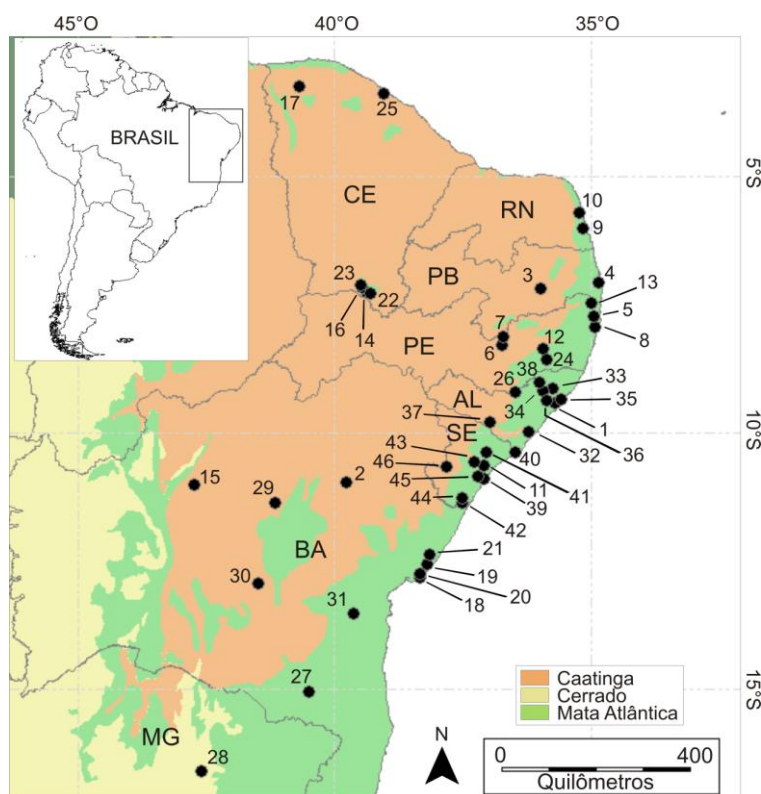


Figura 2 – Exemplar de *Amphisbaena pretrei* proveniente do município de Rio Largo, Alagoas, MUFAL12205.



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3. UNDER THE BRAZILIAN ATLANTIC FOREST: THE RELATIVE ROLE OF GLACIAL REFUGIA AND CURRENT BARRIERS IN THE DIVERSIFICATION OF A FOSSORIAL SQUAMATE

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Abstract

The tropical region harbors the highest number of species in the world, but the evolutionary processes generating that diversity remain obscure. Herein, we test the relative role of the Pleistocene climate oscillations and the presence of the São Francisco River on *Amphisbaena pretrei*, an amphisbaenid widely distributed in the Atlantic Forest, with a fossorial lifestyle, conserved morphology and low vagility. The goals of this study were (1) to identify climatically stable suitable areas for the occurrence of *A. pretrei* and (2) to evaluate whether patterns of morphological and genetic diversity are associated with these areas. The stability model, resulted from the intersection between the models from three different time periods, exhibited a large suitable area located throughout opposite margins of the São Francisco River. Morphology data revealed a distinct group, suggesting that morphological diversification has already occurred. Phylogenetic analyses suggests that diversification within this species started at least 1.31 million years ago, preceding the current location of the river. Nevertheless, genetic differentiation was not correlated with climate, instead, it was strongly correlated with the presence of the São Francisco River and geographic distances, suggesting that current barriers also contribute for diversification in this species.

Keywords: Genetic diversity, South America, Amphisbaenia, morphology, ecological niche modeling.

3.1 INTRODUCTION

The tropical region harbors the highest number of species in the world and several hypotheses have been proposed to explain this remarkable diversity (Moritz *et al.*, 2000; Hillebrand, 2004; Mittelbach *et al.*, 2007). These hypotheses try to explain how historical habitat changes have shaped species diversity through time. They rely mainly on wide macroecological factors, such as temperature and productivity variation, or more specific ones such as competition (Wiens & Donoghue, 2004; Antonelli & Sanmartín, 2011; Wiens, 2015). However, the way in which these factors affect species is largely linked with the species biology, notably its habits, resilience and dispersion capability (Avice, 2000). Nonetheless, since several of these factors and processes are not mutually exclusive, their influence on the species diversification process is still debated topic.

The Pleistocene refugia hypothesis suggests that the climate oscillations in this period could have played a significant role as a driver of species diversification (Haffer, 1969; Haffer, 2008). According to this hypothesis, the dry and cold climate of glacial periods promoted forest contractions, creating islands of forest surrounded by dry open vegetation. According to Haffer (1969), these islands of forest formed climatically stable refugia in areas with higher precipitation, which allowed forest-dependent species to persist, compared to the drier matrix composed by savanna-like vegetation. Populations of forest-dependent species were geographically isolated during glacial periods, resulting in allopatric divergence and speciation (Haffer, 1969). Consequently, species or populations that have undergone such historical isolation process are expected to exhibit a higher degree of genetic structure between separated refugia than within the same refugium. Furthermore, the expansion of forested areas in subsequent warmer periods would allow these populations to expand their range leaving a genetic signature of population expansion (Moritz *et al.*, 2000).

The Pleistocene refugia hypothesis was initially applied in the neotropics to explain diversification in Amazonian birds (Haffer, 1969). However, recent studies in the Brazilian Atlantic Forest (BAF) have found support to the refugia hypothesis (e.g. Cabanne *et al.*, 2007, 2008; Carnaval & Moritz, 2008; Carnaval *et al.*, 2009). Based on paleomodelling analysis coupled with comparison of patterns of genetic diversity across several animal species, Carnaval & Moritz (2008) suggested two areas in the

northern portion of the BAF have remained stable during the climatic oscillations of the Pleistocene and therefore acted as refugia for forest-dependent species. These areas were located at opposite margins of the São Francisco River (SFR), one relatively small area situated at north of the SFR called Pernambuco refugium, and a larger area at south of the SFR called Bahia refugium. Studies on genetic diversity of plants (Ledru *et al.*, 2007; Palma-Silva *et al.*, 2009; Ribeiro *et al.*, 2011), frogs (Carnaval *et al.*, 2009; Fitzpatrick *et al.*, 2009), birds (Cabanne *et al.*, 2008; D'Horta *et al.*, 2011; Amaral *et al.*, 2013; Batalha-Filho & Miyaki, 2016) and mammals (Moraes-Barros *et al.*, 2006; Valdez & D'Elía, 2013) that occur in BAF have demonstrated that these organisms exhibit a genetic pattern that is congruent with their isolation in past refugia and also signals of population expansion, which support the hypothesis of isolation in past refugia.

The validity of this hypothesis to Amazonia has been challenged by palynological data that has indicated that forested areas were probably more persistent during the Pleistocene than what was suggested (Colinvaux & De Oliveira, 2000). Moreover, studies on genetic diversity of high dispersal species such as birds (Aleixo, 2004; Cheviron *et al.*, 2005; Maldonado-Coelho *et al.*, 2013) and mammals (Lessa, Cook, & Patton, 2003) revealed no support to the refugia hypothesis. Instead they present no signals of population expansion (Lessa *et al.*, 2003) and lower genetic diversity in presumed refugia areas (Maldonado-Coelho *et al.*, 2013). Therefore, they suggested that other factors such as current and long-standing geographic features (e.g. the major river systems) are acting as barrier to gene flow in this region. In the same way, patterns of genetic variation in some species in the BAF are better explained by geological events or current geographic barriers, such as the Doce River and the SFR, which are known to be barriers to the gene flow in several species (e.g. Rodrigues, 1996; Passoni *et al.*, 2008; Siedschlag *et al.*, 2010; Werneck *et al.*, 2012; 2015).

