

**INSTITUTO NACIONAL DE PESQUISAS DA AMAZÔNIA -INPA**  
**PROGRAMA DE PÓS-GRADUAÇÃO EM GENÉTICA, CONSERVAÇÃO**  
**E BIOLOGIA EVOLUTIVA**

Conservação, biogeografia e evolução do jacaré-paguá (*Paleosuchus palpebrosus*): um complexo de espécies a ser desvendado

Fábio de Lima Muniz

Manaus

2018

Fábio de Lima Muniz

Conservação, biogeografia e evolução do jacaré-paguá (*Paleosuchus palpebrosus*): um complexo de espécies a ser desvendado

Orientador: Tomas Hrbek, PhD.

Coorientadoras: Izeni Pires Farias, PhD.

Zilca Campos, PhD (Embrapa Pantanal, MS).

Tese apresentada ao Instituto Nacional de Pesquisas da Amazônia como parte dos requisitos para obtenção do título de Doutor em Genética, Conservação e Biologia Evolutiva.

Manaus

2018



PROGRAMA DE PÓS-GRADUAÇÃO  
PPG GCBEv  
GENÉTICA, CONSERVAÇÃO E BIOLOGIA EVOLUTIVA



INSTITUTO NACIONAL DE  
PESQUISAS DA AMAZÔNIA

MINISTÉRIO DA  
CIÊNCIA, TECNOLOGIA,  
INOVAÇÕES E COMUNICAÇÕES



**ATA DA DEFESA PÚBLICA**  
**PROGRAMA DE PÓS-GRADUAÇÃO EM GENÉTICA, CONSERVAÇÃO E BIOLOGIA EVOLUTIVA**

No dia 14 de agosto de 2018, às 09:00 horas, na Auditório do PPG BADPI, campus II – INPA-Aleixo, reuniu-se a Banca Julgadora da DEFESA PÚBLICA de DOUTORADO, composta pelos seguintes Doutores(as), membros titulares: Camila Cherem Ribas, Fernanda de Pinho Werneck, Marina Anciães, Igor Luís Kaefer e Rafael do Nascimento Leite; tendo como membros suplentes: Cleiton Fantin Rezende e José Antonio Alves Gomes, afim de proceder a arguição pública da TESE do estudante FÁBIO DE LIMA MUNIZ, intitulada: "Conservação, biogeografia e evolução do jacaré-pagauá (*Paleosuchus palpebrosus*): um complexo de espécies cripticas a ser desvendado". O estudo foi conduzido sob a orientação do Dr. Tomas Hrbek, da UFAM e coorientação das Dras. Izêni Pires Farias – UFAM e Zilca Campos – EMBRAPA/Pantanal.

Após a exposição da aula, dentro do tempo regulamentar, a discente foi arguida oralmente pelos membros da Banca Julgadora, tendo recebido o conceito final:

Aprovado por unanimidade  
 Aprovado por maioria

Reprovado

**Menção (se meritório):**

Aprovado com "Distinção" (por maioria)  
 Aprovado com "Distinção e Louvor" (por unanimidade)

Foi lavrada a ata e assinada pelos membros presentes da Banca Julgadora.

Camila Cherem Ribas –INPA

Fernanda de Pinho Werneck - INPA

Marina Anciães – INPA

Igor Luis Kaefer – UFAM

Rafael do Nascimento Leite - INPA

Dra. Jacqueline da Silva Batista  
Coordenadora do PPG GCBEv/INPA  
PO. 281/2017 – INPA/MCTIC-PR



Esta Ata não tem efeito de conclusão de curso ou diplomação do estudante. Conforme Regulamento PPG GCBEv Art. 62 "Será conferido ao discente o título de MESTRE ou DOUTOR em Genética, Conservação e Biologia Evolutiva, desde que cumpridas às exigências das Agências de Fomento, dos regulamentos do PPG-INPA e do PPG GCBEv. Para obtenção do título, o estudante deve cumprir, ainda, o exigido nos Arts. 52 ao 55 do Regulamento Geral do INPA e Arts. 61 e 64 do Regulamento PPG GCBEv.

M966

Muniz, Fábio de Lima

Conservação, biogeografia e evolução do jacaré-paguá  
(*Paleosuchus palpebrosus*): um complexo de espécies a ser  
desvendado / Fábio de Lima Muniz. --- Manaus : [s.n.], 2018.  
xiv, 146 f. : il. color.

Tese (Doutorado) --- INPA, Manaus, 2018.

Orientador : Tomas Hrbek.

Coorientadora: Izeni Pires Farias.

Área de concentração : Genética, Conservação e Biologia  
Evolutiva.

1.Jacaré-paguá. 2. *Paleosuchus palpebrosus*. 3. Hibridização. I.  
Título.

CDD 597.980415

Dedico esse trabalho à minha família, que considero  
o bem mais precioso dessa vida e a todos que  
contribuíram para a formação do meu caráter.

## **AGRADECIMENTOS**

Ao meu orientador Tomas Hrbek. Muita gratidão pela oportunidade de compartilhar conhecimentos e alegrias dos trabalhos realizados. Obrigado também por me instruir pacientemente nos momentos de dúvidas. Sua dedicação e inteligência me inspiram a procurar aprender sempre mais.

À minha coorientadora Izeni Farias. Por ser essa mãezona que me acolheu no laboratório há uns anos atrás e sempre me motiva a ser um pesquisador cada vez melhor. Sinto um carinho muito grande e admiro muito como pessoa e pesquisadora.

À minha coorientadora Zilca Campos. Por ser uma amiga/mãe em minha vida. Sem sua presença minha formação como pessoa e pesquisador deixaria muito a desejar do que é hoje. Obrigado por compartilhar tantas experiências e amizade ao longo desses anos. Prometo me dedicar para fazer jus ao grande investimento e confiança depositados em mim.

Ao INPA pela estrutura do curso de pós-graduação e pela estrutura técnico-científica sempre disponível ao longo desses quatro anos de doutorado.

Ao programa de Pós-graduação em Genética, Conservação e Biologia Evolutiva (PPG GCBEv) pela estrutura do curso de doutorado e por ter proporcionado disciplinas fundamentais para desenvolvimento deste trabalho. Em especial, aos professores deste programa interessados em formar profissionais de qualidade.

Às instituições que apoiaram e financiaram esse trabalho: FAPEAM, CNPq/CT-Amazon, Embrapa Pantanal (Macroprogram 3), Fundect Fundação O Boticário, Santo Antônio Energia, Norte Energia, Tractebel, Crocodilian Specialist Group - IUCN.

Aos amigos de laboratório que contribuíram direta e indiretamente para que este trabalho tenha se concretizado. Em especial ao Érico Polo, Joice Farias, Roberto Zamora e Pedro Senna por me apoiarem na reta final com análises e comentários que foram essenciais para o trabalho. Também aos que me apoiaram ao longo da caminhada, ajudando em excursões de coleta ou no laboratório: Aline Ximenes, Priscila Azarak, Valéria Machado, Luciana Frazão, Pedro Senna, Mário Nunes, Sandra Hernandez, José Gregório.

A todos os companheiros de Laboratório (LEGAL) pelas contribuições sobre o trabalho e pelas discussões de artigos e trocas de ideias que enriqueceram muito minha formação acadêmica.

A companheiros valorosos que contribuíram com esforço e dedicação durante as excursões de coleta: Zilca Campos, Dênis Tilcara, Zairo, Reginei (Ney), Roberto (Potó), Pedro Senna, Priscila Azarak, Luciana Frazão, João Danilo e tantos ajudantes locais que tivemos durante essa longa caminhada.

Aos revisores anônimos que deram sugestões e promoveram melhorias nos trabalhos que foram submetidos, bem como, aos professores que participaram da minha banca de qualificação (Serginho Borges, Camila Ribas e Maria Doris) e na minha defesa do doutorado (Camila Ribas, Fernanda Werneck, Marina Anciães, Igor Kaefer e Rafael Leite) e forneceram contribuições valiosas para que este produto final esteja apresentado de maneira satisfatória.

À minha esposa que me apoiou em todos os momentos, tendo paciência nos momentos difíceis e me motivando a persistir e a manter o pensamento positivo até o final. Obrigado por tudo!

Aos meus familiares, que por meio do afeto e dedicação, moldaram minha personalidade e contribuíram diretamente para que eu alcançasse ess objetivo de vida, em especial: Gracilde (minha mãe), Flávio Muniz (meu pai), Márcio Júnio (irmão), José Muniz (avô), Ermelinda Muniz (avó).

Aos cidadãos brasileiros que pagam seus impostos e possibilitam que a ciência permaneça viva nesse país, apesar de tanta corrupção. Que minha dedicação seja suficiente para fazer valer a pena o investimento da sociedade, enquanto tantos apenas sobrevivem e nem o básico possuem.

“A man who dares to waste one hour of time  
has not discovered the value of life.”

**Charles Darwin**

## RESUMO

A região Neotropical é uma zona biogeográfica hiperdiversa e com diversidade subestimada. O recente avanço no uso de ferramentas moleculares contribuiu para a descoberta de que, muitas espécies previamente consideradas com ampla distribuição são, na verdade, complexos de espécies. As espécies amplamente distribuídas são, em geral, classificadas como de menor preocupação para a conservação, entretanto, as espécies pouco estudadas e que ocorrem em uma gama de ambientes heterogêneos e/ou descontínuos são fortes candidatas a abrigar linhagens evolutivas distintas, possivelmente escondendo linhagens ameaçadas de extinção. O jacaré-paguá (*Paleosuchus palpebrosus*), um dos menores crocodilianos existentes, pode ser considerada uma espécie pouco estudada que possui comportamento críptico e ampla distribuição na América do Sul, ocorrendo em diferentes biomas e bacias hidrográficas. Nesse estudo, nós identificamos diversidade críptica em *P. palpebrosus*, delimitando linhagens evolutivas independentes, traçamos um modelo biogeográfico e investigamos os processos evolutivos que atuaram durante o processo de diversificação das linhagens recém-descobertas. Para isso, fizemos uma densa amostragem por quase toda a distribuição da espécie e combinamos o uso do tradicional marcador mitocondrial citocromo *b* com marcadores genômicos SNPs e ddRADs, obtidos por meio de *Next-Generation Sequencing* (NGS). Nós delimitamos três Unidades Evolutivas Significantes (ESUs) e duas Unidades de Manejo (MUs) e sugerimos que *P. palpebrosus* é um complexo de espécies. Verificamos que o rearranjo de drenagem foi o principal promotor da diversificação do complexo *P. palpebrosus* no corredor Paraguai-Madeira-Amazônia, então propusemos um modelo de evolução da paisagem para explicar essa diversificação, discutindo a utilidade desse modelo para outras espécies aquáticas da região. Além disso, testamos diferentes modelos migração e verificamos a ocorrência de Transferência Lateral de Genes (LGT) e contato secundário entre linhagens geograficamente adjacentes.

## ABSTRACT

The Neotropical region is a hyper-diverse biogeographical zone with an underestimated diversity. Recent advances in the use of molecular tools has contributed to the discovery of many species complex in previous broadly distributed species. Broadly distributed species are generally classified as of Least Concern for conservation, however, species that are poorly studied and occur in a range of heterogeneous and/or discontinuous environments are strong candidates to harbor distinct evolutionary lineages, possibly hiding lineages at extinction risk. The Cuvier's dwarf caiman (*Paleosuchus palpebrosus*), one of the smallest living crocodilians, can be considered a poorly studied species with a cryptic behavior and wide distribution in South America, occurring in several different biomes and watersheds. In this study, we identified cryptic diversity in *P. palpebrosus*, delimiting independent evolutionary lineages, elaborated a biogeographic model and investigated the evolutionary processes that acted during the diversification process of the newly discovered lineages. For this, we did dense sampling along almost the entire distribution of the species and combined the use of the traditional mitochondrial cytochrome *b* marker with genomic markers SNPs and ddRADs obtained by Next Generation Sequencing (NGS). We delimit three Evolutionarily Significant Units (ESUs) and two Management Units (MUs) and suggest that *P. palpebrosus* is a species complex. We verified that the drainage rearrangement was the main promoter of the *P. palpebrosus* complex diversification in the Paraguay-Madeira-Amazonia corridor, so we proposed a landscape evolution model to explain this diversification, discussing the usefulness of this model for other aquatic species in the region. In addition, we tested different migration patterns and verified the occurrence of Lateral Gene Transfer (LGT) and secondary contact between geographically adjacent lineages.

## Sumário

AGRADECIMENTOS.....	VI
RESUMO.....	IX
ABSTRACT.....	X
LISTA DE FIGURAS.....	XIII
LISTA DE TABELA.....	XV
INTRODUÇÃO GERAL.....	1
OBJETIVOS.....	9
Objetivo Geral.....	9
Objetivos Específicos.....	9
CAPÍTULO 1.....	10
Acknowledgments.....	13
ABSTRACT.....	14
INTRODUCTION.....	15
METHODS.....	17
Sample and molecular data collection.....	17
Cyt b and ddRADseq analysis.....	19
RESULTS.....	22
DISCUSSION.....	25
Delimiting Cuvier's dwarf caiman units for conservation.....	25
Genetic diversity of mitochondrial lineages.....	27
Implications for the conservation of <i>P. palpebrosus</i> .....	28
REFERENCES.....	33
SUPPLEMENTARY MATERIAL.....	46
CAPÍTULO 2.....	49
ABSTRACT.....	51
INTRODUCTION.....	53
MATERIAL AND METHODS.....	56
Study area and sample collection.....	56
Molecular data collection.....	56

Data analyses.....	57
RESULTS.....	60
DISCUSSION.....	62
Phylogeny and divergence time estimation of lineages.....	63
Biogeographic model for biological diversification across the Madeira basin.....	65
Can this model be applied to other aquatic species?.....	68
REFERENCES.....	74
SUPPORTING INFORMATION.....	85
CAPÍTULO 3.....	87
RESUMO.....	89
INTRODUÇÃO.....	90
MATERIAL E MÉTODOS.....	93
Coleta dos dados genéticos.....	93
Análise dos dados mitocondriais.....	95
Análise dos dados genômicos.....	97
RESULTADOS.....	102
Estruturação genética e demografia histórica com o gene <i>cyt b</i> .....	102
Filogenômica e hibridização no complexo <i>P. palpebrosus</i> .....	104
DISCUSSÃO.....	106
Reconstrução das áreas ancestrais e rota de dispersão.....	108
Filogenômica de <i>P. palpebrosus</i> .....	109
REFERÊNCIAS.....	124
MATERIAL SUPPLEMENTAR.....	129
SÍNTESE GERAL.....	131
REFERÊNCIAS BIBLIOGRÁFICAS.....	133
APÊNDICE A.....	138
APÊNDICE B.....	142

## **LISTA DE FIGURAS**

### **CAPÍTULO 1**

<b>Figura 1.</b> Mapa com todos os sítios de amostragem, resultados do BAPS e rede de haplótipo baseada no gene mitocondrial cyt b.....	43
<b>Figura 2.</b> Árvore baseada no gene cyt b mostrando as relações filogenéticas entre as linhagens de <i>Paleosuchus palpebrosus</i> .....	44
<b>Figura 3.</b> Mapa mostrando a distribuição geográfica dos indivíduos analisados com marcadores ddRAD e resultado do STRUCTURE com correspondência aos clusters de cyt b....	45

### **CAPÍTULO 2**

<b>Figura 1.</b> Mantel Test parcial usado para quantificar a contribuição relativa do isolamento por distância e do isolamento histórico.....	81
<b>Figura 2.</b> Filogenia calibrada com tempo das linhagens de <i>P. palpebrosus</i> com base no gene mitocondrial cyt b.....	82
<b>Figura 3.</b> Árvore de espécies calibrada com tempo inferida com base no SNAPP usando 532 SNPs bialélicos não ligados.....	83
<b>Figura 4.</b> Mudanças geológicas na paisagem Amazônica e do Pantanal durante os últimos 4ma que influenciaram a diferenciação das linhagens de <i>P. palpebrosus</i> .....	84
<b>Figura S1.</b> Redes de haplótipos dos genes Myc, C-Mos e DEN.....	85
<b>Figura S2.</b> Árvore calibrada com tempo alternativa, usando somente calibração fóssil.....	86

### **CAPÍTULO 3**

<b>Figura 1.</b> Distribuição geográfica das localidades amostradas, incluindo a amostragem feita por Muniz et al. (2018) e gráfico do BAPS mostrando o valor de K mais provável, K = 5...115	115
<b>Figura 2.</b> Parâmetros estimados e modelos de isolamento com migração testados usando o programa G-PhoCS (Gronau et al., 2011).....	116
<b>Figura 3.</b> Rede de haplótipos mostrando a relação genealógica entre as linhagens. As cores correspondem aos grupos detectados na análise do BAPS e mostrados na Figura 1.....	117
<b>Figura 4.</b> Demografia histórica de cada uma das linhagens do complexo <i>P. palpebrosus</i> estimada com base em sequências do gene mitocondrial cyt b.....	118

<b>Figura 5.</b> Difusão de Árvore de Espécies no gênero <i>Paleosuchus</i> .....	119
<b>Figura 6.</b> Reconstrução de áreas ancestrais do gênero <i>Paleosuchus</i> realizadas no BioGeoBEARS.....	120
<b>Figura 7.</b> Árvores de espécies reconstruídas no programa STARBEAST2 utilizando dois conjuntos de dados diferentes .....	121
<b>Figura 8.</b> Árvore de espécies reconstruída no programa ASTRAL-III .....	122
<b>Figura 9.</b> Melhores topologias e modelos de migração estimados no programa G-PhoCS ..	123
<b>Figura S1.</b> Árvore filogenética reconstruída com base no cyt b por meio de Inferência Bayesiana realizada no MrBayes.....	129
<b>Figura S2.</b> Análise de suporte dos nós da árvore de espécies reconstruída no programa ASTRAL-III feita com o programa Quartet Sampling.....	130

## LISTA DE TABELA

### CAPÍTULO 1

<b>Table 1.</b> Pairwise $F_{ST}$ between cyt b groups previously established by BAPS cluster analysis .....	40
<b>Table 2.</b> Summary of results of the population analysis using cytochrome b gene and ddRADs genomic marker, the correspondent geographic distribution of each and the type of evolutionary lineages proposed for geographic groups.....	41
<b>Table 3.</b> Population parameters and genetic diversity indexes in <i>P. palpebrosus</i> populations and in other crocodilian species.....	42
<b>Tabela S1.</b> Localidades de estudo mostrando as coordenadas geográficas, o número de indivíduos utilizados com o gene cyt b e o marcador ddRADseq, bem como a correspondência com as localidades da Figura 2.....	47
<b>Tabela S2.</b> Sítios diagnósticos de cada ESU baseado na filosofia de <i>Population Aggregation Analysis</i> (PAA).....	48

### CAPÍTULO 3

<b>Tabela 1.</b> Sumarização das filtragens realizadas com o banco de dados genômicos.....	112
<b>Tabela 2.</b> Comparação entre os valores de log Likelihood para cada modelo do BioGeoBEARS estimados com base nos dados de cyt b.....	113
<b>Tabela 3.</b> Parâmetros demográficos estimados com base nos melhores modelos de migração estimados no program G-PhoCS.....	114

## INTRODUÇÃO GERAL

A região Neotropical é uma zona biogeográfica hiperdiversa que compreende os principais biomas tropicais. Parte dessa biodiversidade permanece não descrita e provavelmente é subestimada (Hughes *et al.*, 2013). Desde o último século, biólogos evolucionistas têm estudado insistenteamente a origem de tanta diversidade nas regiões tropicais e se esforçam para entender os processos biogeográficos e evolutivos que estão por trás dessa diversificação tão alta (Moritz *et al.*, 2000; Bermingham & Dick, 2005).

Muitas teorias foram desenvolvidas na tentativa de encontrar quais dos eventos biogeográficos melhor explicariam o padrão atual de distribuição da biodiversidade neotropical, tais como: “rios como barreiras”, “refúgios”, “arcos estruturais”, entre outras (ver revisão em Leite & Rogers, 2013). No entanto, segundo Moritz *et al.* (2000) todas essas hipóteses podem ser colocadas em duas categorias: as que invocam baixas taxas de extinção (hipótese dos museus) e as que focam em altas taxas de especiação (hipótese dos distúrbios). Atualmente é aceito que somente múltiplas causas atuando em conjunto poderiam gerar uma diversificação de grande magnitude nos trópicos (Bush, 1994; Moritz *et al.*, 2000), em especial porque as teorias não são mutuamente excludentes e são temporalmente distintas.