Although it is becoming clear that these hypotheses are not mutually exclusive, most studies so far have focused on species with relatively high dispersion capability, such as mammals and birds, or species that exhibit a more marked morphological or ecological divergence, such as amphibians. Studies on species with conserved morphology and low vagility, such as fossorial animals, are still scarce precluding interpretations on how these historical processes have affected diversification of species as a whole across the entire biome.

Herein we focus on *Amphisbaena pretrei* Duméril & Bibron, 1839, a fossorial squamate widely distributed throughout the east of Brazil, from Minas Gerais State in southeastern Brazil, to the Ceará State in northeastern Brazil (ca. 1,500km, Fig. 1, Uetz & Hosek, 2015). The distribution of this species is largely associated with the BAF, occurring in forested areas as well as in urban areas near forest fragments throughout the region of the proposed BAF refugia by Carnaval & Moritz (2008). Due to the highly selective pressure imposed by its constrained habitat bellow ground, morphological variation in amphisbaenian species is limited (Gans, 1978; Kearney, 2003; Kearney & Stuart, 2004; Mott & Vieites, 2009). Furthermore, because of this lifestyle, the collection of these animals is not an easy task and consequently there is still a lack of knowledge even on basic aspects of amphisbaenians biology (Andrade, 2006).

Nonetheless, some studies have shown that widely distributed amphisbaenians may exhibit some degree of morphological and genetic structure (Gans, 1965; Gans & Amdur, 1966; Mott & Vieites, 2009). Gans (1965), based on a sampling of 40 specimens, suggested some morphological variation within *A. pretrei* associated to its geographic distribution. However, due to the low sampling, he concluded that those observed variations could be result of sampling artifact.

In this way, the low vagility due to its underground lifestyle, its association with the BAF, and its already known signs of morphological variation, *A. pretrei* seems to be an interesting model to study historical processes in the BAF through its genetic and morphological signatures. Thus, the main goals of this study were (1) to identify climatically suitable areas for the occurrence of *A. pretrei* and (2) to evaluate whether morphological and genetic signatures in *A. pretrei* are associated with these suitable areas or with current geographic or ecologic barrier such as the SFR. If the genetic structure of *A. pretrei* was influenced by the climatic oscillations of the Pleistocene, the diversification processes within the species should take place during the Pleistocene. Furthermore, the pattern of genetic differentiation is expected to coincide with areas of historical habitat suitability. On the other hand, if current ecological barriers are playing a role on the genetic structure of *A. pretrei*, the diversification time would match the changes in the course of the SFR (400.000 year ago) and the genetic differentiation of the species would be related to current ecological suitability.

3.2 MATERIAL AND METHODS

3.2.1. ECOLOGICAL NICHE MODELLING (ENM)

Forty-six distribution records of *Amphisbaena pretrei* throughout its distribution were obtained from the literature and from specimens housed in the following Brazilian Herpetological collections: Museu de Ciências Naturais da Pontifícia Universidade Católica de Minas Gerais (MCNR), Museu de História Natural da Universidade Federal de Alagoas (MUFAL) and Universidade Federal de Sergipe (UFS). Field notes of Miguel Trefaut Rodrigues (MTR), Universidade de São Paulo, São Paulo, were also used. For records that were not georeferenced (77%) the centroid of the municipality was used as its geographical coordinates. Nineteen environmental at 10' spatial resolution variables were downloaded from WorldClim database (<http://worldclim.org>). All variables were cropped to fit the Atlantic Forest in order to avoid prediction errors caused by the size of the calibration area (see Giovanelli et al., 2010).

The reconstruction of current environmental suitability was performed using the machine-learning maximum entropy (MaxEnt) algorithm implemented in MaxEnt v 3.3.3 (Phillips *et al.*, 2006). MaxEnt is a presence-only algorithm that has been shown to have a good performance when compared with other commonly used algorithms (Elith *et al.*, 2006; Hernández *et al.*, 2006). Although the default configuration of MaxEnt was set based on the evaluation of the program performance using several different taxa and conditions, species-specific tuning may be desirable when dealing with a single or few species in order to avoid overfitting (i.e. when the model fits too closely to the data used to generate it) and consequently an unrealistic model (Phillips & Dudík, 2008; Anderson & Gonzalez, 2011). Herein, four parameters of the model were controlled to minimize overfitting: the sampling, the number of predictors and the “features” and “beta regularization” options on MaxEnt.

To avoid sampling bias, which may occur when some areas are more intensively sampled than others, the occurrence records included in the analysis were at least 10km apart. When two records fell within this range, the more precise one was kept (specific geographic coordinates over centroid coordinates). This spatial filtering resulted in the exclusion of three occurrence records. The 19 bioclim variables downloaded from Wordclim were tested for correlation using the Pearson correlation coefficient implemented in ENMTools v1.4.1 (Warren, Glor, & Turelli, 2010).

Whenever the variables were correlated ($r > 0.7$), the ones who represented extreme conditions were chosen, because they are potentially more important in delimiting the species distribution (see appendix A). The final dataset included six environmental variables: mean diurnal range, mean temperature of warmest quarter, precipitation of wettest quarter, precipitation of driest quarter, precipitation of warmest quarter and precipitation of coldest quarter.

The features option on MaxEnt was set in two different ways: first, a set of models was generated with only the linear and the quadratic classes selected, in order to recover simpler models; then a second set of models was generated with autofeatures selected, which allowed the program to choose the more appropriate feature classes based on the number of records included, allowing more complex models. Likewise, the beta regularization option was set to generate models in six different categories with a value range of 0.5 to 3 with a 0.5 interval. Since each feature class option was used to generate models with each beta regularization category, a total of 12 models were generated and the best model was selected based on its capability to minimize omission errors on the test data (see appendix A for details).

After selecting the best configuration, the model was projected to the Last Glacial Maximum (LGM, about 21 ky BP) and the Mid-Holocene (MH, about 6 ky BP) using the earth system model developed by the Max Plank Institute for Meteorology (MPI-ESM), which includes the atmosphere general circulation model ECHAM6 (Stevens et al., 2013), available at the WorldClim database. The analysis was based on 10 bootstrap replicates with auto features option, beta regularization=1, 10,000 background points, 5,000 iterations and a logistic output. The performance of the model was evaluated using the area under the ROC curve (AUC) statistics (Fielding & Bell, 1997). A maximum test sensitivity plus specificity threshold was applied in order to obtain binary presence-absence maps for each period of time, then a stability map highlighting only the suitable areas present in all models, that theoretically remained climatically stable over time, was generated. All GIS manipulations were done using DIVA-GIS v7.5 (<http://www.diva-gis.org/>) and QGIS Desktop v2.16 (<http://www.qgis.org/en/site/>).

3.2.2 MORPHOLOGICAL DATA AND ANALYSES

Seventy-six specimens of *Amphisbaena pretrei* from 17 localities covering nearly its entire distributional range were obtained from the following Brazilian Herpetological collections: Coleção Herpetológica da Universidade Federal da Paraíba (CHUFPB), Coleção Herpetológica da Universidade Federal de Pernambuco (CHUFPE), Universidade Regional do Cariri (URCA), MCNR, MUFAL e UFS. All specimens were characterized following Gans & Alexander (1962) and ten meristic and 25 morphometric characters were analyzed under a Coleman NSZ 405 stereomicroscope. All measurements were taken with 0.1 mm caliper, except for snout-vent length (SVL) and total length, which were measured with a 1 mm precision measuring tape. Sex of each specimen was determined by direct inspection of the gonads.

In order to remove size effect in the traits, correlation between SVL and all other morphometric traits was checked through Pearson's correlation coefficient at a significance level of $\alpha = 0.05$, then correlated traits were regressed to SVL, and the residuals for the independent 25 variables were taken. Data normality was assessed through Shapiro-Wilk test and the homoscedasticity was tested by plotting the fitted values versus the residuals of the analyses. To determine the presence of morphological groupings, principal component analyses (PCA) were performed with the residuals of the linear model analyses. Linear regression and PCA analysis were performed using males and females separately to avoid possible noise caused by sexual dimorphic traits.

3.2.3 MOLECULAR DATA AND ANALYSES

Thirty-six samples of liver or muscular tissues of *A. pretrei* from 14 localities, and two closely related species (*Amphisbaena alba* Linnaeus, 1758 and *A. vermicularis* Wagler, 1839), were included in the molecular analyses (Fig. 1). The samples of *A. pretrei* span over most of the distribution of the species, from Ceará State to Bahia State. The total genomic DNA was extracted using salt or phenol/chloroform protocols (Sambrook & Russel 2001). Subsequently, fragments of the mitochondrial genes cytochrome oxidase subunit I (COI) and NADH dehydrogenase subunit 4 (ND4) were amplified through polymerase chain reaction (PCR), using the primers 7580 and 7582 (Silva, Prudente, & Zaher, 2010) and ND4 and Leu (Arévalo, Davis, & Sites, 1994), for COI and ND4, respectively. Polymerase chain reactions consisted of

twenty-five microliters reactions with 12.5 μ l of Master Mix PCR Buffer with 0.4 mM of each dNTP and 3 mM of $MgCl_2$, 9–10 μ l of nuclease-free water, 0.2–0.5 μ l of Taq DNA polymerase (5U/ μ l), 0.5 μ l of each primer (10pmol) plus 1–2 μ l of DNA template (>20ng/ μ l). Amplifications were performed with the following protocol: initial denaturation at 94°C for 7 min followed by 35–40 cycles of denaturation at 94°C for 30 sec, annealing at 45–48°C for 30 sec, extending at 72°C for 1 min and a final extending at 72°C for 5–7 min. Subsequently, the samples were purified using isopropanol to remove PCR residuals and sequenced using Sanger method at the Laboratório Central da Universidade Federal de Pernambuco.

The sequences were edited and subsequently aligned using the ClustalW algorithm implemented in BioEdit v7.2.5 (Hall, 2011). Saturation in the third codon position was checked using DAMBE v5 (Xia, 2013). As a substantial saturation was found in COI, the third position of the codon was excluded from subsequent analyses. The best-fit partition scheme and evolutionary model for each gene were estimated through Bayesian Information Criterion (BIC) using PartitionFinder v2.1.1 (Lanfear *et al.*, 2012).

Divergence times among lineages were estimated using BEAST 1.8.0 (Drummond *et al.*, 2012), with an uncorrelated lognormal relaxed molecular clock assuming a divergence rate of 2% per million years as suggested by studies on other squamates of similar body size (Thorpe *et al.*, 1994; Paulo *et al.*, 2001; Passoni *et al.*, 2008). The evolutionary models K80 and TrN+I were applied to COI and ND4 datasets, respectively. All parameters were unlinked between genes so they could be estimated for each gene separately. The closely related amphisbaenids *A. alba* and *A. vermicularis* (GenBank accession numbers KY560439–42) were used as outgroups (Dal Vechio *et al.*, 2016). The analysis was constituted of four independent runs of 50 million generations each, sampled every 10,000. The output of the analyses was evaluated using Tracer v1.6 (Rambaut *et al.*, 2014), the log and tree files were combined using LogCombiner v1.8.0 and the maximum clade credibility tree was obtained with TreeAnnotator v1.8.0 (Drummond *et al.*, 2012), after rejecting the first ten percent generations as burn-in. The tree was visualized using FigTree v1.3.1 (<http://tree.bio.ed.ac.uk/software/figtree/>). Nodes support was evaluated through posterior probability values.

The number of polymorphic sites (S), number of haplotypes (h), haplotype diversity (H) and nucleotide diversity (π) were estimated using DnaSP v5.10 (Librado

& Rozas, 2009). Uncorrected p-distances between sampled localities were estimated using MEGA v6.06 (Tamura *et al.*, 2013). Haplotypic differentiation in the ND4 fragment was assessed through a median-joining network (Bandelt, Forster, & Röhl, 1999) using the software PopART v1.7 (<http://popart.otago.ac.nz/index.shtml>). The haplotype network reconstruction was performed only with ND4 samples for a better spatial representation since some localities from the southern part of the distribution (Mucugê and Salvador) were not successfully amplified for COI. In the same way, the ND4 dataset was used to test for population expansion in the groups recovered by the phylogenetic analysis (north and south, see results) through Fu's F_s (Fu, 1997) and Tajima's D (Tajima, 1989) neutrality tests using 1,000 simulated samples. These tests measure how much the mutation rate departs from neutrality, in which large negative values of D and F_s indicate higher frequency of recent mutations which might be a consequence of a bottleneck event or population expansion. These analyses were implemented in Arlequin v. 3.5.2.2 (Excoffier & Lischer 2010).

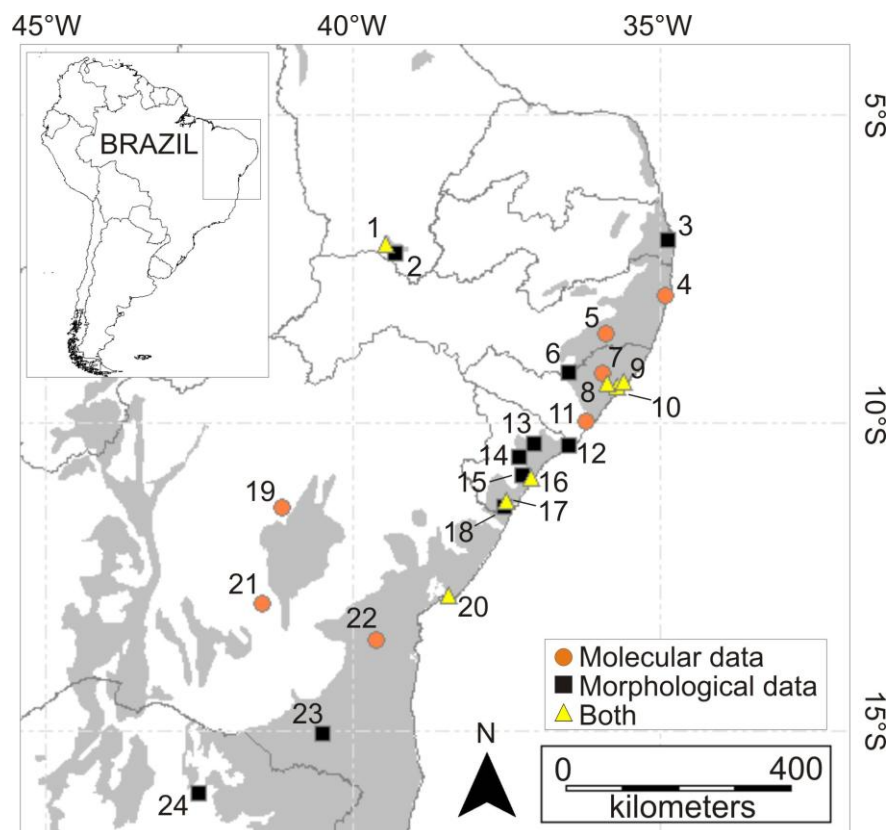


Figure 1. Sampling of morphological and molecular data of *Amphisbaena pretrei* included in this study. Locality names are: 1 = Crato, 2 = Barbalha, 3 = João Pessoa, 4 = Recife, 5 = Lagoa dos Gatos, 6 = Quebrangulo, 7 = Murici, 8 = Rio Largo, 9 = Paripueira, 10 = Maceió, 11 = Coruripe, 12 = Brejo Grande, 13 = Capela, 14 = Malhador, 15 = São Cristóvão, 16 = Aracaju, 17 = Santa Luzia do Itanhi, 18 = Indiaroba, 19 = Morro do Chapéu, 20 = Salvador, 21 = Mucugê, 22 = Wenceslau Guimarães, 23 = Itambé, 24 = Usina Hidrelétrica Irapé, Grão Mogol.