A tendência atual da biogeografia é investigar como eventos paleogeográficos, ocorridos em dado espaço e tempo, afetaram a biota de determinada região, em detrimento da clássica ideia de elaborar uma hipótese geral capaz de explicar a diversificação de todas as espécies. Nesse sentido um campo emergente em biogeografia tem sido a filogeografia comparada, uma subárea da biogeografia que estuda a distribuição geográfica da variação genética de populações naturais em um contexto histórico e comparativo (ver Avise *et al.*, 1987; Avise, 2000; Arbogast & Kenagy, 2001; Riddle & Hafner, 2004). Essa ciência baseia-se

no princípio de que determinado evento histórico pode ter criado padrões semelhantes de distribuição da variabilidade genética em espécies coexistentes e com estilos de vida semelhantes. Em contrapartida, espera-se que espécies que apresentam histórias de vida diferentes respondam diferentemente ao mesmo evento histórico (Moritz *et al.*, 2000; Henle *et al.*, 2004; Ewers & Didham, 2006; Prug *et al.*, 2008). Para a filogeografia comparada, tão importante quanto o estilo de vida é que as espécies a serem comparadas possuam uma distribuição ancestral semelhante.

Ao testar uma hipótese biogeográfica é importante levar em consideração o estilo de vida e a capacidade de dispersão que a espécie estudada possui, dentre outros fatores ecológicos (Dormann *et al.*, 2007; Öckinger *et al.*, 2010). De um modo geral, as hipóteses biogeográficas têm sido testadas através de espécies cujo estilo de vida é estritamente terrestre ou estritamente aquático e, portanto, pouco se sabe sobre a biogeografia de espécies com hábitos semiaquáticos, como é o caso dos crocodilianos. Para dada espécie aquática um evento de captura de cabeceiras pode proporcionar um contato secundário entre linhagens previamente isoladas, mas para outra espécie, que estava ausente na bacia adjacente, o mesmo evento de captura pode favorecer a geodispersão e a ampliação da área de distribuição prévia da espécie.

Do ponto de vista da conservação, em geral, espécies que são amplamente distribuídas são classificadas como de baixa prioridade, principalmente porque são consideradas abundantes e com maiores possibilidades de sobrevivência. No entanto, o desenvolvimento e ampliação do uso de ferramentas moleculares em espécies não-modelos tem revelado que muitas das espécies amplamente distribuídas são, na verdade, complexos de espécies, possivelmente escondendo linhagens que estão sob ameaça de extinção (Frankham

*et al.*, 2010).

É fato que o número de espécies ameaçadas ultrapassa, e muito, os recursos destinados à conservação, gerando a necessidade de se estabelecer prioridades. Portanto, reconhecer espécies crípticas e linhagens evolutivas independentes é fundamental para direcionar esses recursos para espécies ou linhagens prioritárias (Moritz, 1994; Crandall *et al.*, 2000; Allendorf *et al.*, 2013). As linhagens evolutivas mais comumente usadas para fins de conservação são as *Evolutionarily Significant Unit* (ESU) e *Management Unit* (MU). As ESUs podem ser geneticamente estruturadas em MUs (*sensu* Moritz, 1994; Funk *et al.*, 2012), e estas, por sua vez, seriam a unidade lógica para efetuar o monitoramento e o manejo das populações (Moritz, 1994).

O conceito de ESUs e MUs surgiram justamente com o intuito de estabelecer bases racionais para definir prioridades para a conservação em nível intraespecífico (Moritz, 1994; Allendorf & Luikart, 2007). Existe um amplo debate sobre o uso ou não dessas unidades para a conservação, pois os critérios utilizados para definir ESUs são similares aos utilizados para delimitar espécies (Roe & Lydeard, 1998), além disso, cada conceito foca em diferentes etapas de um mesmo processo evolutivo, a especiação (de Queiroz, 2007). Apesar das controvérsias, biólogos conservacionistas advogam que os esforços para a conservação devem centrar-se em unidades de variação genética independentes, com potencial adaptativo para responder aos desafios ecológicos futuros (Fraser & Bernatchez, 2001) independentemente de serem reconhecidas taxonomicamente como espécies. Eles argumentam que, do ponto de vista da conservação, é mais importante manter o potencial evolutivo de populações naturais (Moritz *et al.*, 1995; Waples, 1995), que merecem ser conservadas por possuírem adaptações locais únicas que seriam perdidas caso elas viessem a ser extintas (Haig, 1998).

Fraser & Bernatchez (2001) propuseram um método integrativo que concilia os diferentes pontos de vistas sobre quais critérios deveriam ser utilizados para delimitar ESUs (Waples, 1991; Avise, 1994; Moritz, 1994; Crandall *et al.*, 2000), o *framework Adaptive Evolutionary Conservation* (AEC). Os autores sugerem utilizar os pontos fortes, sozinhos ou em conjunto, de cada critério operacional previamente proposto, de modo que, quanto mais critérios operacionais forem preenchidos maior a evidência de que as populações são linhagens independentes. De acordo com o AEC *framework*, os principais critérios operacionais utilizados para delimitar ESUs são: isolamento reprodutivo (Waples, 1991), ausência de fluxo gênico (Waples, 1991; Moritz, 1994; Fraser & Bernatchez, 2001), monofilia recíproca no mtDNA e diferenças significativas nas frequências alélicas de locus nucleares (Avise, 1994; Moritz, 1994), e ausência de troca genética e ecológica com linhagens irmãs (Vogler & DeSalle, 1994; Crandall *et al.*, 2000).

Como pode ser observado, muitos desses critérios são essencialmente avaliados por meio de ferramentas moleculares, que têm um papel fundamental não só no processo de delimitação de unidades evolutivas, mas também na delimitação de espécies. Há pouco tempo, as ferramentas moleculares mais utilizadas para diversos fins em genética de populações (Avise, 2000, 2008), inclusive na identificação de ESUs, eram as sequências do DNA mitocondrial (mtDNA). Algumas características vantajosas para estudos filogeográficos tornaram esse marcador popular, tais como: (1) ausência de recombinação, que permite avaliar a história evolutiva sem o efeito da recombinação e da hibridização; (2) tamanho efetivo populacional quatro vezes menor que o genoma nuclear, o que permite investigar eventos demográficos mais recentes; (3) e altas taxas evolutivas quando comparadas às do genoma nuclear, tornando-o suficientemente polimórfico para estudos intraespecíficos (ver

Brown *et al.*, 1982; Meyer, 1993; Li, 1997; Nedbal & Flynn, 1998).

Em contrapartida, algumas desvantagens devem ser levadas em conta, como é o caso do viés sexual, da impossibilidade de detectar eventos de hibridização e da saturação entre linhagens filogeneticamente distantes. O viés sexual pode ocorrer quando machos ou fêmeas do táxon estudado possuem comportamentos filopátricos ou ainda capacidades de dispersão diferentes (Hudson & Turelli, 2003), e nesses casos a história evolutiva contada pelo marcador mitocondrial seria referente somente à história das fêmeas. Outro fator importante é a impossibilidade do mtDNA de detectar hibridização, inclusive em casos de captura do mtDNA entre espécies próximas, podendo induzir interpretações evolutivas erradas ou incompletas. A saturação, por sua vez, ocorreria quando são avaliados táxons menos relacionados filogeneticamente, e a taxa de mutação é tão alta que começam a acontecer mutações homoplásicas (em sítios que já haviam mutado antes), saturando o sinal evolutivo.

Os resultados obtidos com o uso de um marcador molecular representam apenas a história evolutiva daquele gene e podem não refletir a história real da espécie (ver revisão em Nichols, 2001). Na busca por reconstruir árvores de espécies em detrimento de árvores gênicas, os biólogos evolucionistas passaram a sequenciar também genes nucleares, que em geral apresentam baixo polimorfismo, e portanto, pouco sinal filogenético em nível intraespecífico. Nessa abordagem é necessário sequenciar não só uma quantidade razoável de indivíduos como também de lócus, sendo necessário um alto consumo de tempo e dinheiro para obtenção desses dados.

A recente revolução tecnológica, com o advento dos sequenciadores de nova geração, ou *Next Generation Sequencing* (NGS), possibilitaram o uso de marcadores amplamente distribuídos no genoma como RADseq (*Restriction site Associated DNA sequencing*) e SNPs

(*Single Nucleotide Polymorphism*). Tais marcadores estão revolucionando os campos de Biogeografia e Genética da Conservação, pois permitem a obtenção de uma grande quantidade de dados representativos do genoma de maneira rápida e custo-efetiva, possibilitando maior poder estatístico, mais eficácia e maior resolução (Allendorf *et al.*, 2010). A aplicabilidade dos marcadores genômicos é ampla, possibilitando inclusive a investigação de processos importantes para desvendar a história evolutiva de espécies não-modelos, tais como: *Incomplete Lineage Sorting* (ILS), *Lateral Gene Transfer* (LGT), hibridização, demografia histórica, tempos de divergência, para citar alguns (Luikart *et al.*, 2003; Allendorf *et al.*, 2010), inaugurando assim um novo campo de pesquisas, a filogenômica.

Nós realizamos um dos primeiros estudos filogenômicos em crocodilianos (Muniz et al., 2018), grupo aparentemente bem estudado devido ao seu tamanho, valor econômico e importância ecológica que desempenham (Grigg & Kirshner, 2015). Pesquisas recentes evidenciam que algumas espécies de crocodilianos com morfologia conservada e ampla distribuição são um complexo de espécies que contém linhagens evolutivas independentes (Eaton *et al.*, 2009; Hekkala *et al.*, 2011; Shirley *et al.*, 2014a). De maneira geral, as principais lacunas do conhecimento sobre o grupo se apresentam em especial nas espécies que ocorrem na região neotropical.

Na América do Sul, análises genéticas revelaram linhagens evolutivas profundas e geograficamente estruturadas em *Caiman yacare* (Godshalk, 2006), *Crocodylus acutus* (Bloor *et al.*, 2015) e em *Paleosuchus trigonatus* (Bittencourt *et al.*, em revisão). Na África, o uso de ferramentas moleculares revelou que três gêneros de crocodilianos são compostos por espécies crípticas (Eaton *et al.*, 2009; Hekkala *et al.*, 2011; Shirley *et al.*, 2014a). Tais estudos

resultaram em mudanças taxonômicas nesse grupo, como: a retomada de nomes previamente reconhecidos para as espécies *Osteolaemus osborni* (Eaton *et al.*, 2009), *Crocodylus suchus* (Hekkala *et al.*, 2011) e para o gênero *Mecistops* (McAliley *et al.*, 2006); a descoberta de novas espécies *Osteolaemus sp. nov. cf. tetraspis* (Eaton *et al.*, 2009; Franke *et al.*, 2013; Shirley *et al.*, 2014b) e *Mecistops sp. nov. cf. cataphractus* (Shirley *et al.*, 2014a); e a identificação de duas linhagens evolutivas independentes em *Osteolaemus tetraspis* (Franke *et al.*, 2013; Shirley *et al.*, 2014b).

Dentre as novas espécies de crocodilianos recém-descobertas, *C. suchus* e *Mecistops sp. nov. cf. cataphractus* apresentam indícios de que estão muito ameaçadas, devido ao declínio populacional ou mesmo à extinção em boa parte da sua distribuição, principalmente devido à caça e perda de habitat em razão de atividades humanas (Hekkala *et al.*, 2011; Shirley *et al.*, 2014a). Fato semelhante ocorreu com *Osteolaemus tetraspis*, que mesmo antes de ser reconhecido como um complexo de espécies crípticas, já era considerado Vulnerável pela lista vermelha da *International Union for Conservation of Nature* (IUCN), principalmente devido à forte pressão de caça e à intensa perda de habitats em sua distribuição (Eaton, 2010). Estudos populacionais ainda estão sendo conduzidos para determinar o *status* e facilitar a conservação independente dessas novas espécies (Smolensky, 2015), porém, não é difícil deduzir que as distribuições geográficas mais restritas e os tamanhos efetivos populacionais menores do que se pensava, colocam-nas em um estado de maior risco de extinção que antes.

O jacaré-paguá (ou Cuvier's dwarf caiman, *Paleosuchus palpebrosus*) é um crocodiliano amplamente distribuído em 10 países da América do Sul. A IUCN o classifica como *Low Concern*, principalmente devido a sua extensa área de distribuição, apesar de

admitir se tratar de uma espécie pouco conhecida (Magnusson & Campos, 2010). As principais ameaças são em decorrência da perda e modificação de seus habitats, como: desmatamento, urbanização, poluição e represamento de rios (Campos *et al.*, 1995; Magnusson & Campos, 2010; Campos *et al.*, 2013; Campos *et al.*, 2015).

*P. palpebrosus* apresenta várias características comuns às espécies do gênero *Osteolaemus*, dentre as quais estão: o pequeno porte, estando entre os menores crocodilianos viventes (Eaton *et al.*, 2009; Campos *et al.*, 2010); a capacidade de viver em pequenos riachos de cabeceira, em ambientes florestais; e apresentarem os hábitos mais terrestres dentre os crocodilianos (Campos *et al.*, 1995; Eaton, 2010; Magnusson & Campos, 2010). Essas características comuns indicam que ambos podem responder de forma semelhante a eventos paleogeográficos e que *Paleosuchus* pode estar tão propenso a cripticidade quanto *Osteolaemus*. Essa possibilidade é acentuada pelo fato de *P. palpebrosus* ter ampla distribuição e ocorrer em várias bacias hidrográficas (Magnusson & Campos, 2010), separadas por barreiras ecogeográficas ou biogeográficas reconhecidas para diversas espécies de vertebrados aquáticos e semi-aquáticos, tais como: peixes, ariranhas, botos e outros crocodilianos (Hubert & Renno, 2006; Hubert *et al.*, 2007; Hrbek *et al.*, 2008; Pickles *et al.*, 2011; Gravena *et al.*, 2014, 2015).

A biologia evolutiva de *Paleosuchus palpebrosus*, permanecia desconhecida até pouco tempo atrás, quando Muniz (2012) investigou a filogeografia da espécie em parte da Amazônia e no Pantanal. O estudo utilizou sequências completas do gene citocromo *b* (*cyt b*) e detectou uma estrutura genética profunda na espécie, lançando luz sobre a distribuição de sua variabilidade genética e levantando muitas outras questões a serem investigadas.

No presente estudo, nós fizemos uso de marcadores amplamente distribuídos no

genoma em conjunto com o marcador mitocondrial para investigar a diversidade críptica em *Paleosuchus palpebrosus*, produzindo importantes contribuições para a biogeografia, conservação e biologia evolutiva desse complexo de espécies.

## OBJETIVOS

### Objetivo Geral

- ✓ Determinar unidades evolutivas independentes no complexo de espécies *Paleosuchus palpebrosus*, avaliando os processos biogeográficos e evolutivos que atuaram na diversificação dessas linhagens.

### Objetivos Específicos

- ✓ Delimitar Unidades Evolutivas Significantes (ESUs – Evolutionary Significant Units) e Unidades de Manejo (MUs - Management Units) em *Paleosuchus palpebrosus*.
- ✓ Avaliar a estruturação genética entre as linhagens encontradas e inferir os níveis de diversidade gênica de cada uma delas, bem como examinar as implicações para a conservação desse complexo de espécies.
- ✓ Inferir a ocorrência de eventos biogeográficos históricos que contribuíram para moldar a distribuição atual das ESUs e MUs identificadas.
- ✓ Propor um cenário hipotético de evolução da paisagem para explicar a diversificação de *Paleosuchus palpebrosus* e de outros taxões aquáticos ao longo do corredor Amazônia-Madeira-Paraguai.
- ✓ Desvendar a história evolutiva do complexo *Paleosuchus palpebrosus* inferindo a distribuição ancestral do grupo e a rota de dispersão de cada linhagem evolutiva.
- ✓ Investigar quais modelos de diversificação das linhagens melhor adequam-se aos sistemas estudados, discriminando retenção de polimorfismo ancestral e fluxo gênico atual ou ancestral nas zonas de contato entre as linhagens evolutivas.

# CAPÍTULO 1

---

Muniz FL, Campos Z, Hernández Rangel SM,  
Martínez JG, Souza BC, De Thoisy B, Botero-  
Arias R, Hrbek T, Farias IP (2018) Delimitation  
of evolutionary units in Cuvier's dwarf caiman,  
*Paleosuchus palpebrosus* (Cuvier, 1807): insights  
from conservation of a broadly distributed  
species. *Conservation Genetics*, 19 (3): 599-610.  
<https://doi.org/10.1007/s10592-017-1035-6>

## Delimitation of evolutionary units in Cuvier's dwarf caiman, *Paleosuchus palpebrosus* (Cuvier, 1807): insights from conservation of a broadly distributed species

F. L. Muniz<sup>1,2</sup>  · Z. Campos<sup>3</sup> · S. M. Hernández Rangel<sup>1,2</sup> · J. G. Martínez<sup>1,4,5</sup> · B. C. Souza<sup>6</sup> · B. De Thoisy<sup>7,8</sup> · R. Botero-Arias<sup>9,10</sup> · T. Hrbek<sup>1</sup> · I. P. Farias<sup>1</sup>

Received: 24 August 2017 / Accepted: 30 November 2017  
© Springer Science+Business Media B.V., part of Springer Nature 2017

### Abstract

An important goal of evolutionary and conservation biology is the identification of units below the species level, such as Evolutionarily Significant Units (ESUs), providing objectively delimited units for species conservation and management. In this study we tested the hypothesis that Cuvier's dwarf caiman (*Paleosuchus palpebrosus*)—a species broadly distributed across several biomes and watersheds of South America—is comprised of different ESUs. We analyzed mitochondrial cytochrome b sequences of 206 individuals and 532 unlinked ddRAD loci of 20 individuals chosen from amongst the mitochondrial haplogroups. Analysis of the cytochrome b sequences revealed four mitochondrial clusters, while STRUCTURE analysis of ddRAD loci detected three genomic clusters with different levels of mixture between them. Using the Adaptive Evolutionary Conservation (AEC) framework we identified three ESUs: “Amazon”, “Madeira-Bolivia” and “Pantanal”; one of them composed of two different Management Units (MUs), “Madeira” and “Bolivia”. In general, based on the comparisons with other crocodilian species, genetic diversity of each lineage was moderate however, the Madeira MU showed fivefold lower genetic diversity than other geographic groups. Considering the particularities of each *Paleosuchus palpebrosus* conservation unit, we recommend that the conservation status of each is evaluated separately. Tropical biodiversity is largely underestimated and in this context the broadly distributed species are the most likely candidates to harbor distinct evolutionary lineages. Thus, we suggest that conservation research should not neglect species that are generally considered of Least Concern by IUCN due to the taxon's broad geographic distribution.

**Keywords** Evolutionarily significant unit · Management unit · Genetic diversity · DdRADseq · Cytochrome b · Gene flow

---

**Electronic supplementary material** The online version of this article (<https://doi.org/10.1007/s10592-017-1035-6>) contains supplementary material, which is available to authorized users.