To calculate the influence of climate stability and the presence of the SFR on the genetic structure of *A. pretrei*, distance matrices were constructed and individually compared to the genetic matrix of uncorrected p-distances through Mantel tests (Mantel, 1967) with 1,000 permutations. The climate distance matrix was constructed based on the stability layer, where a value of 0 was assigned to pairs of localities that were inside the same stable area and a value of 1 was assigned to pairs of localities isolated by a patch of unsuitable areas, indicating that localities inside a same suitable area have a lower climate distance and consequently would have higher gene flow. The same methodology was used to construct the matrix representation of the influence of the SFR, where localities on the same margin of the river were assigned values of 0 and localities on opposite margins were assigned values of 1. After individual Mantel tests, significant matrices were included in a partial Mantel test to evaluate their relative contribution to current genetic distances. These analyses were implemented in Arlequin v. 3.5.2.2 (Excoffier & Lischer 2010).

3.3 RESULTS

3.3.1 ECOLOGICAL NICHE MODELING

The best configuration for the analysis of the *A. pretrei* dataset revealed to be the default configuration of MaxEnt, with auto features option and beta regularization = 1 (see appendix A), which recovered the lowest omission rate on test data (5%) and a good AUC value (0.95). The mean value of the maximum test sensitivity plus specificity threshold was 0.2089, hence the species was considered to be present in areas with suitability values higher than this threshold and absent in areas with suitability values lower than the threshold. All models suggested most of the species distribution as climate suitable areas (black) in the LGM (Fig. 2A), Mid-Holocene (Fig. 2B) and present day (Fig. 2C), however, there was a general tendency to the retraction of these areas through time, especially on the southernmost region. The stability layer suggested a large stable area for the occurrence of *A. pretrei* alongside the coast of northeastern Brazil extending from south of the SFR until the northernmost portion of east Brazil. Most of the species distribution was recovered as suitable, however some localities in the southernmost portion of its distribution, representing records less associated with coastal environments, were not predicted as suitable for the occurrence of the species (Fig. 2D).

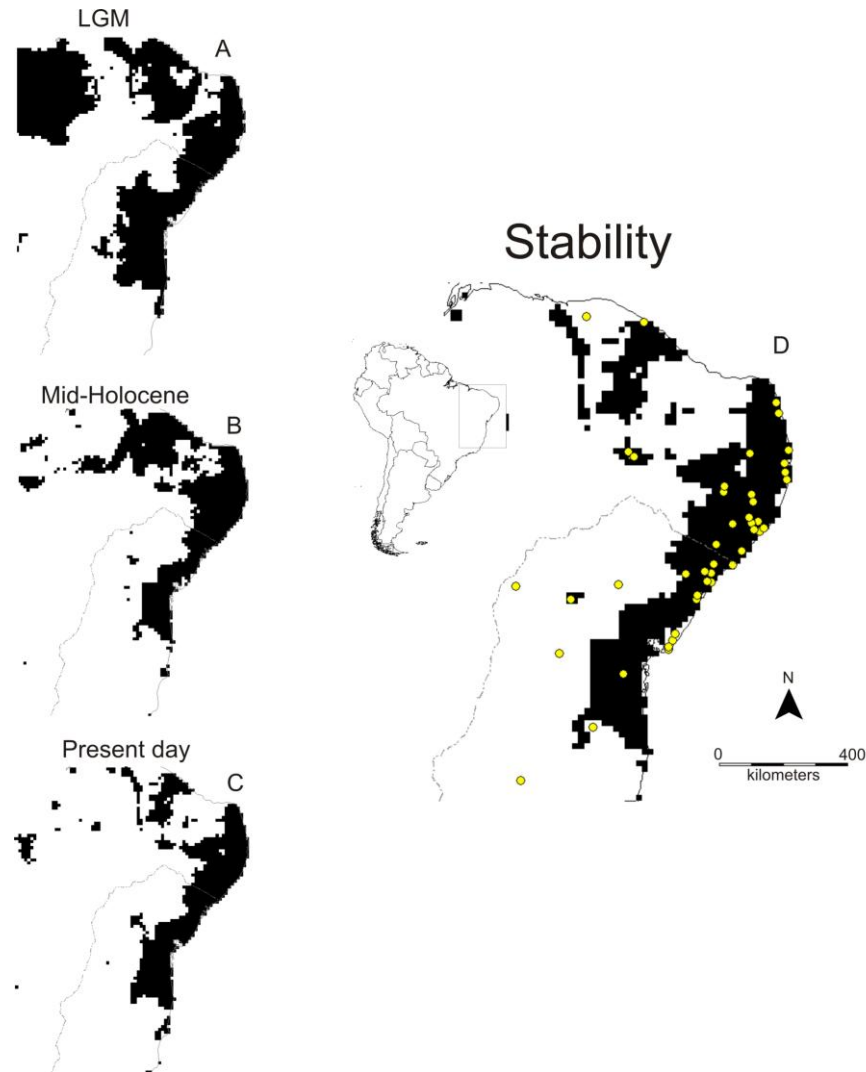


Figure 2. Stability map representing the intersection of three distribution models: present day, Mid-Holocene (6kya) and Last Glacial Maximum (21kya). Areas in black indicate those regions where the species was predicted to be present throughout the three time periods. AUC = 0.95.

3.3.2 MORPHOLOGY

The overall morphology of all specimens of *A. pretrei* is very similar, except for specimens from the southernmost portion of its distribution, corresponding to Grão Mogol (GMO) municipality (Minas Gerais State, in southeastern Brazil, Fig. 3). For instance, in 95% (56 out of 59 specimens) of the specimens from all other localities, the third supralabial is split in two and forms an “infraocular” shield. This modification was seen in only 6% (one out of 16 specimens) of all specimens from GMO. Moreover, the number of body annuli in specimens from GMO is always at the low range of this character. Overall, the range of body and tail annuli was 220–251 and 21–27, respectively, extending the previous known variation in these characters (231–255 and 22–26 body and tail annuli, respectively).

The PCA plots showed no distinct groupings from the two currently suitable areas, north and south of the SFR. The exceptions are the specimens from GMO, which were grouped apart from the rest of the samples by having higher values (positive in males and negative in females) of three variables in the PCA space: Width of the prefrontal shield (WPF), body diameter at the fifth body annulus (BD5) and length of the posterior mental shield (LPM). Furthermore, specimens from the south tended to exhibit a wider range of variation in the analyzed characters, as indicated by the size of the confidence interval ellipses in the PCA plots. Together, PC1 and PC2 explain 44.75% and 48.76% of the variation in the data for males and females, respectively. In males, PC1 and PC2 are more related to body diameter at the fifth annulus and vent length, respectively, while in the females they are more related to width of the prefrontal scale and number of preloacal pores.