 F. L. Muniz  
fabiolm\_bio@yahoo.com.br

<sup>1</sup> Laboratory of Animal Genetics and Evolution (LEGAL), Department of Biology, Federal University of Amazonas (UFAM), Manaus, Amazonas, Brazil

<sup>2</sup> Graduate Program in Genetics, Conservation and Evolutionary Biology, National Institute for Amazonian Research (INPA), Manaus, Amazonas, Brazil

<sup>3</sup> Wildlife Laboratory, Brazilian Agricultural Research Corporation (EMBRAPA) Pantanal, Corumbá, Mato Grosso Do Sul, Brazil

<sup>4</sup> Grupo de Investigación Biociencias, Facultad de Ciencias de la Salud, Institución Universitaria Colegio Mayor de Antioquia, Medellín, Colombia

<sup>5</sup> Grupo de Pesquisa em Genética Molecular e Citogenética, Programa de Pós-Graduação em Biotecnologia e Recursos Naturais (MBT), Escola Superior de Ciências da Saúde, Universidade do Estado do Amazonas, Manaus, Brazil

<sup>6</sup> Chico Mendes Institute for Biodiversity Conservation (ICMBio), Boa Vista, Roraima, Brazil

<sup>7</sup> Institut Pasteur de la Guyane, Cayenne, French Guiana

<sup>8</sup> Association Kwata, Cayenne, French Guiana

<sup>9</sup> Caiman Research in Conservation and Management Program, Instituto Mamirauá para o Desenvolvimento Sustentável, Tefé, Amazonas, Brazil

<sup>10</sup> Department of Wildlife Ecology and Conservation, University of Florida, Gainesville, FL, USA

# **Delimitation of evolutionary units in Cuvier's dwarf caiman, *Paleosuchus palpebrosus* (Cuvier, 1807): insights from conservation of a broadly distributed species**

**FL Muniz<sup>1,2</sup> · Z Campos<sup>3</sup> · SM Hernández Rangel<sup>1,2</sup> · JG Martínez<sup>1,4,5</sup> · BC Souza<sup>6</sup> · B De Thoisy<sup>7,8</sup> · R Botero-Arias<sup>9,10</sup> · T Hrbek<sup>1</sup> · IP Farias<sup>1</sup>**

<sup>1</sup>Laboratory of Animal Genetics and Evolution (LEGAL), Department of Biology, Federal University of Amazonas (UFAM), Manaus, Amazonas, Brazil

<sup>2</sup>National Institute for Amazonian Research (INPA), Graduate Program in Genetics, Conservation and Evolutionary Biology, Manaus, Amazonas, Brazil

<sup>3</sup>Wildlife Laboratory, Brazilian Agricultural Research Corporation (EMBRAPA) Pantanal, Corumbá, Mato Grosso do Sul, Brazil

<sup>4</sup>Grupo de Investigación Biociencias, Facultad de Ciencias de la Salud, Institución Universitaria Colegio Mayor de Antioquia, Medellín, Colombia

<sup>5</sup>Grupo de Pesquisa em Genética Molecular e Citogenética, Programa de Pós-Graduação em Biotecnologia e Recursos Naturais (MBT), Escola Superior de Ciências da Saúde, Universidade do Estado do Amazonas, Manaus, Brazil

<sup>6</sup>Chico Mendes Institute for Biodiversity Conservation (ICMBio), Boa Vista, Roraima, Brazil

<sup>7</sup>Institut Pasteur de la Guyane, Cayenne, French Guiana

<sup>8</sup>Association Kwata, Cayenne, French Guiana

<sup>9</sup>Caiman Research in Conservation and Management Program, Instituto Mamirauá para o Desenvolvimento Sustentável, Tefé, Amazonas, Brazil

<sup>10</sup>Department of Wildlife Ecology and Conservation, University of Florida, Gainesville, FL, USA

**Keywords:** Evolutionarily Significant Unit; Management Unit; genetic diversity; ddRADseq; cytochrome b; gene flow

Corresponding author: Fábio L. Muniz; e-mail: [fabiolm\\_bio@yahoo.com.br](mailto:fabiolm_bio@yahoo.com.br); phone number: +55(92)99248-0328

## Acknowledgments

This study would have been impossible without the people who helped with the field collections: Daniel Martins, Dênis Tilcara, Deyla Oliveira, José Augusto da Silva, Manoel Rodrigues, Pedro Almeida, Tânia Sanaiotti, Valéria Machado and William Vasconcelos; or without Guto Ruffeil who deposited samples in the CTGA/UFAM tissue collection. We are also thankful to Mitchell Eaton for additional information about *Osteolaemus* species. This project was approved by Embrapa ethics committee under the Permit no. 009/2016 and the caimans were captured under License no. 13048-1 granted by the Brazilian Institute of Environment and Renewable Natural Resources (IBAMA). In French Guiana, the species is not protected and sampling does not require license. This study was financed by the following grants: CNPq/CT-Amazon Project no. 575603/2008-9 awarded to IPF, CNPq Project no. 482662/2013-1 to TH, and CNPq Project no. 470383/2007-0 and 479179/2014 to ZC. We are also grateful for the additional financial and logistical support from Embrapa Pantanal (Macroprogram 3), Instituto Nacional de Pesquisas da Amazônia (INPA), Fundect, O Boticário Foundation, Instituto Chico Mendes de Conservação da Biodiversidade (ICMBio) and Santo Antônio Energia. This work is part of FM's thesis in the Genetics, Conservation and Evolutionary Biology program of INPA/UFAM. FM is supported by a grant from FAPEAM, and IPF and TH by a grant from CNPq.

## ABSTRACT

An important goal of evolutionary and conservation biology is the identification of units below the species level, such as Evolutionarily Significant Units (ESUs), providing objectively delimited units for species conservation and management. In this study we tested the hypothesis that Cuvier's dwarf caiman (*Paleosuchus palpebrosus*)—a species broadly distributed across several biomes and watersheds of South America—is comprised of different ESUs. We analyzed mitochondrial cytochrome b sequences of 206 individuals and 532 unlinked ddRAD loci of 20 individuals chosen from amongst the mitochondrial haplogroups. Analysis of the cytochrome b sequences revealed four mitochondrial clusters, while STRUCTURE analysis of ddRAD loci detected three genomic clusters with different levels of mixture between them. Using the Adaptive Evolutionary Conservation (AEC) framework we identified three ESUs: “Amazon”, “Madeira-Bolivia” and “Pantanal”; one of them composed of two different Management Units (MUs), “Madeira” and “Bolivia”. In general, based on the comparisons with other crocodilian species, genetic diversity of each lineage was moderate however, the Madeira MU showed fivefold lower genetic diversity than other geographic groups. Considering the particularities of each *Paleosuchus palpebrosus* conservation unit, we recommend that the conservation status of each is evaluated separately. Tropical biodiversity is largely underestimated and in this context the broadly distributed species are the most likely candidates to harbor distinct evolutionary lineages. Thus, we suggest that conservation research should not neglect species that are generally considered of Least Concern by IUCN due to the taxon’s broad geographic distribution.

## INTRODUCTION

One of the most important goals of evolutionary and conservation biologists is to identify units below the species level for conservation purposes. The concept of the Evolutionarily Significant Unit (ESU) was developed to provide an objective method for identifying such units. The first formulation of this concept by Ryder (1986) gave rise to an intense debate and several alternate definitions (Waples 1991; Avise 1994; Moritz 1994; Crandall et al. 2000; Fraser and Bernatchez 2001). Despite the divergence in operational criteria to delimit ESUs, the fundamental objective of all ESU definitions is to identify intraspecific units of genetic variation that have the potential of adaptively responding to future ecological challenges (Fraser and Bernatchez 2001).

Most of the disagreement between the delimitation of ESUs has its origin in the species concept debate (Roe and Lydeard 1998), ultimately stemming from focusing on different emergent properties of species (de Queiroz 2007). Trying to reconcile these differing viewpoints for the benefit of conservation efforts, Frazer and Bernatchez (2001) proposed the Adaptive Evolutionary Conservation (AEC) integrative framework that uses the strengths, either alone or in combination, of various operational criteria for delimiting ESUs. The main operational criteria used to identify an ESU are thus: reproductive isolation (Waples 1991), absence of gene flow (Waples 1991; Moritz 1994; Frazer and Bernatchez 2001), reciprocal monophyly in mitochondrial DNA (mtDNA), significant differences in allele frequencies of nuclear loci (Avise 1994; Moritz 1994), and ecological and genetic nonexchangeability (Vogler and DeSalle 1994; Crandall et al. 2000).

The AEC framework allows one to consider both historical isolation and adaptive divergence which are extremes along the divergence spectrum that gives rise to species

(Frazer and Bernatchez 2001). Thus, the definition that best summarizes the common point among all ESUs, is that ESUs are intraspecific lineages characterized by a highly restricted gene flow among these lineages.

ESUs can follow semi-independent evolutionary trajectories for different periods of time, and thus satisfy different criteria used for detecting them (Fraser and Bernatchez 2001). In some cases, one species can be composed of only a single ESU, whereas in others, a species can include several ESUs. Furthermore, ESUs may themselves be structured, a hierarchical level generally recognized as Management Units (MUs; *sensu* Moritz 1994). MUs can be logical units for population monitoring and management (Moritz 1994). Overall, recognizing and subsequently delimiting evolutionary units below species level is an important step towards maintaining the evolutionary potential of natural populations (Moritz et al. 1995; Waples 1995).

Cryptic diversity has been discovered recently in crocodilian species both at the intraspecific (Godshalk 2006; Venegas-Anaya et al. 2008; Hekkala et al. 2010, 2011; Cunningham et al. 2016) as well as the interspecific level (Eaton et al. 2009; Franke et al. 2013; Shirley et al. 2014a,b). One important question in crocodilian systematics and the main goal of this study is the mapping of intraspecific genetic diversity of Cuvier's dwarf caiman (*Paleosuchus palpebrosus*). *Paleosuchus palpebrosus* is one of the smallest of all living crocodilians (Campos et al. 2010); it is a forest-associated semi-aquatic taxon that occupies a number of different biomes and watersheds throughout its distribution in northern and central South America (Magnusson and Campos 2010). All these characteristics make it a promising species to test the existence of lineages with independent trajectories and significant adaptive differences.

The International Union for the Conservation of Nature (IUCN) assesses the species as Least Concern (IUCN 2016; last assessment in 1996); however, the Brazilian Chico Mendes Institute of Biodiversity Conservation (ICMBIO) alerts that this species should be monitored due to persistent hunting pressure, habitat loss, fragmentation or modification caused by human activities, including dam construction (Campos et al. 2013). These authors suggested that the species' conservation and long-term persistence is dependent on its access to relatively pristine habitat, located ideally within conservation units.

Given the broad geographic distribution of the species and occurrence in several independent river basins separated by important biogeographic barriers, we aim to test the hypothesis that *P. palpebrosus* is comprised of different ESUs along an environmental gradient extending from the Pantanal to Amazon and the Guianas. Our objectives were as follows: (1) delimit ESUs and Management Units (MUs) in *P. palpebrosus*; (2) evaluate the genetic diversity of the *P. palpebrosus* ESUs and MUs found; and (3) examine the implications of these findings for the conservation of *P. palpebrosus*.

## METHODS

### Sample and molecular data collection

We sampled 206 *P. palpebrosus* individuals from sites throughout its distribution, including in the Amazon Basin, the Madeira River Basin and the upper Paraguay River Basin (Fig. 1; Online Resource 1 – Table S1). We located the individuals through active nocturnal searches and captured them using a noose. We obtained tissue samples by removing a caudal scute from adults, subadults or one hatchling per clutch. Tissues were stored in cryogenic

tubes containing 96% ethanol and deposited in the Animal Genetics Tissue Collection (CTGA) at the Federal University of Amazonas (UFAM), Amazonas, Brazil. We extracted whole genomic DNA using a phenol-chloroform protocol (Green and Sambrook 2012). Subsequently, we amplified the whole cytochrome b (cyt b) gene and sequenced the amplicons on an ABI 3130xl automated sequencer (Applied Biosystems), according to the protocol described by Hrbek et al. (2008).

We also obtained reduced representation genomic sequences using the double digest RAD sequencing protocol (ddRADseq) (Peterson et al. 2012) of five individuals from each identified cyt b cluster of *P. palpebrosus*, totaling 20 specimens (Online Resource 1 – Table S1).

We used an adapted ddRADseq protocol to allow simultaneous digestion and adaptor ligation, with sequencing carried out on a 318 Ion PGM chip (Life Technologies™) using the Ion PGM Sequencing 400 kit (Life Technologies™), in the IonTorrent PGM. Briefly, 200 ng of genomic DNA of each individual was digested with SDAI and CSP6I restriction enzymes (Fermentas) and the IonTorrent P and A adapters were linked to the digested fragments, all in one step. The fragments were enriched via PCR. The A adaptor is a “Y divergent” (Coyne et al. 2004) adaptor which results in enrichment of only those ddRAD fragments that have one P1 and one A adaptor. Furthermore, the A adaptor contains a unique molecular barcode for identification of individuals. The fragments were then size selected on Pippin Prep (Sage Science), and fragments between 320 and 400 bp were used for sequencing. Genome size of alligatorids is approximately  $2.2 \times 10^9$  bp (based on sequenced genomes of *Alligator mississippiensis* and *A. sinensis* deposited in Genbank), resulting in an expectation of ~ 15,000 ddRAD fragments in the range of 300 to 400 bp. The complete protocol is available on

GitHub (<https://github.com/legalLab>).

We converted the raw IonTorrent sequence reads from BAM to FASTQ format using SAMtools v1.3.1 (Li et al. 2009). Sequencing reads were then demultiplexed and assembled into *de novo* loci using the PyRAD v3.0.66 pipeline (Eaton 2014). During *de novo* assembly, we removed reads with errors in barcodes, required that each base in reads have a minimum Phred quality score of 20 else be converted to missing, allowing maximum of four missing nucleotides per read and minor allele frequency greater than 0.01. A minimum read coverage of 5x is desirable for genotyping-by-sequencing, including ddRADseq assemblies (Kenny et al. 2011; Peterson et al. 2012), thus we enforced a minimum coverage of 6x. Sequences were subsequently clustered by similarity at 88% similarity (PyRAD default) using VSEARCH (Rognes et al. 2016) and aligned using MUSCLE (Edgar 2004). In the final STRUCTURE (Pritchard et al. 2000) dataset we only included those loci that were present in at least 50% of the individuals, a cut-off value that appears to maximize phylogenetic informativeness (Streicher et al. 2015).

### Cyt b and ddRADseq analysis

We reconstructed a cyt b haplotype network by first estimating a Maximum Likelihood (ML) topology in Garli v0.95 (Zwickl 2006) using GTR+G evolutionary model estimated in jMODELTEST2 (Darriba et al. 2012) and then using HAPLOVIEWER (Salzburger et al. 2011) to reconstruct the haplotype network.

The cyt b sequences were collapsed into 22 haplotypes, and reciprocal monophyly of cyt b groups was assessed via ML phylogenetic analysis in Garli v2.01 (Zwickl 2006) and Bayesian Inference (BI) in MrBayes v3.2.2 (Ronquist et al. 2012). Both analysis were

performed using the GTR+G model of molecular evolution, with one sequence of the sister species *Paleosuchus trigonatus*, used as outgroup. Two replicate searches using four chains and default priors were performed on each analysis. Support for clades in the ML analysis was estimated by 1,000 bootstrap replicates. BI was performed using  $10^6$  generations, sampling every 1,000 generations and discarding the first 25% of the sampled trees. The topologies were summarized and visualized in FigTree v1.4.2 (Rambaut, 2014).

We also inferred population structure based on nucleotide frequencies of the cyt b gene using the Bayesian approach for assignment of individuals, implemented in the program BAPS v6.0 (Corander et al. 2008). The method provides the posterior probabilities for different numbers of clusters of individuals (K). We performed individual level mixture analysis for multiple defined clusters (K = 5, 10, 15 or 20 clusters), with 10 independent runs for each K values. The K with the highest posterior probability was selected as the most likely data partition.

We delimited mtDNA geographic groups based on haplotype network, phylogenetic tree and population structure analyses. We assessed the degree of genetic structure and the isolation between the geographic groups using Analysis of Molecular Variance (AMOVA) implemented in the program Arlequin v3.5 (Excoffier and Lischer 2010). Significance of the results was assessed via 10,000 permutations. All above analysis allowed us to evaluate the absence of historical gene flow, the genetic non-exchangeability and the reciprocal monophyly between mitochondrial lineages.

In order to verify whether geographical groups show significant differences in allele frequencies of nuclear loci we performed a STRUCTURE analysis using our ddRAD data. We used the admixture and correlated allele frequency model of STRUCTURE to infer

population structure without conditioning on sampling locality (no location prior). We collected 110,000 samples from the MCMC chain of which the first 10,000 were discarded as burn-in and the remaining 100,000 steps were used for this analysis. The number of groups ( $K$ ) varied from 1 to 6, with 10 independent runs for each  $K$  value. The convergence between independent runs was assessed via examination of  $\alpha$  values and profile of posterior probabilities. The  $Q$  values from each of the 10 independent runs for each  $K$  scenario were extracted using the program STRUCTURE HARVESTER 0.6.92 (Earl and VonHoldt 2012) and summarized in the program CLUMPP 1.1.2 (Jakobsson and Rosenberg 2007). Results were visualized in the program DISTRUCT 1.1 (Rosenberg 2004). The most likely number of genomic clusters ( $K$ ) was inferred using the method of Evanno et al. (2005).

We used the program MIGRATE v.3.6 (Beerli 2006) to estimate the effective population size ( $N_e$ ) of each geographic group and the effective number of migrants ( $Nm$ ) between them using both cyt b and ddRAD data. We first estimated the best model of gene flow for “Madeira” and “Bolivia”, as described below, and then we calculated the  $Nm$ . We filtered loci separately for each geographic group (“Amazon”: 593 loci; “Madeira”: 500 loci; “Bolivia”: 361 loci; “Pantanal”: 256 loci) in order to obtain more robust estimative of the  $N_e$ . We did not estimate  $Nm$  between “Amazon” and “Madeira” or “Pantanal” and “Bolivia”, since these clusters represent distinct ESUs (see results).

We verified the existence of asymmetric gene flow by testing four different gene flow models between “Madeira” and “Bolivia” (1- bidirectional gene flow, 2 and 3- unidirectional gene flow in opposite directions, 4- panmitic population with infinite gene flow). We used the thermodynamic integration method to obtain the log marginal likelihood (logML) for each model and the same prior settings for all models. The search strategy for the cyt b dataset used

static heating scheme with four chains at different temperatures (1.0, 1.5, 3.0, 100000.0) and four replicates of one long chain with 40 million generations, discarding a burn-in of 4 million and sampling every 400 generations. To test the same four gene flow models based on ddRAD dataset we used 130 loci filtered in the PyRAD that were present in at least four of the five individuals in both Madeira and Bolivia sampled groups. For this dataset we used a heating scheme, two replicates of one long chain with 400 million generations, burn-in of 20 million and sampling every 1000 generations. We recorded the Bezier approximation value for each model and calculate the posterior probabilities to choose the best model for each dataset.

Considering that genetic diversity is already recognized as important to maintain the capacity of a species to adapt to a changing environment (Frankham et al. 2004), and thus is becoming an important parameter to be monitored in conservation biology, we estimated the genetic diversity based on cyt b dataset for each identified geographic group using Arlequin (Excoffier and Lischer 2010). The estimators used were: number of haplotypes ( $H_p$ ), nucleotide diversity ( $\pi$ ), and genetic diversity (Nei, 1987). We also assessed the Tajima's  $D$  (Tajima 1989) and Fu's  $F_s$  (Fu 1997) historical demography tests by using the same program. We compared the values found for each geographic group in *P. palpebrosus* with those of other crocodilian species in order to assess the relative levels of genetic diversity of the species.

## RESULTS

The cyt b haplotype network showed three geographically restricted haplogroups with 15 to 18 mutational steps between them (Fig. 1C: haplogroup I, haplogroups II+III and haplogroup IV). A Population Aggregation Analysis (PAA) depicting diagnostic sites for each

cyt b haplogroup is provided in the Online Resource 1 (Table S2). The haplogroup I occupies the entire Amazon basin, except the Madeira basin, as well as the Maroni and Oiapoque basins, which drain the Guiana Shield directly into the Atlantic Ocean (Fig. 1A). The haplogroups II+III occupies the entire Madeira River basin including the Bolivian sub-basin (Fig. 1A), and the haplogroup IV is restricted to the Pantanal in the upper Paraguai River basin (Fig. 1A). The haplogroup I is isolated from the haplogroup IV by 18 mutational steps and from the haplogroups II+III by 15 mutational steps while the haplogroup IV is isolated from the haplogroups II+III by 16 mutational steps. The haplogroups II and III are geographically structured (Fig. 1C), so that only three adjacent localities have cyt b haplotypes from both haplogroups, two of these localities are in the upper Madeira River and one in the Mamore River (Fig. 1A).

The ML and BI phylogenetic trees show identical topologies and well supported reciprocal monophyly of the red, green+blue and yellow mitochondrial groups (Fig. 2). The genetic population structure analysis using the cyt b gene indicated that the optimal number of clusters was four ( $K= 4$ ,  $\text{logML}= -670.6851$ ). The BAPS barplot result (Fig. 1B) corresponds to the haplotype network (Fig. 1C) and the geographical distribution of the cyt b clusters were identical to the geographical distribution of the haplogroups (Fig. 1A).

Based on the above analysis we identified three genetic clusters geographically partitioned in the Amazon, Madeira-Bolivia and the Pantanal (Fig. 1). Cyt b haplotypes within the “Madeira-Bolivia” group are non-randomly distributed such that one cluster occurs predominantly in the Madeira and another predominantly in Bolivia. Thus, an AMOVA analysis was performed considering four geographic groups, “Amazon”, “Madeira”, “Bolivia” and “Pantanal”. This analysis revealed strong global genetic structuring ( $\Phi_{ST}$  of 0.959,  $p <$

0.008) as well as strong pairwise differentiation measured as  $F_{ST}$  between geographic groups (Table 1). Even the lowest pairwise  $F_{ST}$  value found in our study ( $\Phi_{ST} = 0.769$ ,  $p < 0.008$ ) indicates strong population genetic differentiation.

Next generation sequencing produced approximately 3.6 million usable reads after processing in PyRAD. Between 2000 to 3000 loci per individual with an average coverage between 7.5-8.5x was obtained before limiting the dataset to those loci that were present in  $\geq 50\%$  of sampled individuals. A total of 722 loci were sampled in  $\geq 50\%$  of sampled individuals. These 722 loci represented 532 unlinked SNPs. The SNP markers detected genetic structuring of *P. palpebrosus* revealing a similar geographical pattern (Fig. 3A) as the mtDNA data.

The STRUCTURE analysis detected three genomic clusters, two of which were concordant with cyt b geographic clusters, the "Amazon" and "Pantanal" (Fig. 3B; Table 2). The third genomic cluster inferred using STRUCTURE occupies the Bolivian basin and the Madeira River, corresponding to the "Madeira-Bolivia" cyt b cluster; however, it shows a significant degree of admixture with the "Pantanal" cluster in the geographic region of Bolivia (Table 2).