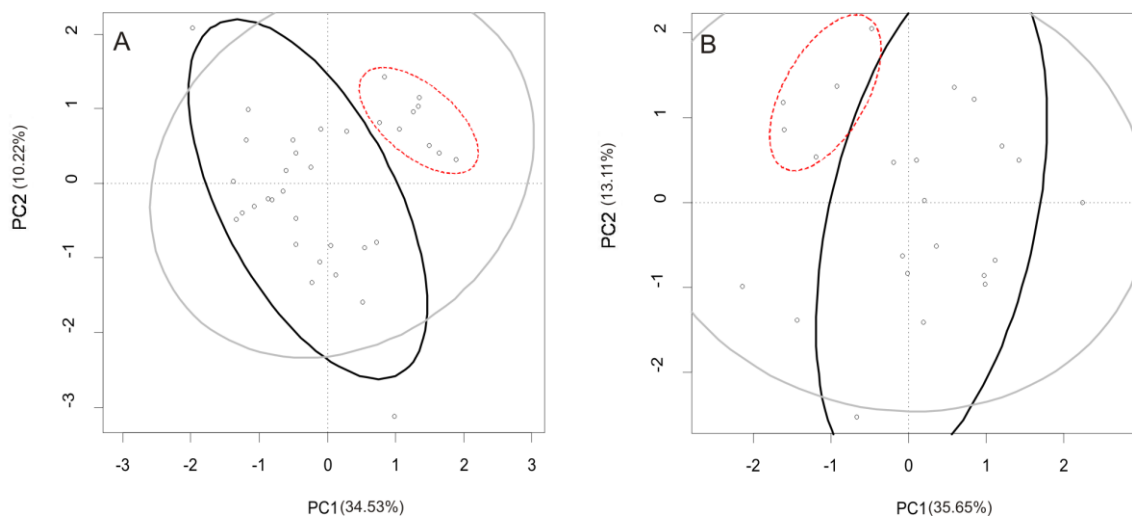


Figure 3. PCA plots of meristic and residuals morphometric data of males (A) and females (B) of specimens of *Amphisbaena pretrei* included in this study. Black and gray ellipses represent 95% confidence intervals for groups from north and south current isolates, respectively. Dashed red ellipse demarks specimens from GMO, Minas Gerais State.

3.3.3 PHYLOGENETIC RELATIONSHIPS AND GENETIC DIVERSITY

The final alignment of 962bp of the mitochondrial dataset encompassed 623bp of ND4 and 339bp (without the third codon position) of COI (GenBank accession numbers in Table 1). The phylogenetic reconstruction successfully recovered a monophyletic *A. pretrei*. There were two main groups, corresponding to samples from north and south of the SFR, although with low statistical support (Fig. 4). The estimated divergence between these two groups was ca. 1.31 (0.87–1.84) million years ago (Myr), dating that split within the Pleistocene, but before the last glacial

maximum (21 ky BP). The north group was subdivided in two subgroups with an estimated divergence of 0.88 (0.50–1.28) Myr, one well-supported (pp=1) group that included samples from Coruripe (COR), Maceió (MAC), Murici (MUR), Paripueira (PAR) and Rio Largo (RLG) in Alagoas State, and another weak-supported (pp=0.44) group that encompassed samples from Lagoa dos Gatos (LAG) and Recife (REC) in Pernambuco State (well-supported, pp=1) and Crato (CRA) in Ceará State. The south group was also subdivided in two subgroups with an estimated divergence time of 0.93 (0.53–1.34) Myr, one well-supported (pp=1) subgroup with samples from Mucugê (MUC), in Bahia State and one relatively well-supported (pp=0.92) subgroup that was further subdivided in another two well-supported subgroups, one encompassing samples from Aracaju (ARA) in Sergipe State and Morro do Chapéu (MCH) in Bahia State (pp=0.99), and other subgroup encompassing samples from Santa Luzia do Itanhi (SLI) in Sergipe State and Salvador (SAL) and Wenceslau Guimarães (WEG) in Bahia State (pp=1, Fig. 4). Nevertheless, neutrality tests revealed no signs of population expansion neither in the north grouping ($D=-1.04259$, $p=0.14$; $F_s=3.16263$, $p=0.89$) nor in the south grouping ($D=0.44332$, $p=0.71$; $F_s=3.57875$, $p=0.93$).

Table 1. Identification, voucher, locality (municipality, state) and Genbank accession numbers of all samples of *Amphisbaena pretrei* included in this study. AL = Alagoas State, BA = Bahia State, CE = Ceará State, PE = Pernambuco State, SE = Sergipe State.

ID	Voucher	Locality	Latitude	Longitude	ND4	COI
Ap1	MUFAL10759	Maceió, AL	-9.5204	-35.7101	KY560373	KY560409
Ap2	MUFAL9057	Maceió, AL	-9.5204	-35.7101	KY560374	KY560410
Ap3	MUFAL10609	Maceió, AL	-9.5204	-35.7101	KY560375	KY560411
Ap4	MUFAL10743	Maceió, AL	-9.5204	-35.7101	KY560376	KY560412
Ap5	MUFAL10792	Maceió, AL	-9.5204	-35.7101	KY560377	KY560413
Ap6	MUFAL10812	Maceió, AL	-9.5204	-35.7101	KY560378	KY560414
Ap7	MUFAL10808	Maceió, AL	-9.5204	-35.7101	KY560402	KY560415
Ap8	MUFAL10809	Maceió, AL	-9.5204	-35.7101	KY560379	KY560416
Ap9	MUFAL10810	Maceió, AL	-9.5204	-35.7101	KY560380	KY560417
Ap10	MUFAL10814	Maceió, AL	-9.5204	-35.7101	KY560381	KY560418
Ap11	MUFAL10815	Maceió, AL	-9.5204	-35.7101	KY560382	KY560419
Ap12	MUFAL11891	Maceió, AL	-9.5204	-35.7101	KY560403	KY560420
Ap13	MUFAL11894	Maceió, AL	-9.5204	-35.7101	KY560383	KY560421
Ap14	MUFAL10595	Maceió, AL	-9.5204	-35.7101	KY560384	KY560422
Ap15	MUFAL10597	Maceió, AL	-9.5204	-35.7101	KY560385	KY560423
Ap16	URCA-G521	Lagoa dos Gatos, PE	-8.6661	-35.8828	KY560386	KY560424
Ap17	MTR14004	Mucugê, BA	-13.0519	-41.4835	KY560387	-

Ap18	MTR11810	Mucugê, BA	-13.0519	-41.4835	KY560388	-
Ap19	C4515	Santa Luzia do Itanhi, SE	-11.3613	-37.5093	KY560389	KY560425
Ap20	MUFAL13409	Maceió, AL	-9.5204	-35.7101	KY560390	KY560426
Ap21	MTR14000	Mucugê, BA	-13.0519	-41.4835	KY560391	-
Ap22	MTR22216	Wenceslau Guimarães, BA	-13.6301	-39.6264	KY560392	KY560427
Ap23	MTR22457	Morro do Chapéu, BA	-11.4709	-41.1498	KY560393	KY560428
Ap24	CHUFPE118	Crato, CE	-7.213	-39.4802	KY560394	KY560429
Ap25	CHUFPE119	Recife, PE	-8.0397	-34.9331	KY560395	KY560430
Ap26	MUFAL10520	Maceió, AL	-9.5204	-35.7101	KY560396	-
Ap27	MUFAL12205	Rio Largo, AL	-9.4767	-35.8635	KY560397	KY560431
Ap28	MUFAL12094	Rio Largo, AL	-9.4767	-35.8635	KY560398	KY560432
Ap29	MUFAL12209	Aracaju, SE	-10.9888	-37.0944	KY560399	KY560433
Ap30	MUFAL10601	Maceió, AL	-9.5204	-35.7101	KY560400	KY560434
Ap31	M324	Murici, AL	-9.2932	-35.9377	KY560401	KY560435
Ap32	MUFAL13410	Salvador, BA	-12.9043	-38.4371	KY560404	-
Ap33	MUFAL13411	Salvador, BA	-12.9043	-38.4371	KY560405	KY560436
Ap34	TM262	Salvador, BA	-12.9043	-38.4371	KY560406	-
Ap35	MUFAL12323	Paripueira, AL	-9.4351	-35.5935	KY560407	KY560437
Ap36	MUFAL12333	Paripueira, AL	-9.4351	-35.5935	KY560408	KY560438

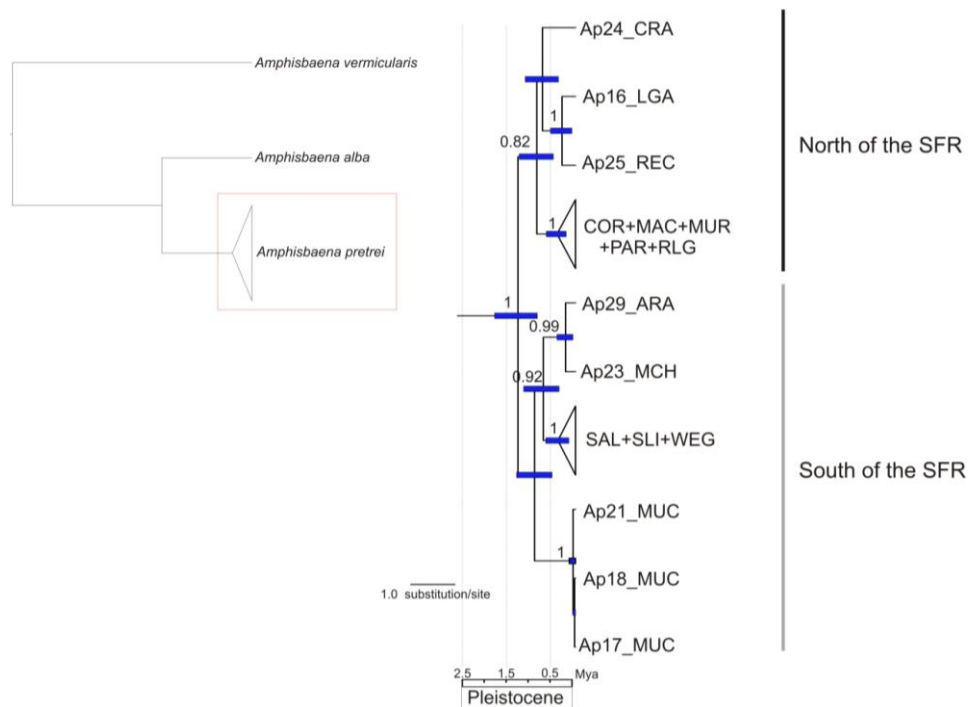


Figure 4. Bayesian phylogenetic tree of *Amphisbaena pretrei* based on 623bp of ND4 and 339bp of COI mitochondrial genes. Posterior probability values are shown near the nodes. Geological scale is plotted on the bottom, assuming a divergence rate of 2% per million years. ARA = Aracaju, COR = Coruripe, CRA = Crato, LAG = Lagoa dos Gatos, MAC = Maceió, MCH = Morro do Chapéu, MUC = Mucugê, MUR = Murici, REC = Recife, RLG = Rio Largo, SAL = Salvador, SLI = Santa Luzia do Itanhi, PAR = Paripueira.

The mitochondrial alignment resulted in 14 and 13 haplotypes of ND4 and COI, respectively (Table 2, Fig. 5). In the ND4 dataset, there was haplotype sharing between MAC and MUR, MAC and PAR, and MAC and RLG, all very close localities (<60km) in Alagoas State. In the COI dataset there was haplotype sharing only between MUR and RLG. Mean uncorrected p-distances between COI and ND4 haplotypes were 1.5% (0–4.3%) and 2.6% (0.2–5.3%) respectively (Table 3). Besides, samples from ARA and SLI (<60km), two close locations in Sergipe State, were more similar genetically to samples from MCH and SAL, two locations in Bahia State ca. 195km apart. The haplotype network exhibited some structure within *A. pretrei*. Six mutational steps separate the north and south groups, however there is a huge variability inside these two groups (Fig. 5).

Table 2. Molecular diversity parameters of fragments of the mitochondrial genes ND4 and COI in *Amphisbaena pretrei*. N = number of samples, h = number of haplotypes, H = Haplotype diversity, S = number of polymorphic sites, π = nucleotide diversity.

Marker	N	S	h	H	π
ND4	36	77	14	0.902	0.026
COI	30	38	13	0.867	0.015

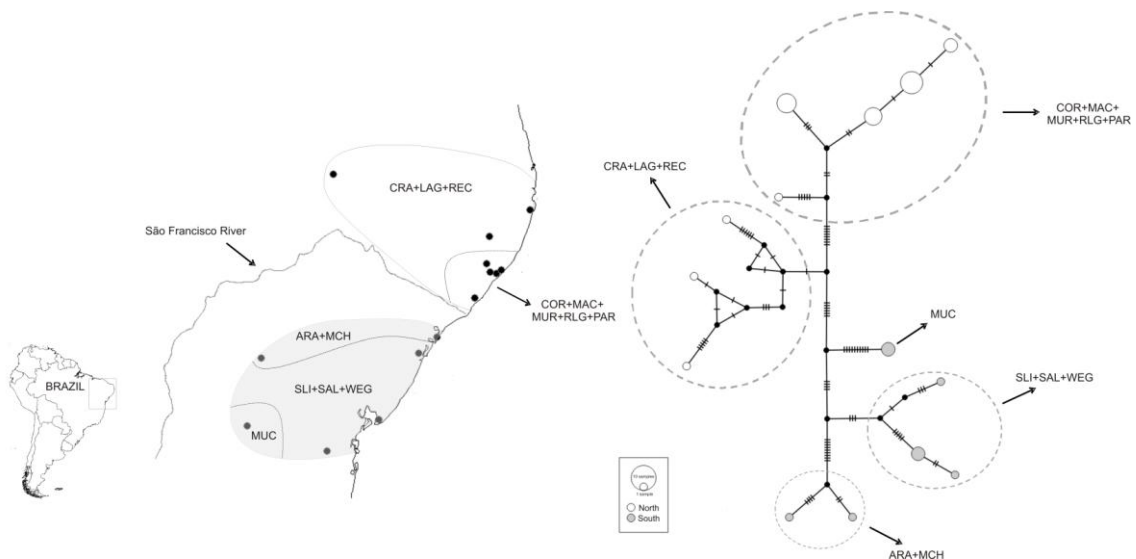


Figure 5. Median-joining haplotype network of *Amphisbaena pretrei* reconstructed based on 623bp of the mitochondrial ND4. Size of the circles is proportional to the number of haplotypes. Dashes on the branches indicate number of mutations. ARA = Aracaju, COR = Coruripe, CRA = Crato, LAG = Lagoa dos Gatos, MAC = Maceió, MCH = Morro do Chapéu, MUC = Mucugê, MUR = Murici, REC = Recife, RLG = Rio Largo, SAL = Salvador, SLI = Santa Luzia do Itanhi, PAR = Paripueira.

Table 3. Uncorrected p-distances between localities sampled for *Amphisbaena pretrei*. Values for COI and ND4 are indicated above and below diagonal, respectively.