Unidirectional gene flow from "Bolivia" to "Madeira" ( $Nm = 0.97$ ; 95% HPD 0.02 – 3.05) had the highest support ( $\text{logML} = -1574.38$ ) and was the most probable model (52% probability) based on the cyt b sequence dataset compared to the full model with gene flow in both directions ( $\text{logML} = -1574.47$ ; 47% probability). In the full migration model,  $Nm$  from "Bolivia" to "Madeira" was 1.08 (95% HPD 0.01 – 5.71) while  $Nm$  from "Madeira" to "Bolivia" was 0.86 (95% HPD 0.00 – 5.34). In contrast, bidirectional gene flow between "Madeira" and "Bolivia" had the highest support ( $\text{logML} = -64267.32$ ) as was the most

probable mode (99% probability) based on analysis of ddRAD dataset, showing a similar number of effective migrants from “Bolivia” to “Madeira” ( $Nm = 1.77$ ; 95% HPD 1.28 – 2.33) and from “Madeira” to “Bolivia” ( $Nm = 1.98$ ; 95% HPD 1.49 – 2.52). The effective population size ( $Ne$ ) was estimated based on both the cyt b gene (“Amazon”:  $Ne = 351,000$ ; “Madeira”:  $Ne = 209,000$ ; “Bolivia”:  $Ne = 185,000$ ; “Pantanal”:  $Ne = 142,000$ ) and ddRAD data (“Amazon”:  $Ne = 740,740$ ; “Madeira”:  $Ne = 833,333$ ; “Bolivia”:  $Ne = 668,276$ ; “Pantanal”:  $Ne = 688,405$ ).

In general *P. palpebrosus* had high levels of genetic diversity, comparable to other crocodilian species (Table 3). Comparisons between intraspecific clusters revealed that the “Madeira” cluster had five fold lower genetic diversity (Nei’s haplotype diversity) than the other clusters (Table 3). Both Tajima’s  $D$  and Fu’s  $F_s$  tests indicated recent population expansion of the “Madeira” geographic cluster (Table 3).

## DISCUSSION

### **Delimiting Cuvier’s dwarf caiman units for conservation**

It is important to identify intraspecific units such as ESUs and MUs so that limited conservation resources can be utilized optimally (Ryder 1986). The lack of gene flow, reciprocal monophyly, and high estimates of pairwise  $F_{ST}$  using cyt b sequence data, suggest long-term isolation between clusters of *P. palpebrosus*. The ddRAD data show a similar pattern in the geographic distribution due significant differences in the allele frequencies. This population structure allowed for the identification of three genetic clusters isolated in the Amazon, Madeira-Bolivia basin and the Pantanal, which are sufficiently differentiated to be considered ESUs according the multiple criteria summarized by the AEC framework (Frazer

and Bernatchez 2001).

The “Amazon” and “Pantanal” ESUs form reciprocally monophyletic mitochondrial groups (Fig. 2), and display no evidence of admixture with another genetic deme. Based on cyt b analysis, the “Madeira-Bolivia” ESU is comprised of two geographically structured subgroups, “Madeira” and “Bolivia” ( $F_{ST} = 0.769$ ), with a contact zone in the upper Madeira River (haplotype sharing at three localities). These two groups are connected by restricted gene flow with approximately 1 effective individual per generation migrating in either direction ( $Nm \sim 1$ ). These results together with the analysis of genomic data in STRUCTURE indicates that individuals from the Madeira River and the Bolivian basin comprise just one biological group; however, this “Madeira-Bolivia” ESU is best viewed as comprising two MUs, “Madeira” and “Bolivia”, according to the original definition of Moritz (1994). The boundary between “Madeira” and “Bolivia” MUs is not sharp, with a transition zone between these two MUs occurring in the region of the Madeira River rapids. Therefore, we propose that the “Madeira” and “Bolivia” MUs occur downstream and upstream of the Madeira River rapids, respectively.

Dwarf caimans from the Bolivian basin also showed evidence of substantial nuclear admixture with individuals from the Pantanal, while dwarf caimans from the Madeira River showed small amount of nuclear admixture with individuals from the Pantanal and Amazon (Fig. 3). In general the  $N_e$  estimated using ddRAD was at least twice that estimated using cyt b gene. The difference in the magnitude of the estimated  $N_e$  is possibly a result of the difference in inheritance that the cyt b and ddRAD markers have, maternal and biparental, respectively. Thus, mating system, imbalance in sex ratio, sex biased dispersal or other biological characteristic of the species may have been the promoters of the observed

differences in the estimated  $N_e$ . Moreover, the high levels of admixture found between "Madeira-Bolivia" and adjacent ESUs may have contributed to elevated  $N_e$  estimate the "Madeira" and "Bolivia" MUs.

In addition to our genetic data, Campos et al. (2015a) also found an ecological difference between Amazon and Pantanal *P. palpebrosus* populations, which may indicate ecological isolation between them. The authors observed that breeding season of *P. palpebrosus* from the Amazon begins in September (dry season) while that *P. palpebrosus* from the Pantanal begins in November (wet season). The reproductive period of *P. palpebrosus* from the Madeira River initiates in June/July, thus earlier than that of the "Amazon" ESU (ZC, unpublished data). This difference in the breeding season occurs even though the rainy season regime, which is thought to regulate reproduction of the crocodilians (Campos et al., 2015a), is identical. Campos et al. (2015a) suggest other environmental variables such as differences in vegetation, the intensity and the daily-minimum temperatures during the dry-season, and not just the rainy season regime are potential limiting factors causing differences in breeding regimes. Whatever the proximal cause, the reproductive periods of *P. palpebrosus* from the Amazon, the Pantanal and the Madeira are distinct, and may limit gene flow between these two lineages.

### **Genetic diversity of mitochondrial lineages**

*Paleosuchus palpebrosus* has high level of genetic diversity if treated as only one panmictic group, and this diversity is comparable with that of other crocodilian species (Table 2). However, species structured into ESUs and MUs, such as the case of *P. palpebrosus*, necessitate separate monitoring and/or management of each unit (Frankham et al. 2004).

ESUs and MUs are the logical units for species monitoring since preserving the genetic diversity of each unit will also conserve genetic diversity and evolutionary potential of the species as a whole (Moritz 1994). Ignoring independent evolutionary lineages could lead to the incorrect inference about a species' resilience and adaptive potential in a changing environment by implicitly or explicitly assuming the functional and evolutionary equivalence of these lineages.

Analyzing the genetic diversity of each lineage separately, the "Amazon" and "Pantanal" ESUs, and the "Bolivia" MU have moderate levels of genetic diversity based on the comparisons with other crocodilians (Table 2). However, the "Madeira" MU, the most well sampled group, has a fivefold lower genetic diversity than the other *P. palpebrosus* groups. The low genetic diversity may have resulted from a founder effect followed by population expansion, as suggested by the Tajima's *D* (Tajima 1989) and Fu's *Fs* (Fu 1997) tests (Table 2). This expansion may have occurred because the "Madeira" group probably originated via a founder effect from "Bolivia" group during the formation of the Madeira River. Irrespective of how this MU originated, the "Madeira" MU has the smallest geographical distribution, occurs in a very anthropogenically perturbed area, has the lowest genetic diversity, and is therefore at greatest demographic and genetic risk of extinction.

### **Implications for the conservation of *P. palpebrosus***

Conservation efforts must focus on evolutionary lineages as they are a manifestation of evolutionary history, reflecting current and past adaptive responses to environmental challenges as well as neutral divergences. Conservation recommendations and actions based solely on nominal species (named taxonomic entities) will lead us to the false sense of

conservation health of that nominal species, while in reality many lineages of that species may be threatened and may be in a need to lineage-specific conservation measures. ESUs and MUs are increasingly being recognized as appropriate units of conservation (see Guia and Saitoh 2007). Their identification takes into account the evolutionary history and potential of the species, helping to prioritize conservation efforts (Frankham et al. 2004). ESUs often represent lineages adapted to local environmental conditions (Funk et al. 2012) that once extinct may not be replaced by emigrants. The three ESUs of the broadly distributed *P. palpebrosus* are each subject to different and distinct threats stemming from differences between the area occupied by each lineage and levels of habitat degradation within these areas; therefore, they are natural candidates for differentially optimized management strategies.

Based on IUCN criteria used for the evaluation of extinction risk, the main threats for *P. palpebrosus* as a whole are habitat destruction, local subsistence hunting, dams, urbanization and pollution (Magnusson and Campos 2010). Nevertheless this species is classified as “Least Concern”, mainly due to its large distribution and local abundance in some areas (IUCN 2016; last assessment in 1996). *Paleosuchus palpebrosus* “Amazon” ESU occupies broad geographical distribution including much of the Amazon basin and non-Amazonian drainage basins such as the Maroni and Oiapoque River basins, which flow directly into the Atlantic. From a conservation perspective, the “Amazon” ESU possesses relatively high levels of genetic diversity indicating that the population does not require immediate conservation attention. In addition, the Amazon basin has many available pristine habitats for the species to occupy, many of them within federal and state conservation units and Amerindian reservations, as well as relatively small anthropogenic impact compared to

the areas of occurrence of the other ESUs.

In contrast, the “Madeira-Bolivia” and “Pantanal” ESUs are exposed to ever increasing anthropogenic pressure and the “Madeira-Bolivia” ESU is genetically depauperate. In particular the "Madeira" MU is genetically the most vulnerable of all *P. palpebrosus* lineages as it has a very low genetic diversity and the most restricted geographic distribution. The upstream located “Bolivia” MU has higher levels of genetic diversity and may contribute, in part, to maintaining the diversity for the “Madeira” MU. In the future, it may also be an important source population for the active management of the “Madeira” MU, if necessary.

The Madeira River is one of the most important tributaries of the Amazon basin formed from the confluence of Bolivian rivers that arise in the Andean piedmonts and on the Brazilian Shield. Historically, a set of 18 waterfalls and rapids on the upper Madeira River marked the transition between the Bolivian basin and the Madeira River (Cella-Ribeiro et al. 2013). Most of the waterfalls and rapids have now been submerged by the holding dams of the Jirau and Santo Antonio hydroelectric power plants. This region represents an important historical barrier to gene flow in different aquatic and semi-aquatic Amazonian taxa including fishes, the giant river otter, the pink river dolphin and caimans (e.g. Hubert and Renno 2006; Hubert et al. 2007; Hrbek et al. 2008; Pickles et al. 2011; Gravena et al. 2014, 2015). We found that this region is also a transition zone between two *P. palpebrosus* MUs. Flooding of this area resulting from the construction of the Santo Antonio and the Jirau hydroelectric power plants resulted in the loss of areas suitable for reproduction (Campos and Magnusson 2016) and other important habitat for the species (Campos et al. 2013). This, in turn, effectively isolated the “Bolivia” and “Madeira” MUs, augmenting extinction risk of the “Madeira” MU due to its increased isolation coupled with greater anthropogenic impact being

experienced by the Madeira River basin.

The “Pantanal” ESU shows levels of genetic diversity equivalent to the “Amazon” ESU. Its geographic distribution in the Paraguay River basin is restricted to areas surrounding the Pantanal wetland (Campos et al. 1995; Campos and Mourão 2006; Campos et al. 2013). Although the genetic diversity of “Pantanal” ESU is on par with other ESUs of *P. palpebrosus* and crocodilians in general, it is affected by a combination of anthropogenic threats, such as the removal of riparian forests, alteration of springs, pollution, poaching, intense human occupation and above all the installation of hydroelectric power plants (Campos and Mourão 2006; Campos et al. 2013; Campos et al. 2015b). There are already 178 hydroelectric dams installed or planned in the upper Paraguay River (available on <http://sigel.aneel.gov.br/sigel.html>). The principal problem of dam construction in these urbanized or agriculturally intensive regions is the complete loss of riparian habitat resulting from the flooding of remnants of riparian vegetation, and consequently the elimination of nesting in addition to foraging habitat.

It is possible that these three independent lineages currently recognized as a single widely distributed *P. palpebrosus* represent in reality a complex of cryptic species. The type locality of *P. palpebrosus* is Cayenne in French Guiana (Magnusson 1992). According to our analysis, individuals from French Guiana belong to the “Amazon” ESU which would then be the nominal species. Therefore, additional studies examining the “Madeira-Bolivia” and “Pantanal” ESUs as candidate species using the integrative taxonomy framework (Padial et al. 2010) would be welcome. Potential available epithets for the non-“Amazon” ESUs are the two junior synonyms *Jacaretinga moschifer* Spix 1825:1 and *Champsa gibbiceps* Natterer, 1841:324.

This study highlights the importance of understanding the spatial patterns of genetic variation for improving conservation and management of broadly distributed species, especially in tropical regions where biodiversity is largely underestimated. In such a context, broadly distributed species are the most likely candidates to harbor distinct evolutionary lineages (Scheffers et al. 2012). Recent studies have inferred extensive population structuring in broadly distributed mammals as well – e.g. African elephants (Rohland et al. 2010), giraffes (Brown et al. 2007; Fennessy et al. 2016), orangutan (Nater et al. 2017), river dolphins (Hrbek et al. 2014), and whales (Jackson et al. 2014) as well as in broadly distributed crocodilian species (Eaton et al. 2009; Hekkala et al. 2010, 2011; Franke et al. 2013; Shirley et al. 2014a,b; Cunningham et al. 2016; Milián-García et al., in press). In all of these cases, just like in *P. palpebrosus*, the nominal species were not just broadly distributed in the tropical regions, but also spanned a range of environments and/or discontinuous habitats. It would thus appear that broadly distributed species, in particular, need to be subject to population and conservation genetic analysis. Thus, we suggest that conservation research should not neglect species that are generally considered of Least Concern by IUCN criteria.

## REFERENCES

- Avise JC (1994) Molecular Markers, Natural History, and Evolution. New York, Chapman & Hall
- Beerli P (2006) Comparison of Bayesian and maximum-likelihood inference of population genetic parameters. *Bioinformatics* 22:341-345. doi:10.1093/bioinformatics/bti803
- Brown DM, Brenneman RA, Koepfli KP, Pollinger JP, Milá B, Georgiadis NJ et al. (2007). Extensive population genetic structure in the giraffe. *BMC biol.* 5:57 doi:10.1186/1741-7007-5-57
- Campos Z, Coutinho M, Abercrombie C (1995) Size structure and sex ratio of dwarf caiman in the Serra Amolar, Pantanal, Brazil. *Herpetological Journal* 5:321-322
- Campos Z, Magnusson WE (2016) Density and Biomass Estimates by Removal for an Amazonian Crocodilian, *Paleosuchus palpebrosus*. *PLoS One* doi:10.1371/journal.pone.0156406
- Campos Z, Marioni B, Farias IP, Verdade LM, Bassetti L, Coutinho ME et al. (2013) Avaliação do risco de extinção do jacaré-paguá *Paleosuchus palpebrosus* (Cuvier, 1807) no Brasil. *Biodiversidade Brasileira* 3:40-47.
- Campos Z, Mourão G (2006) Conservation status of the dwarf caiman, *Paleosuchus palpebrosus*, in the region surrounding Pantanal. *Crocodile Specialist Group Newsletter* 25:9-10
- Campos Z, Muniz FL, Farias IP, Hrbek T (2015b) Conservation status of the dwarf caiman *Paleosuchus palpebrosus* in the region of the Araguaia-Tocantins basin, Brazil. *Crocodile Specialist Group Newsletter* 34:4-8
- Campos Z, Sanaiotti T, Magnusson W (2010) Maximum size of dwarf caiman, *Paleosuchus palpebrosus* (Cuvier, 1807), in the Amazon and habitats surrounding the Pantanal, Brazil. *Amphib-reptil* 31:439-442. doi:10.1163/156853810791769392
- Campos Z, Sanaiotti T, Marques V, Magnusson WE (2015a) Geographic Variation in Clutch Size and Reproductive Season of the Dwarf Caiman, *Paleosuchus palpebrosus*, in Brazil. *J Herpetol* 49:95-98. doi:10.1670/11-224
- Cella-Ribeiro A, Torrente-Vilara G, Hungria DB, Oliveira M (2013) As corredeiras do Rio Madeira. In: Queiroz LJ, Torrente-Vilara G, Ohara WM, Pires T, Zuanon J, Doria CRC

- (eds) Peixes do Rio Madeira, 1st edn. Dialeto Latin American Documentary, São Paulo, pp 56-63
- Corander J, Marttinen P, Sirén J, Tang J (2008) Enhanced Bayesian modelling in BAPS software for learning genetic structures of populations. BMC Bioinformatics 9:539. doi: 10.1186/1471-2105-9-539
- Coyne KJ, Burkholder JM, Feldman RA, Hutchins DA, Cary SC (2004) Modified serial analysis of gene expression method for construction of gene expression profiles of microbial eukaryotic species. Appl Environ Microbiol 70:5298-5304. doi:10.1128/AEM.70.9.5298
- Crandall KA, Bininda-Emonds ORP, Mace GM, Wayne RK (2000) Considering evolutionary processes in conservation biology. Trends Ecol Evol 15:290-295. doi:10.1016/S0169-5347(00)01876-0
- Cunningham SW, Shirley MH, Hekkala ER (2016) Fine scale patterns of genetic partitioning in the rediscovered African crocodile, *Crocodylus suchus* (Saint-Hilaire 1807). PeerJ 4:e1901:1-20. doi: 10.7717/peerj.1901
- Darriba D, Taboada GL, Doallo R, Posada D (2012) jModelTest 2: more models, new heuristics and parallel computing. Nat Methods 9:772. doi:10.1038/nmeth.2109
- Earl DA, VonHoldt BM (2012) STRUCTURE HARVESTER: a website and program for visualizing STRUCTURE output and implementing the Evanno method. Conserv Genet Resour 4:359-361. doi:10.1007/s12686-011-9548-7
- Eaton DAR (2014) PyRAD: assembly of de novo RADseq loci for phylogenetic analysis. Bioinformatics 30:1844-1849. doi: 10.1093/bioinformatics/btu121
- Eaton MJ, Martin A, Thorbjarnarson J, Amato G (2009) Species-level diversification of African dwarf crocodiles (Genus *Osteolaemus*): a geographic and phylogenetic perspective. Mol Phylogenet Evol 50:496-506. doi:10.1016/j.ympev.2008.11.009
- Edgar RC (2004) MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Research 32:1792-1797. doi:10.1093/nar/gkh340
- Evanno G, Regnaut S, Goudet J (2005) Detecting the number of clusters of individuals using the software structure: a simulation study. Mol. Ecol. 14:2611-2620. doi:10.1111/j.1365-294X.2005.02553.x
- Excoffier L, Lischer HEL (2010) Arlequin suite ver 3.5: A new series of programs to perform

- population genetics analyses under Linux and Windows. Mol Ecol Resour 10:564-567. doi:10.1111/j.1755-0998.2010.02847.x
- Farias IP, Silveira R, Thoisy B, Monjeló LA, Thorbjarnarson J, Hrbek T (2004) Genetic diversity and population structure of Amazonian crocodilians. Anim Conserv 7:265-272. doi:10.1017/S136794300400143X
- Fennessy J, Bidon T, Reuss F, Kumar V, Elkan P, Nilsson MA et al. (2016) Multi-locus analyses reveal four giraffe species instead of one. Curr. Biol. 26:2543-2549. doi: 10.1016/j.cub.2016.07.036
- Franke FA, Schmidt F, Borgwardt C, Bernhard D, Bleidorn C, Engelmann WE, Schlegel M (2013) Genetic differentiation of the African dwarf crocodile *Osteolaemus tetraspis* Cope, 1861 (Crocodylia: Crocodylidae) and consequences for European zoos. Org Divers Evol 13:255-266. doi:10.1007/s13127-012-0107-1
- Frankham R, Ballou JD, Briscoe DA (2004) A Primer of Conservation Genetics. Cambridge University Press, New York
- Fraser DJ, Bernatchez L (2001) Adaptive evolutionary conservation: towards a unified concept for defining conservation units. Mol Ecol 10:2741-2752. doi:10.1046/j.1365-294X.2001.t01-1-01411.x
- Fu YX (1997) Statistical tests of neutrality of mutations against population growth, hitchhiking and background selection. Genetics 147:915-925
- Funk WC, McKay JK, Hohenlohe PA, Allendorf FW (2012) Harnessing genomics for delineating conservation units. Trends Ecol Evol 27:489-496. doi:10.1016/j.tree.2012.05.012
- Godshalk R (2006) Phylogeography and conservation genetics of the yacare caiman (*Caiman yacare*) of South America. PhD thesis, University of Florida at Gainesville
- Gravena W, Farias IP, Da Silva MNF, Da Silva VMF, Hrbek T (2014) Looking to the past and the future: Were the Madeira River rapids a geographical barrier to the boto (Cetacea: Iniidae)? Conserv Genet 15:619-629. doi:10.1007/s10592-014-0565-4
- Gravena W, da Silva VMF, da Silva MNF, Farias IP, Hrbek T (2015) Living between rapids: genetic structure and hybridization in boto (Cetacea: Iniidae: *Inia* spp.) of the Madeira River, Brazil. Biol J Linn Soc 114:764-777. doi:10.1111/bij.12463
- Green MR, Sambrook J (2012) Molecular Cloning: A Laboratory Manual: Three-volume set,