	CRA	REC	LGA	MUR	RLG	MAC	PAR	COR	ARA	SLI	MCH	SAL	MUC	WEG
CRA	-	0.018	0.022	0.022	0.022	0.025	0.029	-	0.035	0.043	0.037	0.041	-	0.041
REC	0.027	-	0.008	0.016	0.016	0.017	0.020	-	0.026	0.033	0.028	0.031	-	0.031
LGA	0.021	0.011	-	0.024	0.024	0.025	0.028	-	0.029	0.041	0.028	0.039	-	0.039
MUR	0.034	0.035	0.032	-	0.000	0.008	0.012	-	0.026	0.033	0.028	0.033	-	0.033
RLG	0.030	0.035	0.032	0.010	-	0.008	0.012	-	0.026	0.033	0.028	0.033	-	0.033
MAC	0.033	0.035	0.032	0.003	0.007	-	0.004	-	0.027	0.032	0.029	0.032	-	0.032
PAR	0.032	0.034	0.03	0.002	0.008	0.003	-	-	0.029	0.033	0.031	0.033	-	0.033
COR	0.034	0.034	0.029	0.016	0.016	0.016	0.014	-	-	-	-	-	-	-
ARA	0.046	0.043	0.038	0.050	0.053	0.05	0.048	0.050	-	0.024	0.010	0.024	-	0.024
SLI	0.037	0.032	0.032	0.043	0.043	0.043	0.042	0.043	0.032	-	0.022	0.010	-	0.008
MCH	0.043	0.040	0.035	0.046	0.050	0.047	0.045	0.046	0.010	0.029	-	0.022	-	0.022
SAL	0.042	0.037	0.037	0.045	0.045	0.045	0.043	0.045	0.034	0.014	0.030	-	-	0.002
MUC	0.037	0.037	0.037	0.046	0.045	0.046	0.045	0.046	0.040	0.034	0.037	0.035	-	-
WEG	0.045	0.040	0.040	0.048	0.048	0.048	0.046	0.048	0.037	0.018	0.034	0.003	0.038	-

The Mantel tests revealed that climate conditions were not significantly correlated to the genetic variability of *A. pretrei* ($r=0.08$, $p=0.14$). Therefore, it was not included in the partial Mantel test analyses. On the other hand, the presence of the SFR was significantly correlated with the genetic diversity ($r=0.72$, $p=0.002$). Likewise, the geographic distances were found to be significantly correlated with genetic distances ($r=0.57$, $p<0.001$). Then, the partial Mantel analysis was used to evaluate the relative contribution of the presence of the SFR and the geographic distances to the genetic structure in *A. pretrei*. The partial Mantel test suggested that together the SFR and geographic distances explain 55% ($r=0.63$, $p=0.03$) of the genetic variance in *A. pretrei*, however the presence of the SFR alone explains 44% of the genetic variance.

3.4 DISCUSSION

3.4.1 ECOLOGICAL NICHE MODELING

Amphisbaena pretrei is a species associated with the Atlantic Forest and ENM analyses were successful by recovering current and past suitable habitats for the species mostly restricted to this biome. All models recovered a large area of suitable habitats in northeastern Brazil. Contrary to the expectations, the LGM model exhibited a slightly wider suitable area to the occurrence of *A. pretrei*, which is unexpected because of the cold and dry climatic conditions of the LGM (Van Der Hammen & Hooghiemstra, 2000), which theoretically should reduce the range of distribution of forest-dependent species as a consequence of forest retractions (Haffer, 1969; Carnaval & Moritz, 2008; Carnaval *et al.*, 2009). Moreover, the distribution of reptiles is often associated with temperature (Hawkins *et al.*, 2003), so it would be expected that suitable areas would be wider in warmer periods.

3.4.2 MORPHOLOGY

In spite of the conserved morphology in *A. pretrei*, it was possible to detect cryptic, yet significant, morphological divergence in the species. Specimens from the extreme southern range of the species distribution (GMO) have larger values of WPF, BD5 and LPM relative to other localities (Fig. 3). These results are in agreement with the preliminary study by Gans (1965) that, based on limited sampling, suggested fewer body annuli in specimens from the southern of Bahia and from Minas Gerais. Herein

the number of body annuli of *A. pretrei* specimens from the southernmost portion of its distribution, corresponding to Minas Gerais was generally lower (220–231, n=16) than other localities (229–251, n=59). In addition, the third supralabial modification was much less frequent in these specimens (6% versus 95% in other localities).

The constraints imposed by the underground environment resulted in homoplastic evolution of a set of characteristics (limb reduction and loss and some cranial features) in many fossorial vertebrates, including amphisbaenians, which can many times mask the real diversity of the group (Kearney & Stuart, 2004; Mott & Vieites, 2009). Therefore, even in longtime isolated populations the degree of morphological differentiation is expected to be low and the results found here suggest a potential cryptic diversity in *A. pretrei*. Nonetheless, although these results clearly show a morphological divergence in the extreme southern distribution of *A. pretrei*, the lack of tissue samples from this region prevents us from testing hypotheses regarding the processes underlying such morphologic divergence.

3.4.3 MOLECULAR ANALYSES

The phylogenetic analysis recovered the monophyly of *A. pretrei* (Fig. 4) and indicated that *A. pretrei* diversification started in the middle Pleistocene (approximately 1.3 Myr, Fig. 4), which indicates that the climatic oscillations of that period caused the initial periods of vicariance between lineages of this species. This result suggests that the species started its diversification before the LGM, modeled here (Fig. 2A). This is congruent with studies on other species co-distributed throughout the northern portion of the BAF (Cabanne et al., 2008; Carnaval et al., 2009; D'Horta et al., 2011), suggesting that diversification of the endemic species from this biome is relatively old and that lineages within species may have survived throughout several glacial cycles.

Nevertheless, neutrality tests suggested the absence of population expansion in *A. pretrei*, which is in contrast to the expectations of the refugia hypothesis. Furthermore, the Mantel tests suggested that the SFR have a bigger influence on the genetic structure of the species, which is in agreement with some studies in the BAF that suggested the SFR as a significant barrier to the gene flow in some species (e.g. Pellegrino et al., 2005; Cazé et al., 2016). However, geological evidence supported by molecular studies postulates that the SFR used to flow north to the Atlantic Ocean in the equator, but ca. 400–450 kya it changed its course to the present configuration

where it flows north until the northern region of Bahia State and then turns to the east across the Atlantic Forest meeting the Atlantic Ocean between the states of Alagoas and Sergipe (Potter, 1997; Faria *et al.*, 2013; Nascimento *et al.*, 2013). This change in the course of the SFR might have acted as a vicariant event separating populations and promoting further genetic structure. This imposes a puzzling scenario for the diversification of *A. pretrei* since the estimated divergence time found here (1.3 Myr) suggests that the main diversification process had already started by then.

Both the phylogenetic and the network analyses revealed marked genetic structure within *A. pretrei* (Figs. 4 and 5). The Bayesian coalescent tree recovered two groups corresponding to two lineages with distribution at north and south of the SFR (Fig. 4). However, the support of these clades was relatively low (pp=0.78 and pp=0.71). Although there are no phylogeographic studies on amphisbaenians in South America, some studies with morphological (Gans, 1965; 1966) and molecular data (Mott & Vieites, 2009) have suggested that widespread South American amphisbaenians can exhibit geographical structure and thus cryptic diversity.

Genetic divergences found in *Amphisbaena pretrei* throughout its distributional range were slightly high (0–4.3% in COI and 0.2–5.3% in ND4). This is congruent with other amphisbaenians analyzed with the same ND4 fragment and that likely suffered similar contractions and expansions during glacial cycles (Vaconcelos *et al.*, 2006; Albert *et al.*, 2007; Sampaio *et al.*, 2014). Studies with species of the genus *Blanus* (Blanidae) from northern Africa and southern Europe recovered a range of intra-specific genetic divergence ca. 0.6–4.9% while the range of inter-specific divergence was about 6.7–17%. In the same way, genetic divergence in codistributed taxa, using fast-evolving mitochondrial genes, were similar, for example for frogs (Carnaval *et al.*, 2009; Thomé *et al.*, 2010) and lizards (Pellegrino *et al.*, 2005).