- 4th ed. Cold Spring Harbor Laboratory Press, New York
- Guia APO, Saitoh T (2007) The gap between the concept and definitions in the Evolutionarily Significant Unit: The need to integrate neutral genetic variation and adaptive variation. *Ecol Res* 22:604-612. doi:10.1007/s11284-006-0059-z
- Hekkala ER, Amato G, DeSalle R, Blum MJ (2010) Molecular assessment of population differentiation and individual assignment potential of Nile crocodile (*Crocodylus niloticus*) populations. *Conserv Genet* 11:1435-1443. doi:10.1007/s10592-009-9970-5
- Hekkala ER, Shirley MH, Amato G, Austin JD, Charter S, Thorbjarnarson J et al. (2011) An ancient icon reveals new mysteries: mummy DNA resurrects a cryptic species within the Nile crocodile. *Mol Ecol* 20:4199-4215. doi:10.1111/j.1365-294X.2011.05245.x
- Hrbek T, Silva VMF, Dutra N, Gravena W, Martin AR, Farias IP (2014) A new species of river dolphin from Brazil or: how little do we know our biodiversity. *PLoS One* 9:e0083623. doi:10.1371/journal.pone.0083623
- Hrbek T, Vasconcelos WR, Rebêlo GH, Farias IP (2008) Phylogenetic relationships of south american alligatorids and the caiman of Madeira River. *J Exp Zool A Ecol Genet Physiol* 309:588-599. doi:10.1002/jez.430
- Hubert N, Duponchelle F, Nuñes J, Garcia-Davila C, Paugy D, Renno JF (2007) Phylogeography of the piranha genera *Serrasalmus* and *Pygocentrus*: implications for the diversification of the Neotropical ichthyofauna. *Mol Ecol* 16:2115-2136. doi:10.1111/j.1365-294X.2007.03267.x
- Hubert N, Renno JF (2006) Historical biogeography of South American freshwater fishes. *J Biogeogr* 33:1414-1436. doi:10.1111/j.1365-2699.2006.01518.x
- IUCN (2016) The IUCN red list of threatened species. Version 2016.3. <http://www.iucnredlist.org>. Accessed 20 December 2016
- Jackson JA, Steel DJ, Beerli P, Congdon BC, Olavarria C, Leslie MS et al. (2014) Global diversity and oceanic divergence of humpback whales (*Megaptera novaeangliae*). In *Proc R Soc B* 281:20133222. doi:10.1098/rspb.2013.3222
- Jakobsson M, Rosenberg NA (2007) CLUMPP: a cluster matching and permutation program for dealing with label switching and multimodality in analysis of population structure. *Bioinformatics* 23:1801-1806. doi:10.1093/bioinformatics/btm233
- Kenny EM, Cormican P, Gilks WP, Gates AS, O'Dushlaine CT, et al. (2011) Multiplex target

- enrichment using DNA indexing for ultra-high throughput SNP detection. *DNA Research* 18:31-38. doi:10.1093/dnaresearch/dsq029
- Li H, Handsaker B, Wysoker A, Fennell T, Ruan J, Homer N, Marth G, Abecasis G, Durbin R, 1000 Genome Project Data Processing Subgroup (2009) The Sequence alignment/map (SAM) format and SAMtools. *Bioinformatics* 25:2078-2079. doi:10.1093/bioinformatics/btp352
- Magnusson WE (1992) *Paleosuchus palpebrosus*. Cat Am Amphib Rept 554.1-554.2.
- Magnusson WE, Campos Z (2010) Cuvier's Smooth-fronted Caiman *Paleosuchus palpebrosus*. In: Manolis SC, Stevenson C (eds). *Crocodiles: Status Survey and Conservation Action Plan*, 3rd edn. Crocodile Specialist Group, Darwin, pp 40-42
- Milián-García Y, Castellanos-Labarcena J, Russello M A, Amato G (2018) Mitogenomic investigation reveals a cryptic lineage of *Crocodylus* in Cuba. *Bull Mar Sci* 94: (in press). doi: 10.5343/bms.2016.1134
- Moritz C (1994) Defining "Evolutionarily Significant Units" for conservation. *Trends Ecol Evol* 9:373-375. doi:10.1016/0169-5347(94)90057-4
- Moritz C, Lavery S, Slade R (1995) Using allele frequency and phylogeny to define units for conservation and management. In: Nielsen JL, Powers GA (eds) *Evolution and the Aquatic Ecosystem: Defining Unique Units in Population Conservation, Symposium*. American Fisheries Society, Maryland, pp 249-262
- Nater A, Mattle-Greminger MP, Nurcahyo A, Nowak MG, Manuel M de, Desai T, et al. (2017) Morphometric, Behavioral, and Genomic Evidence for a New Orangutan Species. *Curr Biol* 27: 3487-3498. doi:10.1016/j.cub.2017.09.047
- Nei M (1987) *Molecular evolutionary genetics*. Columbia University Press, New York
- Padial JM, Miralles A, De la Riva I, Vences M (2010) The integrative future of taxonomy. *Front Zool* 7:16. doi:10.1186/1742-9994-7-16
- Peterson BK, Weber JN, Kay EH, Fisher HS, Hoekstra HE (2012) Double digest RADseq: An inexpensive method for de novo SNP discovery and genotyping in model and non-model species. *PLoS One* 7:e37135. doi:10.1371/journal.pone.0037135
- Pickles RSA, Groombridge JJ, Zambrana VDR, Van Damme P, Gottelli D, Kundu S (2011) Evolutionary history and identification of conservation units in the giant otter, *Pteronura brasiliensis*. *Mol Phylogenetic Evol* 61:616-627.

- doi:10.1016/j.ympev.2011.08.017
- Pritchard JK, Stephens M, Donnelly P (2000) Inference of population structure using multilocus genotype data. *Genetics* 155:945-959. doi:10.1111/j.1471-8286.2007.01758.x
- de Queiroz K (2007) Species concepts and species delimitation. *Syst Biol* 56:879-886. doi:10.1080/10635150701701083
- Rambaut A (2014) Figtree 1.4. 2 software. <http://tree.bio.ed.ac.uk/software/figtree>
- Roe KJ, Lydeard C (1998) Species Delineation and the Identification of Evolutionarily Significant Units : Lessons from the Freshwater Mussel Genus *Potamilus* (Bivalvia : Unionidae). *J Shellfish Res* 17:1359-1363
- Rognes T, Flouri T, Nichols B, Quince C, Mahé F (2016) VSEARCH: a versatile open source tool for metagenomics. *PeerJ* 4:e2584. doi:10.7717/peerj.2584
- Rohland N, Reich D, Mallick S, Meyer M, Green RE, Georgiadis NJ et al. (2010) Genomic DNA Sequences from Mastodon and Woolly Mammoth Reveal Deep Speciation of Forest and Savanna Elephants. *PLOS Biol* 8:e1000564. doi:10.1371/journal.pbio.1000564
- Ronquist F, Teslenko M, van der Mark P, Ayres DL, Darling A, Hohna S et al. (2012) MrBayes 3.2: efficient Bayesian phylogenetic inference and model choice across a large model space. *Syst Biol* 61:539-554. doi:10.1093/sysbio/sys029
- Rosenberg NA (2004) DISTRUCT: a program for the graphical display of population structure. *Mol Ecol Notes* 4:137-138. doi:10.1046/j.1471-8286.2003.00566.x
- Ryder OA (1986) Species conservation and systematics: the dilemma of subspecies. *Trends Ecol Evol* 1:9-10. doi:10.1016/0169-5347(86)90059-5
- Salzburger W, Ewing GB, Von Haeseler A (2011) The performance of phylogenetic algorithms in estimating haplotype genealogies with migration. *Mol Ecol* 20:1952-1963. doi:10.1111/j.1365-294X.2011.05066.x
- Scheffers BR, Joppa LN, Pimm SL, Laurance WF (2012) What we know and don't know about Earth's missing biodiversity. *Trends Ecol Evol* 27:501-510. doi:10.1016/j.tree.2012.05.008
- Shirley MH, Villanova VL, Vliet KA, Austin JD (2014b) Genetic barcoding facilitates captive and wild management of three cryptic African crocodile species complexes. *Anim*

- Conserv 18:322-330. doi:10.1111/acv.12176
- Shirley MH, Vliet KA, Carr AN, Austin JD (2014a) Rigorous approaches to species delimitation have significant implications for African crocodilian systematics and conservation. Proc R Soc B 281:20132483. doi:10.1098/rspb.2013.2483
- Streicher JW, Schulte II JA, Wiens JJ (2016) How should genes and taxa be sampled for phylogenomic analyses with missing data? An empirical study in iguanian lizards. Syst Biol 65:128-145. doi:10.1093/sysbio/syv058
- Tajima F (1989) Statistical method for testing the neutral mutation hypothesis by DNA polymorphism. Genetics 123:585-595
- Vasconcelos WR, Hrbek T, Da Silveira R, De Thoisy B, Marioni B, Farias IP (2006) Population genetic analysis of *Caiman crocodilus* (Linnaeus, 1758) from South America. Genet Mol Biol 29:220-230. doi:10.1590/S1415-47572006000200006
- Vasconcelos WR, Hrbek T, Da Silveira R, De Thoisy B, Ruffeil LAAS, Farias IP (2008) Phylogeographic and Conservation Genetic Analysis of the Black Caiman (*Melanosuchus niger*). J Exp Zool Part A Ecol Genet Physiol 309A:600-613. doi:10.1002/jez.452
- Venegas-Anaya M, Crawford AJ, Galván AHE, Sanjur OI, Densmore III LD, Bermingham E (2008) Mitochondrial DNA phylogeography of *Caiman crocodilus* in Mesoamerica and South America. J Exp Zool 309A:614-627. doi:10.1002/jez.502
- Vogler AP, Desalle ROB (1994) Diagnosing Units of Conservation Management. Conserv. Biol. 8:354-363. doi:10.1046/j.1523-1739.1994.08020354.x
- Waples RS (1991) Pacific salmon, *Oncorhynchus* spp. & the definition of “species” under the Endangered Species Act. Mar Fish Rev 53:11-22
- Waples RS (1995) Evolutionarily significant units and the conservation of biological diversity under the Endangered Species Act. In: Nielsen JL, Powers GA (eds) Evolution and the Aquatic Ecosystem: Defining Unique Units in Population Conservation. American Fisheries Society, Maryland, pp 17:8-27
- Zwickl DJ (2006) Genetic algorithm approaches for the phylogenetic analysis of large biological sequence datasets under the maximum likelihood criterion. Dissertation, The University of Texas, Austin.

**Table 1.** Pairwise  $F_{ST}$  between cyt b groups previously established by BAPS cluster analysis.  
All values are significant at the  $p < 0.008$  level after Bonferroni correction.

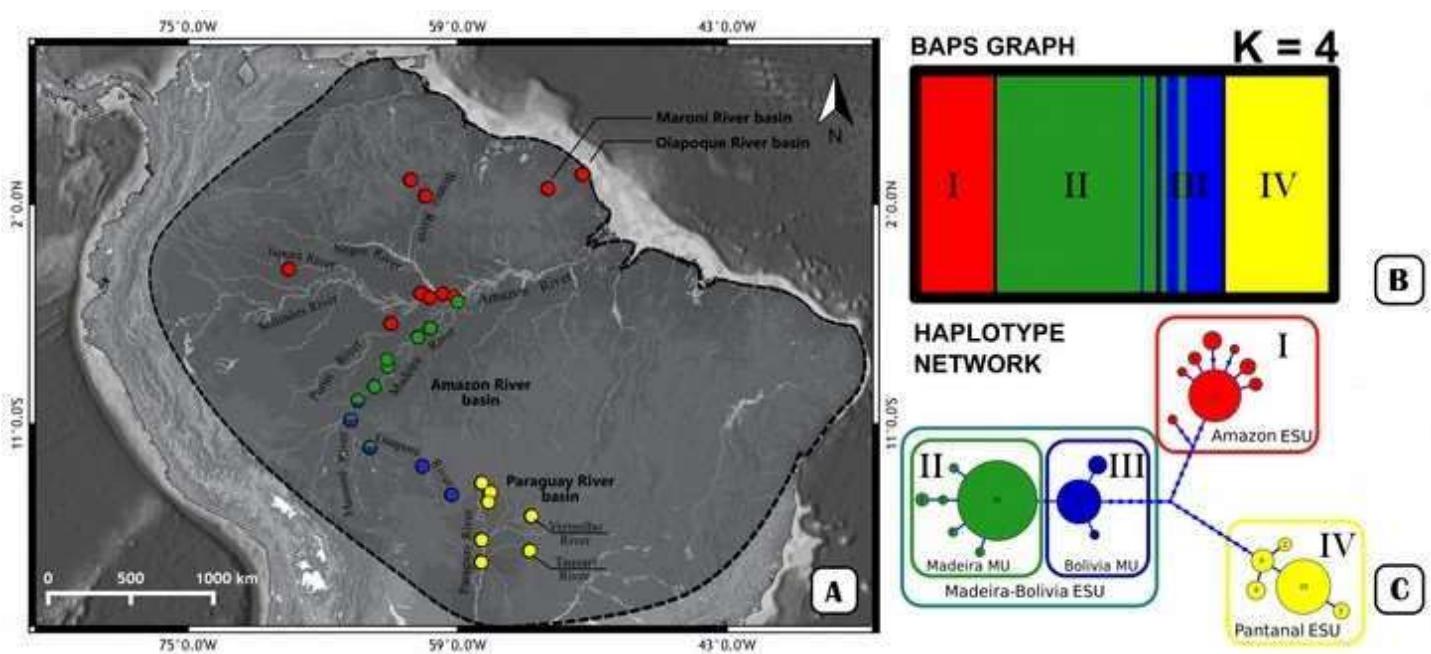
Pairwise $F_{ST}$	Amazon	Madeira	Bolivia	Pantanal
<b>Amazon</b>	---			
<b>Madeira</b>	0.972	---		
<b>Bolivia</b>	0.929	0.769	---	
<b>Pantanal</b>	0.957	0.981	0.952	---

**Table 2.** Summary of results of the population analysis using cytochrome b gene and ddRADs genomic marker, the correspondent geographic distribution of each and the type of evolutionary lineages proposed for geographic groups. The colors of each cluster are correspondent to figure 1 and 2.

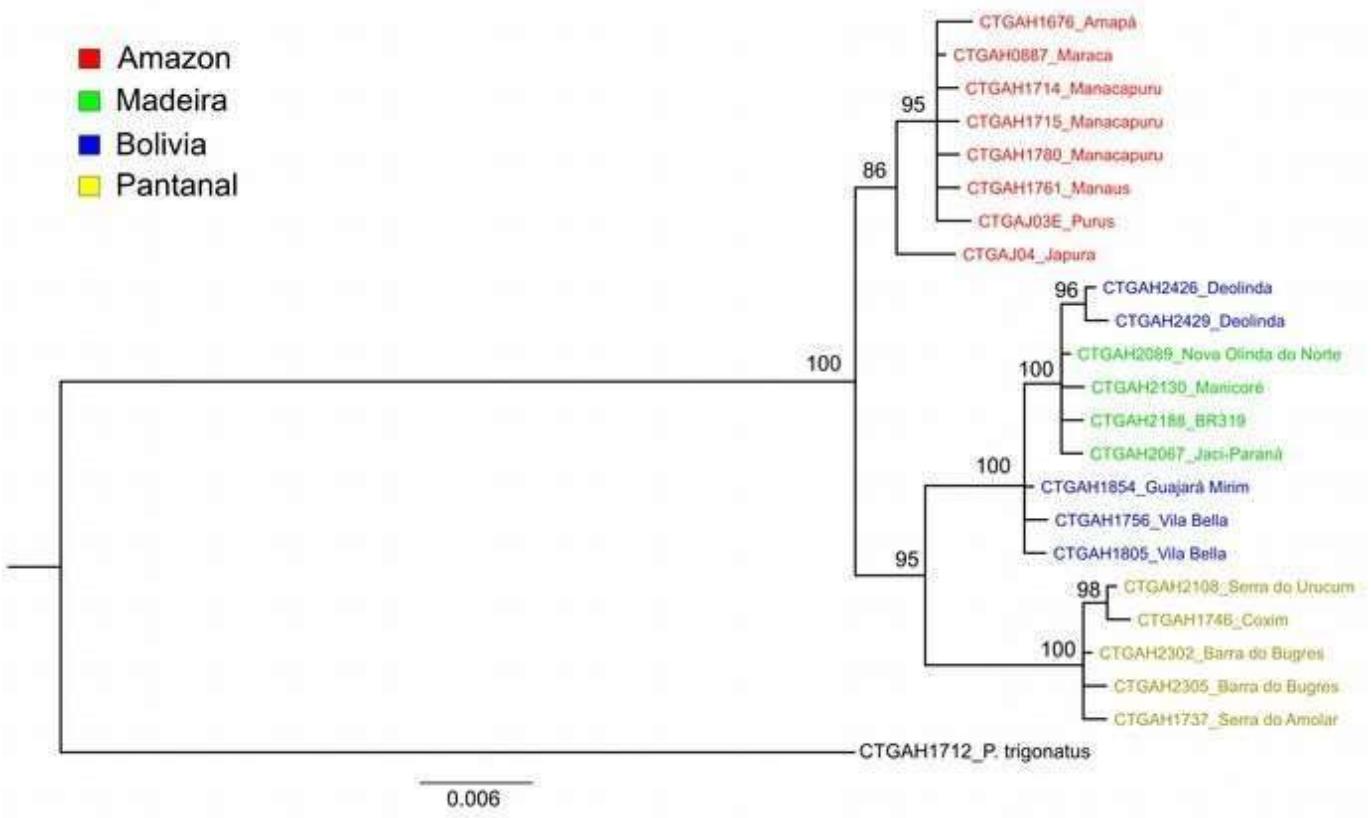
Nº		Cyt b clusters /Geographic distribution		ddRAD clusters /Geographic distribution		Evolutionarily Significant Units (ESU)	Management Units (MU)
1	I	Amazon, Maroni and Oiapoque basins		I	Amazon and Maroni basins	Amazon	---
2	II	Madeira basin		II+III	Madeira and Bolivia basins	Madeira-Bolivia	Madeira
3	III	Bolivia basin					Bolivia
4	IV	Upper Paraguay basin	IV	Upper Paraguay basin		Pantanal	---

**Table 3.** Population parameters and genetic diversity indexes in *P. palpebrosus* populations and in other crocodilian species. N: number of samples; Hp: number of unique haplotypes; cyt b: number of sequenced base pairs (bp) of cyt b gene;  $\pi$ : Nei's nucleotide diversity and standard deviation (SD); Nei's gene diversity (1987) and standard deviation (SD); and Tajima's *D* (1989) and Fu's *Fs* (1997) neutrality tests. (\*) Indicates significant *p* values (<0.05).

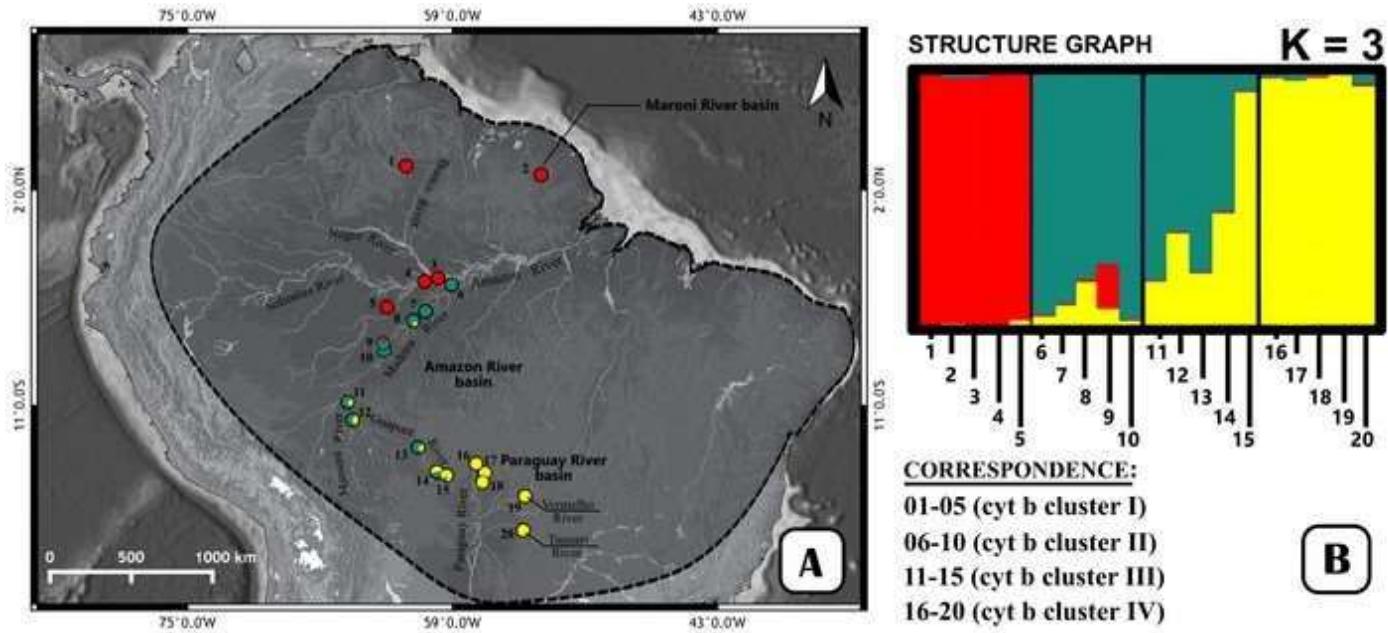
Species	Population	N	Hp	cyt b (bp)	$\pi \pm SD$	Nei's gene diversity $\pm SD$	Tajima's <i>D</i> (p)	Fu's <i>Fs</i> (p)	Reference
<i>Paleosuchus palpebrosus</i>	Amazon	47	08	1,102	0.0007 $\pm$ 0.0006	0.544 $\pm$ 0.093	-1.155 (0.117)	-1.966 (0.081)	This study
	Madeira	77	05	1,102	0.0001 $\pm$ 0.0002	0.101 $\pm$ 0.047	-1.943 (0.001)*	-5.881 (0.000)*	This study
	Bolivia	32	06	1,102	0.0010 $\pm$ 0.0007	0.518 $\pm$ 0.100	-0.680 (0.264)	-1.200 (0.231)	This study
	Pantanal	50	05	1,102	0.0006 $\pm$ 0.0005	0.494 $\pm$ 0.080	-0.449 (0.380)	-1.020 (0.258)	This study
	ALL	206	22	1,102	0.0101 $\pm$ 0.0051	0.791 $\pm$ 0.021	1.651 (0.957)	6.914 (0.922)	This study
<i>Caiman crocodilus</i>	Amazon + Fr. Guiana	32	09	1,142	---	0.692 $\pm$ 0.079	-1.612	-4.885	Farias et al. (2004)
	Amazon + Fr. Guiana	125	38	1,085	0.0011 $\pm$ 0.0008	0.733 $\pm$ 0.042	-2.568*	-28.829*	Vasconcelos et al. (2006)
<i>Melanosuchus niger</i>	Amazon + Fr. Guiana	47	10	1,142	---	0.715 $\pm$ 0.049	-0.449*	-1.330*	Farias et al. (2004)
	Amazon + Fr. Guiana	130	39	1,027	0.0017 $\pm$ 0.0011	0.911 $\pm$ 0.023	-2.523*	-27.602*	Vasconcelos et al. (2008)
<i>Caiman yacare</i>	Bolivian basin	143	16	1,143	0.0017 $\pm$ 0.0010	0.573 $\pm$ 0.033	-0.956	-4.638	Godshalk (2006)
	Pantanal	65	9	1,143	0.0013 $\pm$ 0.0009	0.778 $\pm$ 0.031	-1.089	-1.714	Godshalk (2006)
<i>Osteolaemus osborni</i>	Congo Basin	24	1	781	0.0000 $\pm$ 0.0000	0.000 $\pm$ 0.000	0.000	0.000	Eaton et al. (2009)
<i>Osteolaemus tetraspis</i>	Ogooue Basin	46	7	781	0.0014 $\pm$ 0.00103	0.515 $\pm$ 0.077	-1.335	-1.879	Eaton et al. (2009)
	West Africa	10	7	781	0.0048 $\pm$ 0.0029	0.866 $\pm$ 0.107	-0.229	-1.494	Eaton et al. (2009)
<i>Mecistops cataphractus</i>	Central Africa	92	7	935	0.00042 $\pm$ 0.00045	0.295 $\pm$ 0.061	-1.721*	-5.091*	Shirley et al. (2014a)
	West Africa	13	5	935	0.0023 $\pm$ 0.0015	0.782 $\pm$ 0.079	-0.637	0.116	Shirley et al. (2014a)



**Fig. 1** a Map showing all the sites sampled in this study, b results of the BAPS analysis and c haplotype network based on the mitochondrial cyt b gene. The colors in the BAPS graph, haplotype network and at every point on the map represent the cluster to which the analyzed individuals belong based on BAPS assignments. The points with two colors on the map indicate the sites where haplotypes assigned to different groups were found. Each group is also highlighted in the haplotype network, indicating which type of conservation unit it represents, i.e., Evolutionarily Significant Unit or Management Unit. These assignments also took into account the results of the STRUCTURE analysis of ddRAD data. The dotted lines delimit distribution of the *P. palpebrosus* based on IUCN. The map was constructed in QGIS program and final graphic artwork was made in Inkscape



**Fig. 2** Phylogenetic tree reconstructed by Bayesian Inference (BI) approach depicting the relationships among the *P. palpebrosus* from Amazon, Madeira, Bolivia and Pantanal, estimated from 1102 bp of cyt b gene sequence data (mtDNA). Terminals are individuals with representative sequence of each cyt b haplotype found, named according with tissue collection number and their locality of origin. Colors indicate to which evolutionary lineage each individual has been assigned. Maximum-likelihood (ML) tree showed similar topology and equivalent node supports to BI and so was not presented



**Fig. 3** (A) Map showing the geographic distribution of the individuals analyzed and (B) the results of STRUCTURE analysis using ddRAD data as well as correspondence with the cyt b clusters shown in the figure 1. Each location shown in the figure is represented by only one individual. In the results of STRUCTURE and at every point on the map, the colors represent the groups to which each analyzed individual belongs. The points with two colors on the map indicate individuals with genomic composition of two different groups and therefore the result of admixed ancestry. The proportion of colors represents the proportion of SNPs in each group contained in the interbred individual. The dotted lines delimit distribution of the *P. palpebrosus* based on IUCN. The map was constructed in QGIS program and final graphic artwork was made in Inkscape

## SUPPLEMENTARY MATERIAL

### **Delimitation of evolutionary units in Cuvier's dwarf caiman, *Paleosuchus palpebrosus* (Cuvier, 1807): insights from conservation of a broadly distributed species**

*Conservation Genetics*

**FL Muniz<sup>1,2</sup> · Z Campos<sup>3</sup> · SM Hernández Rangel<sup>1,2</sup> · JG Martínez<sup>1,4,5</sup> · BC Souza<sup>6</sup> · B De Thoisy<sup>7,8</sup> · R Botero-Arias<sup>9,10</sup> · T Hrbek<sup>1</sup> · IP Farias<sup>1</sup>**

<sup>1</sup>Laboratory of Animal Genetics and Evolution (LEGAL), Department of Biology, Federal University of Amazonas (UFAM), Manaus, Amazonas, Brazil

<sup>2</sup>National Institute for Amazonian Research (INPA), Graduate Program in Genetics, Conservation and Evolutionary Biology, Manaus, Amazonas, Brazil

<sup>3</sup>Wildlife Laboratory, Brazilian Agricultural Research Corporation (EMBRAPA) Pantanal, Corumbá, Mato Grosso do Sul, Brazil

<sup>4</sup>Grupo de Investigación Biociencias, Facultad de Ciencias de la Salud, Institución Universitaria Colegio Mayor de Antioquia, Medellín, Colombia

<sup>5</sup>Grupo de Pesquisa em Genética Molecular e Citogenética, Programa de Pós-Graduação em Biotecnologia e Recursos Naturais (MBT), Escola Superior de Ciências da Saúde, Universidade do Estado do Amazonas, Manaus, Brazil

<sup>6</sup>Chico Mendes Institute for Biodiversity Conservation (ICMBio), Boa Vista, Roraima, Brazil

<sup>7</sup>Institut Pasteur de la Guyane, Cayenne, French Guiana

<sup>8</sup>Association Kwata, Cayenne, French Guiana

<sup>9</sup>Caiman Research in Conservation and Management Program, Instituto Mamirauá para o Desenvolvimento Sustentável, Tefé, Amazonas, Brazil

<sup>10</sup>Department of Wildlife Ecology and Conservation, University of Florida, Gainesville, FL, USA

**Corresponding author:** Fábio de Lima Muniz · e-mail: [fabiolm\\_bio@yahoo.com.br](mailto:fabiolm_bio@yahoo.com.br)

**Table S1** Study sites in the Brazilian Amazon, Guiana Shield and Pantanal biome. All sample locations were in Brazil less one (\*) that was collected in French Guyana. In the table are shown the coordinate for samples, the number of individuals used in the analysis with cyt b gene and ddRADseq as well as the correspondence of the localities with the figure 2

Locality	Coordinates	cyt b	ddRADseq	Correspond to figure 2
1. Japura River	1°50'00.01"S 69°02'00.02"W	1	0	-
2. Maracá Island	3°26'53.11"N 61°48'38.54"W	6	1	1
3. Mucajáí	2°28'20.87"N 60°55'42.12"W	2	0	-
4. Tanpok River*	2°55'26.43"N 53°38'50.32"W	1	1	2
5. Uaçá River	3°45'56.06"N 51°36'56.19"W	1	0	-
6. Careiro da Várzea	3°18'35.00"S 59°52'26.67"W	7	1	3
7. Cururu lake	3°30'46.40"S 60°41'18.78"W	13	1	4
8. Caapiranga	3°17'02.76"S 61°13'07.67"W	8	0	-
9. Purus River	5°04'17.82"S 62°58'00.02"W	7	1	5
10. Autazes	3°36'14.06"S 59°09'01.13"W	1	0	-
11. Nova Olinda do Norte	3°43'26.03"S 59°04'00.75"W	12	1	6
12. Novo Aripuanã	5°19'25.80"S 60°38'27.89"W	10	1	7
13. Manicoré	5°59'20.55"S 61°29'20.32"W	12	1	8
14. Humaitá, BR-319, km 57	7°22'28.37"S 63°12'36.42"W	8	1	9
15. Humaitá, BR-230, km 20	7°35'15.51"S 63°09'09.51"W	12	1	10
16. Porto Velho	8°47'28.37"S 63°56'36.42"W	11	0	-
17. Among rapids	9°38'14.51"S 64°55'57.89"W	12	0	-
18. Guajará-Mirim	10°48'08.35"S 65°19'52.60"W	7	1	11
19. Surpresa	11°51'55.97"S 65° 2'53.70"W	3	1	12
20. Costa Marques	12°24'23.27"S 64°12'14.42"W	4	0	-
21. Pimenteiras do Oeste	13°30'53.27"S 61° 5'09.80"W	9	1	13
22. Vila Bella da Santíssima Trindade	15°01'07.92"S 59°56'52.24"W	9	1	14
23. Pontes e Lacerda	15°12'13.87"S 59°21'18.56"W	0	1	15
24. Tangará da Serra	14°30'16.65"S 57°34'59.04"W	2	1	16
25. Barra do Brugres	15°04'0.12"S 57°04'42.33"W	6	1	17
26. Serra das Araras	15°37'36.29"S 57°12'5.97"W	11	1	18
27. Rondonópolis	16°28'12.64"S 54°37'57.63"W	9	1	19
28. Coxim	18°31'02.39"S 54°44'24.13"W	11	1	20
29. Serra do Amolar	17°52'38.86"S 57°36'5.94"W	5	0	-
30. Serra do Urucum	19°11'43.34"S 57°36'45.45"W	6	0	-
TOTAL		206	20	

**Table S2** Diagnostic sites for each Evolutionarily Significant Unit (ESU) based on Population Aggregation Analysis (PAA) phylosophy. The position of each cytochrome b site fixed for at least one Evolutionarily Significant Unit (22 sites) and the number of individuals sharing these sites (N) are shown

ESU	Nucleotide position																						N
	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
	0	0	0	1	2	3	3	3	5	5	5	6	6	6	6	7	7	8	8	8	9	0	0
	0	7	7	4	2	0	0	2	1	6	6	4	7	7	1	2	6	7	9	7	3	8	
	8	4	7	9	1	2	9	9	5	6	9	4	1	4	0	2	7	3	6	4	9	9	
Amazon	C	G	C	A	A	A	A	A	G	G	G	T	A	A	C	C	G	G	G	G	C	T	47
Madeira-Bolivia	A	G	A	A	C	G	A	A	A	G	G	C	A	G	C	T	A	G	A	A	C	A	111
Pantanal	A	A	C	G	C	G	G	G	A	A	A	T	G	A	T	C	G	A	G	A	T	A	50

# CAPÍTULO 2

---

Muniz FL, Campos Z, Farias IP, Hrbek T (2018)  
What drives diversification along the Amazon–  
Paraguay basin transition? A biogeographic model  
for semi-aquatic and aquatic taxa. In prep. to  
*Journal of Biogeography*.

Original Article

What drives diversification along the Amazon–Paraguay basin transition? A biogeographic model for semi-aquatic and aquatic taxa

Muniz, Fábio<sup>1,2</sup>; Campos, Zilca<sup>3</sup>; Farias, Izeni Pires<sup>2,4</sup>; Hrbek, Tomas<sup>2,4</sup>

<sup>1</sup>*National Institute for Amazonian Research (INPA), Graduate Program in Genetics, Conservation and Evolutionary Biology, Manaus, Amazonas, Brazil*

<sup>2</sup>*Laboratório de Evolução e Genética Animal, Departamento de Genética, Instituto de Ciências Biológicas, Universidade Federal do Amazonas, Manaus, AM, Brazil*

<sup>3</sup>*Empresa Brasileira de Pesquisa Agropecuária (Embrapa) Pantanal, Corumbá, MS, Brazil*

<sup>4</sup>*Brigham Young University, Provo, UT*

\*Correspondence: Tomas Hrbek, e-mail: hrbek@evoamazon.net

**Keywords:** South America, Evolutionary Significant Units, dwarf caiman, Madeira River, Fitzcarrald Arch

**RH:** Divergence and hybridization in the Madeira basin

## ABSTRACT

**Aim** Rivers act as corridors for dispersal of aquatic taxa, so we may expect that species are largely panmictic or show a pattern of isolation-by-distance along a basin. Cuvier's dwarf caiman (*Paleosuchus palpebrosus*) is constituted by evolutionary lineages deeply genetic structured along the Paraguay-Madeira-Amazon corridor. Their geographical structuring could be explained by both, isolation-by-distance and historical isolation promoted by drainage rearrangements, and such competitive hypothesis were tested in the present study. Here, we propose a biogeographical model for *P. palpebrosus* diversification and discuss the utility of this model in other co-occurring aquatic taxa.

**Location** Neotropics, Amazon basin, Madeira River, Paraguay basin

**Methods** We sequenced total cytochrome *b* gene for 206 individuals from 28 localities in the Amazon and Pantanal basins. We also sequenced three nuclear genes for a subset of individuals and generate SNPs from a ddRADseq data for 20 individuals sampled from 20 localities. We reconstructed an haplotype network and performed Bayesian Inferences to infer clusters. We performed a partial Mantel test to quantify in a partitioned way the contribution of Isolation by Distance (IBD) and of historical processes to explain the patterns of genetic diversity observed. We also inferred phylogenetic relationships and estimated divergence times among lineages.

**Results** We found that historical isolation best explain the spatial distribution of the genetic diversity within *P. palpebrosus*. We detect four cytochrome *b* clusters, concordant with haplotype network, and three genomic clusters, with geographical congruence between

markers. However, we found incongruences in the phylogenetic relationships and in the divergence time estimates using these markers, indicating that historical mixture events may have occurred.

**Main conclusions** We propose a biogeographic model that can be summarized as follows: 1) isolation of the Bolivian sub-basin and the Pantanal from the Amazon basin 3-2 mya by the rise of the Fitzcarald Arch; 2) isolation of the Bolivian basin from the Pantanal by the compression event along the Andean belt 2.5 mya; 3) formation of the current course of the Madeira River when it began to drain the Bolivian basin and connecting itself to the lower course of the Aripuana River at 1-2 mya; 4) previously allopatric populations that come into secondary contact in the Madeira River hybridize and 5) the upper Madeira rapids restrict gene flow or delimit species distributions. Finally, we discuss that the diversification of some co-occurring aquatic vertebrate species (e.g. *Caiman crocodilus* and *C. yacare*, lineages in *Pteronura brasiliensis*, *Inea geoffrensis* and *I. boliviensis*) should be explained in light of the biogeographical model we proposed.

## INTRODUCTION

The origin of Neotropical biodiversity and the biogeography of South American fauna have fascinated biologists since Wallace's expedition to the Amazon and Negro river basins. Many hypotheses have been proposed, and it is clear that many different processes have contributed to the origin of this biodiversity (Leite & Rogers, 2013; Pyron & Wiens, 2013). The River barrier hypothesis, proposed by Wallace (1852), is one of the most frequently invoked theory to explain this high biodiversity, especially in the Amazon, so that formation of rivers will lead to vicariant speciation in terrestrial taxa (e.g. Pounds & Jackson, 1981; Ribas et al., 2012; Boubli et al., 2014; Oliveira et al., 2016). Rivers also play an important, if different role for aquatic taxa and taxa associated with aquatic environments. They act as corridors for the dispersal of species (e.g. Pounds & Jackson, 1981; Barthem & Goulding, 1997; Fernandes, 1997; Hrbek et al., 2005; Santos et al., 2007), so we may expect that aquatic species are largely panmictic or show a pattern of isolation-by-distance along the length of their distributions.

On the other hand, the current configuration of most river networks themselves were geologically assembled in stages (Latrubesse & Franzinelli, 2005), resulting in structuring of species occupying them (e.g. Schneider et al., 2012). In general, historical events of landscape changes result in drainage rearrangements or headwater captures, which may promote dispersion of aquatic-dependent taxa to an area beyond its original distribution (geodispersal) or even a secondary contact between previously isolated populations (Albert & Carvalho, 2011; Crampton, 2011). Furthermore, such rearrangements are expected to have affected several species with similar life histories at the same time frame and in a similar way, producing congruent spatial-temporal distribution patterns that can be used to make

biogeographic inferences (Avise, 2000; Smith et al., 2014).

Multiple geological rearrangements occurred in the contact zone between Amazon and Paraguay river basins, promoting headwaters capture events and consequently aquatic fauna exchange between these basins (Carvalho & Albert, 2011). The last headwater capture event that occurred between these basins was due the compression event along the Andean belt (~2,5 Ma) which gave rise to the modern Pantanal wetland (Assine, 2004). One of the most important transition zone between aquatic vertebrates occupying both Amazon and Pantanal River basins is represented by the entire Madeira River sub-basin. Understanding what led to the structuring of species and species groups of the Madeira River sub-basin is particularly challenging, mainly because the observed pattern of spatial structure can be explained by both ecological or vicariant speciation processes.

The Madeira River sub-basin is characterized by gradients in vegetational communities (Schietti et al., 2016; Ortiz et al., 2018); it also is a transitional zone between the Amazonian rainforest, and the drier biomes to the south of it including the Cerrado, the Beni savanna and the Chaco plain, thus clinal divergences may be important, especially to terrestrial taxa. In the case of caimans, which despite being a semi-aquatic species uses the terrestrial habitat to forage and build their nests, we may expect a pattern of isolation-by-distance. In contrast, the Madeira River sub-basin would have undergone drainage rearrangements due to the uplift of the Fitzcarrald Arch, which would have isolated populations that were later reconnected with the rest of Amazon basin. If this isolation of Madeira sub-basin has lasted long enough, we may expect that aquatic-dependent species show a pattern of genetic structure incompatible with isolation-by-distance scenario in the Madeira River, thus reinforce the historical isolation scenario.

There are few aquatic taxa that occur in the Madeira basin and also in the Paraguay and the rest of Amazon basin, and thus Cuvier's smooth-fronted caiman (*Paleosuchus palpebrosus*), the smallest Neotropical Alligatoridae (Campos et al., 2010), would represent an ideal systems to test such diversification scenarios. *Paleosuchus palpebrosus* is semi-aquatic and can be found in flooded forests surrounding small tributary rivers and lakes of the Amazon basin (Campos et al., 2010); however, it also occurs in the upper Paraguay basin where it is restricted to small rivers at high elevations before they enter the Pantanal floodplain (Campos & Mourão, 2006).

Muniz et al. (2018) delimited evolutionary lineages deeply genetic structured along the Paraguay-Madeira-Amazon River, that were hidden in the nominal species *Paleosuchus palpebrosus*. Given the congruence between genetic structuring and geographical distribution, the authors have named such lineages as ESUs (Evolutionary Significant Units) and MUs (Management Units) as follows: ESU "Amazon", ESU "Madeira-Bolivia" (subdivided into MU "Madeira" and MU "Bolivia") and ESU "Pantanal". The pattern of geographical structuring these authors found could be explained for both, isolation-by-distance and historical isolation, and such competitive hypothesis were tested in the present study.

The aim of this study was to test the role of the drainage rearrangements along the Paraguay-Madeira-Amazon River corridor for the structuring of the *P. palpebrosus*. We further propose a biogeographical model to explain the diversification of *P. palpebrosus* lineages along these areas and discuss the utility of this model for other co-occurring aquatic and semi-aquatic taxa.