As fossorial animals with limited dispersal capability, makes sense that the individuals from distant sites are less related genetically, however this does not seem to be a linear trend for *A. pretrei*, since some samples from distant localities (e.g. ARA and MCH) were more related to each other than to samples from nearest sites (e.g. ARA and SLI, Table 2). The results found here indicate that *A. pretrei* has a complex evolutionary history, initiated by multiple episodes of vicariance and likely enhanced by current barriers. The main diversification of this clade is estimated to

initiate during the Pleistocene climatic oscillations. In addition to showing two main phylogenetic clades, genetic data suggests further substructuring that is likely related to IBD. Finally, although the genetic structure of the species was found to be significantly older than the current location of the SFR, the river seems to have a significant influence on the genetic structure of the species. This suggests that it may also play a significant role on the genetic structure of other fossorial animals distributed in this area as a barrier to dispersion.

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APPENDIX A – SAMPLING BIAS, FEATURE CLASSES AND BETA REGULARIZATION

One of the assumptions of several modeling techniques, including MaxEnt, is that the study area was randomly sampled (Kramer-Schadt et al., 2013). However, the dataset available for most species is often a result of local sampling effort and many times better represent those areas that are more accessible or were more intensively studied. This sampling bias may lead to an environmental bias, since the areas that will be better represented in the dataset do not necessarily represent the true preferences of the species (Kramer-Schadt et al., 2013). This bias can be minimized by manipulating the input data (spatial filtering) or by setting the background sampling (points randomly sampled from the calibration area to represent environmental conditions) on MaxEnt to represent these intensively sampled areas (PHILLIPS; DUDÍK, 2008).

Other sources of overfitting are the correlation between the predictors and the number of predictors included in the analysis, which can increase model complexity and overparameterization, which can also increase model complexity and overfitting. The correlation between the predictors can be minimized by applying dimensionality reduction techniques, such as a principal component analysis, or by removing highly correlated predictors (MEROW; SMITH; SILANDER, 2013). Overparameterization can be controlled by setting the “features” option on MaxEnt, which determines how the predictors will be transformed and included in the analysis (MEROW; SMITH; SILANDER, 2013; PHILLIPS et al., 2006; PHILLIPS; DUDÍK, 2008). The inclusion of several different feature classes in the model can increase the model complexity. Likewise, model complexity can be controlled using the beta regularization option, which is a measure that penalizes the model proportionally to overparameterization (MEROW; SMITH; SILANDER, 2013; PHILLIPS et al., 2006; PHILLIPS; DUDÍK, 2008).

In order to determine the best configuration to reconstruct the suitability models, two features configurations were applied: first, the features option was set to apply the linear and quadratic features (LQ), which would generate simpler models with less parameters; secondly, the features option was set to autofeatures allowing the MaxEnt to apply more feature classes and consequently generate more complex models. With the autofeatures option selected, MaxEnt determines which feature

classes to use according with the number of records included (PHILLIPS; DUDÍK, 2008). The beta regularization value was set to vary between 0.5 and 3 with a 0.5 interval. Then a model was generated for each combination of feature class and beta regularization value, which resulted in a total of 12 models. The models performance was evaluated based on AUC values and on its capability to minimize omission errors on the test data (Figs. 1, 2).

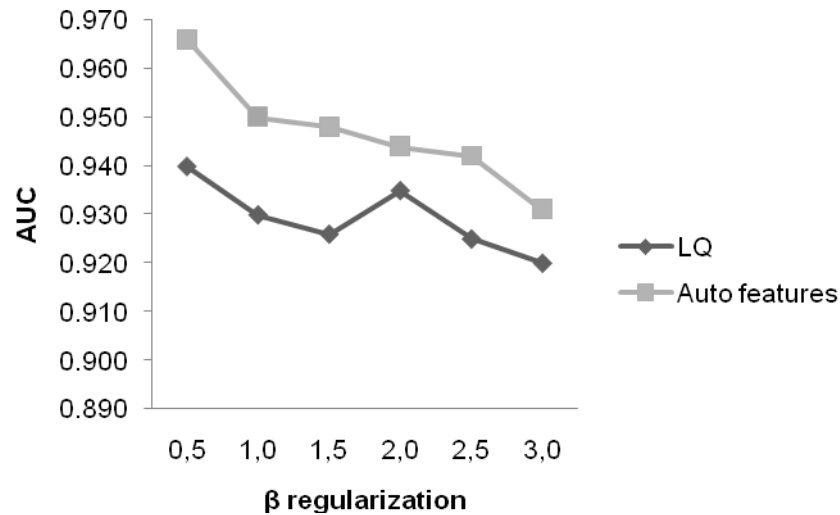


Figure 1. Model performance based on AUC values taking into account different beta regularization values and two different feature classes sets. LQ = linear and quadratic feature classes.

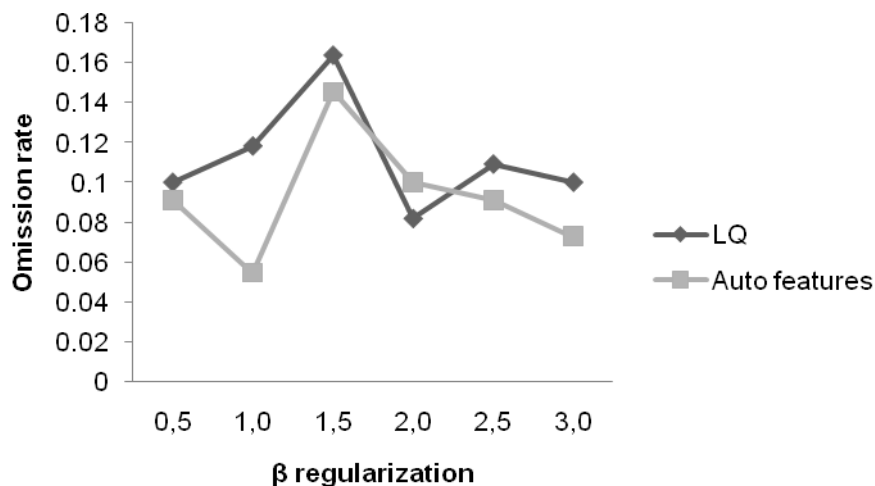


Figure 2. Model performance based on omission rates values considering a threshold of maximum sensitivity plus specificity and taking into account different beta regularization values and two different feature classes sets. LQ = linear and quadratic feature classes.

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4 CONCLUSÕES

A análise dos dados morfológicos dos 76 espécimes de *Amphisbaena pretrei* inclusos neste estudo revelou a existência de um agrupamento morfológicamente divergente, o que foi inesperado devido à morfologia conservada encontrada em anfisbênias. A presença deste grupo morfológicamente divergente foi reforçado pelo fato dos modelos de nicho ecológico não terem recuperado a região de onde esses espécimes são provenientes (norte do Estado de Minas Gerais) como adequadas para a ocorrência da espécie. Esses resultados sugerem que esses espécimes podem representar uma linhagem divergente dentro de *A. pretrei*. Apesar dessa hipótese não ter sido testada devido à falta de amostras de tecido de exemplares dessa região, estes resultados apontam a necessidade da inclusão destes espécimes em uma abordagem molecular a fim de avaliar se eles de fato representam uma linhagem evolutiva divergente.

A análise dos dados moleculares mostrou que a diversificação dentro da espécie provavelmente se iniciou a cerca de 1,31 milhões de anos, o que sugere uma influência das oscilações climáticas do Pleistoceno. Além disso, a comparação entre as matrizes de distância climática, geográfica, ecológica (rio) e genética mostrou que há uma maior correlação entre a estruturação genética na espécie com a distância geográfica e com a presença de rio São Francisco, sugerindo que o rio pode estar atuando como um fator gerador do processo de diversificação dentro de *A. pretrei*.