## MATERIAL AND METHODS

### Study area and sample collection

The sample strategy used in this study is the same we used in Muniz et al. (2018; thesis chapter 1). We sampled individuals along a transect starting in the upper Paraguay basin ( $19^{\circ}11' S$  and  $57^{\circ}36' W$ ) and continuing through the Bolivian sub-basin to the mouth of the Madeira River ( $03^{\circ}43' S$  and  $59^{\circ}04' W$ ). Additionally we sampled individuals from the central Amazon, the lower Madeira-Purus interfluve, the upper Japura River and the Guiana Shield. In total, we sampled 206 individuals from 28 localities (Fig. 1a). Number of individuals per locality ranged from one to 14. Samples were obtained by removing one or more scutes from adults, sub-adults or juveniles during standard field marking procedures. Individuals were captured at night, and collected scutes were stored in 95% ethanol and deposited at the Animal Genetics Tissue Collection (CTGA) of the Federal University of Amazonas (UFAM), Brazil. Caimans were captured under license #13048-1/IBAMA Brazil.

### Molecular data collection

Total DNA was isolated using a standard proteinase K/phenol-chloroform extraction protocol (Sambrook et al., 1989). The mitochondrial cytochrome b gene (1102 bp) and the nuclear *MYC* (myelocitomatosis, 966pb), *DEN* (dentin, 570pb) and *MOS* (c-mos, 520pb) genes were amplified and sequenced following the protocol of Hrbek et al. (2008). The generated sequences were assembled, edited and aligned in the software Geneious 6.1.8 (Drummond et al., 2014).

We also generated a reduced representation library of 20 individuals of *P. palpebrosus*, five individuals from each of the mitochondrial groups (see below), and five individuals of *P.*

*trigonatus*. Library preparation followed in spirit the protocol of Peterson et al. (2012), but included modifications such that sample digestion and linker ligation was done simultaneously. From each individual, 200 ng of genomic DNA was digested with *SdaI* and *Csp6I* restriction enzymes (Fermentas), and the P and A linker were ligated to the fragments. The number of expected fragments in the tight range of fragments selected was estimated based on genomes of *Alligator mississippiensis* and *A. sinensis* deposited in Genbank (approximately  $2.2 \times 10 \times 9$  bp). Following PCR enrichment and size selection of fragments between 320 and 400 bp (producing  $\sim 15,000$  fragments) in the PippinPrep (Sage Science), all sampled were pooled at equimolar quantities, and sequenced on an IonTorrent PGM using the 400 bp sequencing chemistry and the 318 chip. The complete protocol is available on GitHub (<https://github.com/legalLab>). Reads were processed in the pyRAD v3.0.5 pipeline (Eaton, 2014) and were used to generate a matrix of unlinked SNPs and of concatenated sequences. All alleles had to have minimum coverage of 5x to be called, and in the final datasets, a locus had to be present in at least 50% of the individuals to be included.

## Data analyses

We evaluate hypothesis of isolation-by-distance and historical isolation by testing if the current patterns of genetic diversity are explained by an equilibrium between gene flow and genetic drift among the sampled localities of the transect, or if additional historical processes are necessary to explain the patterns of distribution of genetic diversity. For this we carried out both a simple and a multiple matrix regression (partial Mantel test). For the traditional Mantel test, we analyzed two matrices: 1) genetic distance matrix (mtDNA data), based on pair-wise  $\Phi_{ST}$  between localities; 2) matrix of geographical distances between

localities following the course of rivers, except in the Pantanal where *P. palpebrosus* is restricted to the periphery of the basin. For the partial Mantel test, in addition to the two matrices above, we included a binary matrix representing biological groups/phylogenetic lineages. The binary matrix was derived from the evolutionary lineages identified in the program B<sub>APS</sub> representing connectivity (1) or lack thereof (0) between individuals (Sokal et al., 1997). Linear regressions and correlations between the matrices were carried out in Spacial Analysis in Macroecology (SAM) software (Rangel et al., 2010) with statistical significance being assessed via 5000 permutations.

In order to estimate gene flow, and its historical components, between geographically adjacent populations, we analyzed the mtDNA data within the IM<sub>A2</sub> framework (Hey & Nielsen, 2007). Each population pair we ran 15 independent chains with heating and chain-swapping following an exponential probability. Following a burn-in of 10 thousand topologies, we generated 30 million topologies, sampling each 10<sup>th</sup> topology for a total of 300 thousand topologies.

We used the program BEAST v2.3.2 (Bouckaert et al., 2014) to generate a time calibrated mtDNA phylogenetic hypothesis of the divergence of the main lineages of *P. palpebrosus*. We used one sequence from the most frequent haplotype of each of the four mtDNA haplogroup, as well as one sequence of *P. trigonatus* from genbank (EU161679.1) as outgroup (Hrbek et al., 2008). We fixed the topology (Amazon(Pantanal(Madeira, Bolivia))) to correspond the well supported Bayesian inference using 22 individuals representative of all haplotypes (see Muniz et al., 2018; thesis chapter 1). We also considered the divergence time of *P. palpebrosus* and *P. trigonatus* (mean = 9.53 my; 95% HPD = 7.54–11.31 my), based on species tree estimated by Oaks (2011), as a secondary calibration point. An alternative cyt *b*

time-calibrated tree was generated using only fossil records following Oaks (2011) with an additional calibration point at ~12 mya, corresponding to the first non-ambiguous *Paleosuchus* fossil (Salas-Gismondi et al., 2015).

The sampling strategy consisted of 10 million generations with topologies sampled every thousands generation, starting from a random tree. The Birth-Death process was applied on all the uncalibrated divergence times (Gernhard, 2008) and default values for remaining priors. We also used a lognormal relaxed-clock, that assumes independent rates of substitution on each branch of the tree (Drummond et al., 2006). To improve the accuracy of phylogenetic reconstruction (Castoe et al., 2004) we partitioned the data *a priori* by codon position. The topologies were used to calculate posterior clade probabilities and divergence times. Convergence of all parameters was assessed by inspection in the program TRACER (Drummond et al., 2012). Estimates of parameter values were based on an effective sample size (ESS) of at least 200. Of the 1000 topologies, the first 50 were discarded as burn-in, and the remaining tree were summarized in TREEANNOTATOR – component of the BEAST package. The resulting temporally calibrated topology was visualized in FIGTREE v1.4.2 (Rambaut, 2014).

To reconstruct phylogenetic relationships using the nuDNA (ddRADseq) data, and to test for concordance in phylogenetic relationships based on the mtDNA and nuDNA data, we ran an analysis on SNAPP (Bryant et al., 2012) under the default priors ( $\lambda = 1/X$ ;  $\mu = 1/X$ ;  $v = 1/X$ ;  $snapprior = \gamma$  with parameter values determined empirically). The analysis consisted of 10 million generations with topologies sampled every thousands generation. Divergence time calibrations were also based on the species tree divergence estimate of *P. palpebrosus* and *P. trigonatus* (Oaks, 2011).

## RESULTS

Of the four genes analyzed, the mitochondrial cytochrome *b* gene had the most phylogenetic information. In a total of 1102 bp, sequenced in 206 individuals, we observed 42 variable sites of which 37 were parsimony informative. The three nuclear genes contained at most two alleles and thus were not used in subsequent analyses (see supplementary material, Figure S1).

Next generation sequencing produced approximately 3.6 million usable reads after processing in PYRAD (Eaton, 2014). On average, between 17 to 21 thousand unique loci with an average coverage of 3.5x were observed in each individual in the 300-400 bp range. Filtering loci to a minimal of 5x coverage, on average 2-3 thousand loci per individual with an average coverage of 7.5-8.5x coverage were retained. In the final dataset, where a locus had to be present in at least 50% of the individuals, 722 loci were retained. These 722 loci represented 532 unlinked SNPs.

We investigated the pattern of geographical structuring in the Amazon-Madeira-Paraguay corridor, using both simple and multiple Mantel tests. The traditional Mantel test analysis between genetic and geographic distances indicated that the two matrices are correlated ( $r = 0.621$ ;  $p < 0.001$ ), showing that 38.6% of the variation in genetic distance is explained by geographic distance between pairs of adjacent localities. The partial Mantel test allowed us to evaluate the contribution of each predictive variable, historical divergence (History) between groups and isolation-by-distance (IBD), for the observed patterns of population structuring of genetic diversity (Fig. 1). The binary matrix containing information about whether pairs of individuals belong to the same or different biological groups obtained from BAPS was significantly correlated with the genetic distance matrix ( $r = 0.906$ ;  $p <$

0.001) such that historical processes explained 82.0% (a+b, Fig. 1) and isolation-by-distance 38.6% (b+c, Fig. 1) of the variation in the distribution of genetic diversity along the transect. However, while 44.7% (a, Fig. 1) of the total variation is explained exclusively by historical differentiation, only 1.3% (c, Fig. 1) of the total variation is explained by ongoing restricted gene flow (isolation-by-distance). As expected, it was not possible to completely separate historical from ongoing components given that the binary matrix is also spatially structured, i.e. correlated with the geographic distance matrix. Thus 37.3% (b, Fig. 1) of the total variation could be explained by either historical or current processes.

The IM<sub>A</sub>2 analyzes based on cyt *b* dataset showed absence of gene flow between pairs of populations: Amazon / Madeira-Bolivia / Pantanal. However, non-zero gene flow was observed between Madeira and Bolivia mitochondrial clusters, likely restrictely in the localities downstream and upstream of the Teotônio rapids. The rate of gene flow was low in both directions, however, slightly greater in the direction of Bolivia to Madeira ( $2Nm = 1.655$  individuals per generation) than in the opposite direction ( $2Nm = 0.629$  individuals / generation).

Using cyt<sub>b</sub> data we estimated the age of the first split within *P. palpebrosus* species complex at 1.36 mya (95% HPD, 0.48 – 3.15 mya), when the “Amazon” diverged from the others. The divergence time between “Pantanal” and “Madeira+Bolivia” was 1.06 mya (95% HPD, 0.33 – 2.26 mya), while the divergence found between the populations of “Madeira” and “Bolivia” was 0.13 mya (95% HPD, 0.01 – 0.44 mya; see Fig. 2). Using SNAPP analysis with genomic dataset we estimated the age of the root of *P. palpebrosus* species complex at 3.49 mya (95% HPD, 3.08 – 4.03 mya). The first diverging lineage was the genomic cluster from the Pantanal, with divergence time estimated at 2.79 mya (95% HPD, 2.57 – 3.18 mya).

Geographic regions were not monophyletic, and individuals from the “Amazon” were nested within the “Madeira+Bolivia” genomic clusters. The age of the root of the “Amazon” plus “Madeira+Bolivia” clade was estimated at 1.55 mya (95% HPD, 1.28 – 1.95 mya; see Fig. 3). An alternative time-tree estimated with fossil record calibration points only are provided in the supplementary material (Fig. S2).

## DISCUSSION

Before we discuss some aspects of our results it is important to know the current macro-geographic features of the Amazon-Madeira-Pantanal corridor. These regions consists of river basins and sub-basins separated by well-defined geological features, with the exception of the Amazon macro-geographic region (occupied by the Amazon group/cluster) which is not separated by any evident physical barrier from the Madeira River macro-geographic region (occupied by the Madeira and Bolivian group/cluster). Further south, the Madeira River is separated by a 290 km stretch of rapids and waterfalls (Cella-Ribeiro et al., 2013) from the Bolivian basin macro-region (occupied by the Madeira and Bolivian group/cluster) which in turn is separated by the Parecis mountains from the Pantanal macro-region (occupied by the Pantanal group/cluster); the Pantanal encompasses the headwaters of the Paraguay River basin while the Bolivian basin encompasses the headwaters of the Madeira River basin.

Muniz et al. (2018) noticed that the spatial distribution of genetic structure they found in *P. palpebrosus* is congruent with the geological features described above. The authors argued that *P. palpebrosus* is clearly comprised of at least three distinct evolutionary lineages along the transect from the Pantanal to the Amazon. Such pattern of geographical structuring

would be explained by both, isolation-by-distance (IBD) and historical isolation, and we tested these competitive hypothesis in the present study. We found significant correlation between genetic and geographical distances using traditional Mantel test, that not necessarily should be interpreted as IBD, because historical events are also spatial autocorrelated (see Diniz-Filho et al., 2009; Legendre & Legendre, 2012). We applied a Partial Mantel test to quantify the relative contribution of both current isolation and deep historical events to explain the observed genetic patterns. We found that historical isolation best explain the spatial distribution of the genetic diversity within *P. palpebrosus* (see Fig. 1).

### **Phylogeny and divergence time estimation of lineages**

Muniz et al. (2018) found a geographical congruence between mitochondrial and genomic clusters using population genetic structure analyses. However, the phylogenetic reconstruction using SNPs dataset was different from that made with the mitochondrial marker. The SNAPP reconstruction indicated that "Pantanal" was the sister group of the other lineages within *P. palpebrosus* instead of "Amazon", whose samples were phylogenetically nested within the "Madeira" and "Bolivia" (Fig. 3).

We also found subtle incongruities in the divergence times estimated by the different markers. Analysis of the mtDNA data suggests that the oldest divergence dated at 1.36 mya (95% HPD, 0.48 – 3.15 mya) is between individuals from the Amazon and all other individuals. This split was followed by the divergence of the "Pantanal" lineage from the "Madeira+Bolivia" lineage at 1.06 mya (95% HPD, 0.33 – 2.26 mya). Older divergence times were observed in SNAPP analyses of the ddRADseq SNP data, the root of the SNAPP topology was dated at 3.49 mya (95% HPD, 3.08 – 4.03 mya). Given that individuals from

other localities are phylogenetically nested within the Pantanal group, the coalescent event directly preceding allopatric divergence between the Pantanal and the other samples, was dated at 2.79 mya (95% HPD, 2.57 – 3.18 mya). Despite the subtle incongruities on time of divergence between markers the confidence intervals from basal diversification overlaps and are compatible with the biogeographic model we proposed below.

Such incongruities between mtDNA and nuclear data indicate that historical mixture events could have occurred between "Amazon" and "Bolivia" via Madeira River. This mixture would have been maintained for an extended period until reproductive isolation mechanisms were established, producing a current preferential mating even in the absence of an explicit geographical barrier. This hypothesis would plausibly explain how the allele frequencies of "Amazon" and "Madeira+Bolivia" were maintained different in the STRUCTURE results ( $k = 3$ ; Muniz et al., 2018), despite individuals from "Amazon" were phylogenetically nested within the "Madeira+Bolivia" in the SNAPP reconstruction (Fig. 3).

Furthermore, the STRUCTURE analysis evidenced an unidirectional introgression of "Pantanal" to "Bolivia" as result of a male mediated dispersion, since "Pantanal" remains monophyletic in mtDNA (maternally inherited) and with differentiated allele frequencies in nuclear genome. Interesting that an individual in the Guaporé River that has mtDNA of "Bolivia" was included in the ddRADseq data and showed > 90% of the genome from "Pantanal" (Muniz et al., 2018), representing certainly a hybrid. This individual from Bolivian basin was nested within "Pantanal" clade in the SNAPP reconstruction, thus corroborating the hybrid condition.

The multispecies coalescent analysis on SNAPP incorporates the effect of Incomplete Lineage Sorting (ILS) but not of Lateral Gene Transfer (LGT), so that the presence of LGT

violates one of the SNAPP assumptions (Bryant et al., 2012). The LGT would produce a reticulation of character states incompatible with Hennigian cladistic inferences and consequently produce an unreliable estimate of the phylogeny. Since the mtDNA marker is free of the effect of hybridization we believe that its topology better represents the phylogenetic relationships within *P. palpebrosus* and thus the biogeographic model we will propose below is based on that topology. It is important to highlight that the mtDNA and nuclear markers show complementary histories and thus our ddRADseq data will be useful to investigate the evolutionary processes such as ILS, LGT, gene flow and demographic changes that occurred along the diversification of *P. palpebrosus*.

### **Biogeographic model for biological diversification across the Madeira basin**

The Amazon River is formed by the confluence of the Ucayali and Marañon Rivers and has the Madeira River its main tributary, both in volume and sediment delivery. The current pattern of the Amazon River running into the Atlantic, and the formation of the Madeira River drainage as we know it today occurred after the breach of the Purus Arch. Theories explaining the evolution of the drainage in the Amazonian landscape divide scientists depending on whether data sets (marine- or land-derived) or the type of analytic methods (stratigraphic, geochemistry, sedimentology or phylogeography) are used (Hoorn et al., 2017). Two alternative time frames are debated to the transcontinental origin of Amazon River: A Late Miocene hypothesis (Hoorn et al., 1995, 2010; Figueiredo et al., 2009; Antoine et al., 2016) and a Plio-Pleistocene hypothesis (Campbell et al., 2006, 2010; Latrubesse et al., 2010; Ribas et al., 2012). However, all authors agree that the Bolivian basin was continuous with the western Amazon basin before the formation of the transcontinental Amazon River. As

these competitive hypotheses for the current conformation of the Amazon River predate our biogeographic model we could use any of them. So we chose the map proposed by Hoorn et al. (2010) to represent the landscape before the divergence of *P. palpebrosus* (Fig. 6A).

The Paraguay and Amazon basins were primarily isolated because of the formation of Chapare Buttress, a structural divide between paleo-Amazonas-Orinoco and Paraguay basins by about 30-20 mya (Lundberg et al., 1998). The first event of capture of headwaters happened 11.8-10 mya when the boundary between these basins moved south to the Michicola Arch and headwaters of Upper Paraguay basin were captured by Amazon basin (Lundberg et al., 1998). Subsequently, many headwater capture events happened and the precise limits of the two watersheds have shifted numerous times (Wilkinson et al., 2006).

The uplift of the Fitzcarrald Arch in early middle Pliocene commenced to isolate the Bolivian basin (southern Amazonian foreland basin sensu Espurt et al. 2007) from the Amazon basin (northern Amazonian foreland and eastern Amazon basin sensu Espurt et al. 2007) no earlier than 4 mya (Espurt et al., 2007, 2010). This uplift was gradual and continuous until the principal outflow of the Bolivian basin, the proto-Purus River, was severed (probably 3-2 mya), resulting in major remodeling of drainage patterns within the Bolivian basin, turning the Bolivian basin into an endorheic basin (Menezes, 1988) dominated by swamps and lakes (Fig. 4B). Headwater capture events may have allowed faunal exchange between the Bolivian endorheic basin and Pantanal at this time.

The formation of the modern Pantanal wetland occurred in the Late Pliocene (~2.5 Ma) following the orogenesis of the Parecis mountains (Ussami et al., 1999) and the last compression event along the Andean belt (Assine, 2004) which also represents the last major headwater capture event (Fig. 4C). This headwater capture would have continuously

distributed species, which posteriorly would have undergone vicariant divergence. Currently, the divide between these basins is not noticeable, encompassing several large fluvial fans (megafans) and lowland divide that provide a suitable landscape for movement of aquatic taxa across basins (Carvalho & Albert, 2011), that is, currently the basins are partially connected. For about 1,400 years the Parapeti River (in the headwater of Mamoré River) flows into the Izozog swamp and eventually exchanges water with the Timané River (a tributary of the Paraguay River), during the rainy season (Iriondo, 1993), thus allowing for faunal interchange between these basins and possibly promoting a secondary contact between deeply diverged lineages.

Subsequent to the separation of the Paraguay basin, the Bolivian endorheic basin channeled through the escarpment of the Brazilian Shield, creating a series of rapids now characteristic of the upper Madeira River. Eventually the outflow from the Bolivian basin connected with the lower portion of the Aripuana River, creating the current Madeira River (Räsänen et al., 1987; Hoorn et al., 1995; Campbell et al., 2001), and reconnecting the aquatic fauna of the Bolivian basin with that of the Amazon basin (Fig. 4D). The age of the Madeira River is supported by the divergence of sister lineages of trumpeter birds of the genus *Psophia* (Ribas et al., 2012) and the antbirds of the genus *Rhegmatorhina* (Ribas et al., 2018).

This sequence of events had important consequences for the Madeira basin fauna evolution. First, it would have transported Bolivian fauna down the Madeira River until its encounter with the Aripuanã River. Second, this fauna would have come into secondary contact with allopatrically diverged fauna of the Amazon basin. Third, the descend of the

Madeira River over the Brazilian Shield resulted in the formation of the upper Madeira River rapids which subsequently partially or completely limited bidirectional gene flow.

The model can thus be summarized this way: 1) isolation of the Bolivian basin and the Pantanal from the Amazon basin 3-2 mya by the rise of the Fitzcarald Arch (Fig. 4B); 2) isolation of the Bolivian basin from the Pantanal by the compression event along the Andean belt 2.5 mya (Fig. 4C); 3) formation of the current course of the Madeira River when it began to drain the Bolivian basin, transcending the Brazilian shield at the region of the upper Madeira rapids, and connecting itself to the lower course of the Aripuana River at 1-2 mya (Fig. 4D); 4) previously allopatric populations that come into secondary contact in the Madeira River hybridize (Fig. 4D); 5) the upper Madeira rapids restrict gene flow or delimit species distributions (Fig. 4D).

### **Can this model be applied to other aquatic species?**

Drainage rearrangements are expected to produce a congruent spatial-temporal distribution in aquatic species, that can be used to make biogeographic inferences. Species with different dispersal capacity and ancestral distribution can respond differently to the same historical event, thus producing a spatial-temporal distribution partially inconsistent, that not necessarily invalidate the model. Here we discuss some examples of aquatic vertebrates whose diversification could be explained in light of the biogeographical model we proposed.

Pickels et al. (2011), using mitochondrial marker, found a spatial-temporal pattern in the semi-aquatic giant otter (*Pteronura brasiliensis*), very similar to what we find in *P. palpebrosus* using the same molecular marker. The authors found four deeply structured phylogenetic groups distributed in “Amazon+Orinoco+Guianas”, “Madeira+Madre de Dios”,

“Itenez” (or Guaporé River corresponding to our denomination of “Bolivia”) and “Pantanal”; thus showing an entirely congruent spatial pattern with *P. palpebrosus* diversification. Despite the relationships between these phylogroups are uncertain due to the low support of the nodes, the divergence time of the basal split at 1.24–1.69 mya (0.72–3.1 mya 95% HPD) and subsequent divergence at 0.88 mya (0.2–1.6 mya 95% HPD) also demonstrate a temporal congruence with *P. palpebrosus*.

The uplift of the Fitzcarrald Arch, and thus the isolation of the western Amazon basin from the Bolivian basin would have led to the vicariant divergence of many of the species previously shared between them. This event corresponds not only to the split between *P. palpebrosus* lineages from the Amazon and the Bolivian-Pantanal basin, but is also reflected in the sister taxon relationships of caimans (*Caiman crocodilus* and *C. yacare*), dolphins (*Inia geoffrensis* and *I. boliviensis*), frogs (*Allobates femoralis* and *A. hodli*), and fishes (*Cichla monoculus* and *C. pleiozona*). Divergence of the caiman was estimated at 3.28 mya (2.09–4.79 mya 95% HPD, species tree) (Oaks, 2011), of the dolphins at 2.87 mya (1.31–4.90 mya 95% HPD) (Hrbek et al., 2014), the frogs at 2.8 mya (1.4–4.3 mya 95% HPD) (Simões, 2010) and the fishes at 10% pair-wise divergence in the mtDNA control region suggesting approximately 2-3 my divergence (Willis et al., 2012).

Multiple headwater capture events occurred between Pantanal and the Bolivian basin, so it is observed in the divergence of the “Pantanal” and “Madeira+Bolivia” lineages of *Paleosuchus palpebrosus* and the deep phylogenetic divergence of *Caiman yacare* lineages (~1.5 mya) reported by Godshalk (2006). While no species of *Inia* occurs in the Paraguay basin, iniid dolphins (*Inia* congeners) occurred in the Paraguay basin until at least the late Miocene (Gutstein et al., 2014). Fish genera that are widespread in the Paraná-Paraguay basin, but with

only one species with restricted distribution in the Amazon, support the headwater capture as a promoter of aquatic fauna exchange between them (e.g. Menezes, 1988; Wimberger et al. 1998; Kullander, 2003; Bockmann & Miquelarena, 2008). Other case, involve fish species that occur in both basins and are restricted to high elevations, as *Cetopsis starnesi* and the *Ixinandria steinbachi* (see Vari et al., 2005; Rodriguez et al. 2008, respectively). In these cases, the dispersal through megafan dynamics seems unlikely and stream capture in the Sub-Andean region is the plausible explanation. More recently Tagliacollo et al. (2015) used a species-dense time-calibrated phylogeny to investigate the pimelodid catfishes and found biogeographical signature of river capture in the spatial and phylogenetic distributions of these taxa. Characiform taxa of the genera *Prochilodus* (Sivasundar et al., 2001), and *Serrasalmus* and *Pygocentrus* (Hubert et al., 2007) occurring on either side of the Parecis mountains are estimated to have diverged 4.1–2.3 mya,  $1.76 \pm 0.2$  mya and  $1.77 \pm 0.3$  mya, respectively. All these divergence times were related to events of headwater capture.

Downstream of the confluence of the Madeira and Aripuana rivers, one observes the substitution of lineages of dolphins of the genus *Inia* (Gravena et al., 2014), lineages of the clade B of peacock cichlids (Willis et al., 2012), as well as several terrestrial taxa (Geurgas & Rodrigues, 2010; Simões et al., 2013; Ortiz et al., 2018). Additionally, in this region, there is an abrupt change in fish communities, one characteristic of the middle and upper Madeira River, and the other of the Amazon basin (Torrente-Vilara & Zuanon, pers. com.). Upstream of this contact zone, a discordance in the mtDNA and nuDNA phylogenies of *Paleosuchus palpebrosus* is observed. This is best explained by hybridization of the “Bolivian+Madeira” lineage with the Amazon lineage resulting in the replacement of the former by the later’s nuDNA. The same pattern of hybridization between and replacement of *Inia boliviensis*

nuDNA by *I. geoffrensis* nuDNA is observed in *Inia* river dolphins (Gravena et al., 2015), and in the clade B of the peacock cichlids (Willis et al., 2012).

In *Paleosuchus palpebrosus* (Fig. 3), and in the clade B of the peacock cichlids (Willis et al., 2012) this discordance is observed throughout the Madeira River and the Bolivian basin, while in the *Inia* dolphins it only reaches the first set of major rapids on the upper Madeira River. Being fully aquatic, the Teotônio waterfalls are an unsurmountable barrier in the upstream direction for *Inia* dolphins, while the forested banks of the Madeira River act as a dispersal corridor to dispersal for *P. palpebrosus*. The effect of the Teotônio waterfalls on structuring mtDNA of both *Paleosuchus palpebrosus* and *Inia boliviensis* is the same, however. Gene flow is primarily, or in the case of *Inia boliviensis* exclusively in the downstream direction (Gravena et al., 2015), with an estimated 0.13 my divergence (0.01–0.44 mya 95% HPD) observed in *P. palpebrosus* and a similar divergence age of 0.12 mya (0.03–0.28 mya 95% HPD) of *Inia boliviensis* groups upstream and downstream of the rapids (Gravena et al., 2014).

Although our model explains why hybridization occurs in the Madeira River, it does not explain why discordances in mitochondrial and nuclear DNA patterns persist. It is intriguing to observe concordance in the discordance of mitochondrial and nuclear DNA patterns across multiple taxa occupying the Madeira River basin, which leads us to suspect that the commonly invoked explanation of female philopatry is unlikely mechanism in maintaining this pattern. Thus, future studies evaluating such question will be welcome.

**Biosketch.** F.M. is a PhD student using field and laboratory based approaches to study the evolution of *Paleosuchus* crocodilians. All authors have had long term research interest in South American crocodilian taxa, and use these taxa to understand evolutionary processes that generate observed biodiversity patterns in crocodilian and other Amazonian taxa. All authors are also avid field biologists and seek to contribute to the conservation of these enigmatic species. Laboratory website is at <http://evoamazon.net>.

**Authors' contributions.** F.M., I.P.F. and T.H. conceived the study; All contributed in the design of this study; F.M. and Z.C. collected samples; F.M. and T.H. collected sequence data; F.M., I.P.F. and T.H. participated in data analysis and all authors drafted and gave final approval for publication.

**Competing interests.** We declare that we have no competing interests.

**Funding.** Financial support was provided by grants CNPq/CT-Amazon 575603/2008-9 to IPF, CNPq 482662/2013-1 to TH and CNPq 470383/2007-0 to ZC. We thank Embrapa Pantanal (Macroprograma 3), Fundect, Fundação O Boticário and Santo Antônio Energia for providing additional financial support. This work forms a portion of FM's PhD thesis at the Genetics, Conservation and Evolutionary Biology program of INPA/UFAM. FM is supported by a fellowship from FAPEAM, and IPF and TH by a CNPq fellowship.

**Acknowledgments.** This study would not have been possible without the many colleagues who partook in fieldwork and contributed with samples, members of the LEGAL lab who

help optimize the ddRAD protocol and Sandra Hernandez who helped generate the ddRADseq data. We thank Jack Sites, and members of the Sites lab for helpful comments on earlier versions of this MS.

## REFERENCES

- Antoine, P. O., Abello, M. A., Adnet, S., Ali, J. A. S., Patrice, B., .... Salas-Gismondi, R. (2016). A 60-million-year Cenozoic history of western Amazonian ecosystems in Contamana, eastern Peru. *Gondwana Research*, 31, 30–59.
- Assine, M. L. (2004). A bacia sedimentar do Pantanal Mato-Grossense. In V. Mantesso-Neto, A. Bartorelli, C.D.R. Carneiro, & B.B. Brito-Neves (Eds.), *Geologia do Continente Sul-Americano - Evolução da Obra de Fernando Flávio Marques de Almeida* (pp. 61–74). Beca, São Paulo.
- Avise, J. C. (2000). Phylogeography: the history and formation of species. Harvard university press, Cambridge, MA.
- Barthem, R. B., & Goulding, M. J. (1997). The catfish connection: ecology, migration, and conservation of Amazon predators. Columbia University Press, New York.
- Bockmann, F. A. & Miquelarena, A. M. (2008). Anatomy and phylogenetic relationships of a new catfish species from northeastern Argentina with comments on the phylogenetic relationships of the genus *Rhamdella* Eigenmann and Eigenmann 1888 (Siluriformes, Heptapteridae). *Zootaxa*, 54, 1-54.
- Boublí, J. P., Ribas, C. C., Lynch-Alfaro, J. W., Alfaro, M. E., da Silva, M. N. F., Pinho, G.M., & Farias, I. P. (2014). Spatial and temporal patterns of diversification on the Amazon: A test of the riverine hypothesis for all diurnal primates of Rio Negro and Rio Branco in Brazil. *Molecular Phylogenetics and Evolution*, 82, 400–412.
- Bouckaert, R. R., Heled, J., Kühnert, D., Vaughan, T., Wu, C. H., Xie, D., Suchard, M. A., Rambaut, A., & Drummond, A. J. (2014). BEAST 2: A software platform for Bayesian evolutionary analysis. *PLoS Computational Biology*, 10, e1003537.
- Bryant, D., Bouckaert, R. R., Felsenstein, J., Rosenberg, N. A., & Roychoudhury, A. K. (2012). Inferring species trees directly from biallelic genetic markers: Bypassing gene trees in a full coalescent analysis. *Molecular Biology and Evolution*, 29, 1917–1932.
- Campbell, Jr. K. E., Frailey, C. D., & Romero Pittman, L. (2006). The Pan-Amazonian Ucayali Peneplain, late Neogene sedimentation in Amazonia, and the birth of the modern Amazon River system. *Palaeogeography, Palaeoclimatology, Palaeoecology*, 239, 166–219.
- Campbell, Jr. K. E., Heizler, M., Frailey, C. D., Romero Pittman, L., & Prothero, D. R. (2001). Upper Cenozoic chronostratigraphy of the southwestern Amazon Basin. *Geology*, 29, 595-598.

- Campos, Z., & Mourão, M. P. G. (2006). Conservation status of the dwarf caiman, *Paleosuchus palpebrosus*, in the region surrounding Pantanal. *Crocodile Specialist Group Newsletter*, 25, 9–10.
- Campos, Z., Sanaïotti, T. M., & Magnusson, W. E. (2010). Maximum size of dwarf caiman, *Paleosuchus palpebrosus* (Cuvier, 1807), in the Amazon and habitats surrounding the Pantanal, Brazil. *Amphibia-Reptilia*, 31, 439-442.
- Carvalho, T. P. & Albert, J. S. (2011). The Amazon-Paraguay Divide. In J. S. Albert & R. E. Reis (Eds.), *Historical Biogeography of Neotropical Freshwater Fishes* (pp. 192-202). University of California Press, Los Angeles.
- Castoe, T. A., Doan, T. M., & Parkinson, C. L. (2004). Data partitions and complex models in Bayesian analysis: The phylogeny of gymnophthalmid lizards. *Systematic Biology*, 53, 448-469.
- Cella-Ribeiro, A., Torrente-Vilara, G., Hungria, D. B., & Oliveira, M. de (2013). As corredeiras do Rio Madeira. In L. J. de Queiroz, G. Torrente-Vilara, W. M. Ohara, T. H. S. Pires, J. Zuanon, & C. R. C. Doria (Eds.), *Peixes do Rio Madeira* (pp. 47–53). Santo Antonio Energia.
- Crampton, W. G. R. (2011). An Ecological Perspective on Diversity and Distributions. In J. S. Albert & R. E. Reis (Eds.), *Historical Biogeography of Neotropical Freshwater Fishes* (pp. 165–189). University of California Press, Los Angeles.
- Diniz-Filho, J. A. F., Nabout, J. C., Telles, M. P. C., Soares, T. N., & Rangel, T. F. L. V. B. (2009). A review of techniques for spatial modeling in geographical, conservation and landscape genetics. *Genetics and Molecular Biology*, 32, 203-211.
- Drummond, A.J., Ashton, B., Buxton, S., Cheung, M., Cooper, A., Duran, C., Field, M., Heled, J., Kearse, M., Markowitz, S., Moir, R., Stones-Havas, S., Sturrock, S., Thierer, T., & Wilson, A.C. (2014). Geneious v6.1.8. available at <http://www.geneious.com/>.
- Drummond, A. J., Ho, S. Y. W., Phillips, M. J., & Rambaut, A. (2006). Relaxed phylogenetics and dating with confidence. *PLoS Biology*, 4, e88.
- Drummond, A. J., Suchard, M. A., Xie, D., & Rambaut, A. (2012). Bayesian phylogenetics with BEAUti and the BEAST 1.7. *Molecular Biology and Evolution*, 29, 1969-1973.
- Eaton, D. A. R. (2014). PyRAD: assembly of de novo RADseq loci for phylogenetic analyses. *Bioinformatics*, 30, 1844-1849.
- Espurt, N., Baby, P., Brusset, S., Roddaz, M., Hermoza, W., & Barbarand, J. (2010). The Nazca Ridge and uplift of the Fitzcarrald Arch: implications for regional geology in northern South America. In C. Hoorn & F.P. Wesselingh (Eds.), *Amazonia: Landscape*

- and Species Evolution: A Look into the Past* (pp. 89–100). Wiley-Blackwell.
- Esprt, N., Baby, P., Brusset, S., Roddaz, M., Hermoza, W., Regard, V., Antoine, P. O., Salas-Gismondi, R., & Bolaños, R. (2007). How does the Nazca Ridge subduction influence the modern Amazonian foreland basin? *Geology*, 35, 515-518.
- Fernandes, C. C. (1997). Lateral migration of fishes in Amazon floodplains. *Ecology of Freshwater Fish*, 6, 36–44.
- Figueiredo, J. P., Hoorn, C., van der Ven, P., & Soares, E. C. (2009). Late Miocene onset of the Amazon River and the Amazon deep-sea fan: Evidence from the Foz do Amazonas Basin. *Geology*, 37, 619-622.
- Gernhard, T. (2008). The conditioned reconstructed process. *Journal of Theoretical Biology*, 253, 769-778.
- Geurgas, S. R. & Rodrigues, M. T. U. (2010). The hidden diversity of *Coleodactylus amazonicus* (Sphaerodactylinae, Gekkota) revealed by molecular data. *Molecular Phylogenetics and Evolution*, 54, 583-593.
- Godshalk, R. (2006). Phylogeography and conservation genetics of the yacare caiman (*Caiman yacare*) of South America. Ph.D. thesis, University of Florida, Gainesville, FL.
- Gravena, W., Farias, I. P., da Silva, M. N. F., da Silva, V. M. F., & Hrbek, T. (2014). Looking to the past and the future: were the Madeira River rapids a geographic barrier to the boto (Cetacea: Iniidae)? *Conservation Genetics*, 15, 619-629.
- Gravena, W., da Silva, V. M. F., da Silva, M. N. F., Farias, I. P., & Hrbek, T. (2015). Living between rapids: Genetic structure and hybridization in botos (Cetacea: Iniidae: *Inia* spp.) of the Madeira River, Brazil. *Biological Journal of the Linnean Society*, 114, 764-777.
- Gutstein, C. S., Cozzuol, M. A., & Pyenson, N. D. (2014). The antiquity of riverine adaptations in Iniidae (Cetacea, Odontoceti) documented by a humerus from the Late Miocene of the Ituzaingó Formation, Argentina. *Anatomical Record*, 297, 1096-1102.
- Hey, J. & Nielsen, R. (2007). Integration within the Felsenstein equation for improved Markov chain Monte Carlo methods in population genetics. *Proceedings of the National Academy of Sciences*, 104, 2785-2790.
- Hoorn, C., Bogotá-A, G. R., Romero-Baez, M., Lammertsma, E. I., Flantua, S. G. A., Dantas, E. L., Dino, R., do Carmo, D. A., & Chemale, F. (2017). The Amazon at sea: Onset and stages of the Amazon River from a marine record, with special reference to Neogene plant turnover in the drainage basin. *Global and Planetary Change*, 153, 51-65.
- Hoorn, C., Guerrero, J., Sarmiento, G. A., & Lorente, M. A. (1995). Andean tectonics as a cause for changing drainage patterns in Miocene northern South America. *Geology*, 23,

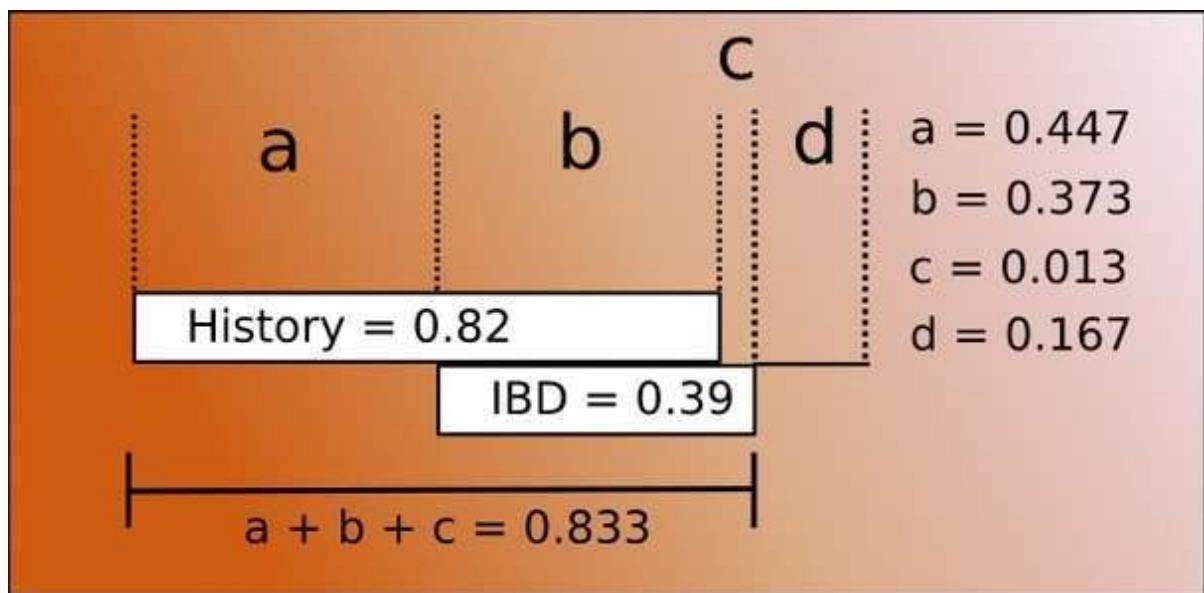
237-240.

- Hoorn, C., Wesselingh, F. P., ter Steege, H., Bermudez, M. A., Mora, A., Sevink, J., Sanmartín, I., Sanchez-Meseguer, A., Anderson, C. L., Figueiredo, J. P., Jaramillo, C., Riff, D., Negri, F. R., Hooghiemstra, H., Lundberg, J. G., Stadler, T., Särkinen, T., & Antonelli, A. (2010). Amazonia through time: Andean uplift, climate change, landscape evolution, and biodiversity. *Science*, 330, 927-931.
- Hrbek, T., Farias, I. P., Crossa, M., Sampaio, I., Porto, J. I. R., & Meyer, A. (2005). Population genetic analysis of *Arapaima gigas*, one of the largest freshwater fishes of the Amazon basin: implications for its conservation. *Animal Conservation*, 8, 297-308.
- Hrbek, T., da Silva, V. M. F., Dutra, N., Gravena, W., Martin, A. R., & Farias, I. P. (2014). A new species of river dolphin from Brazil or: how little do we know our biodiversity. *PLoS ONE*, 9, e0083623.
- Hrbek, T., Vasconcelos, W. R., Rebêlo, G. H., & Farias, I. P. (2008). Phylogenetic relationships of South American alligatorids and the *Caiman* of Madeira River. *Journal of Experimental Zoology Part A: Ecological Genetics and Physiology*, 309A, 588-599.
- Hubert, N., Duponchelle, F., Nuñes, J., Garcia Davila, C., Pauly, D., & Renno, J. F. (2007). Phylogeography of the piranha genera *Serrasalmus* and *Pygocentrus*: implications for the diversification of the Neotropical ichthyofauna. *Molecular Ecology*, 16, 2115-2136.
- Iriondo, M. (1993). Geomorphology and late Quaternary of the Chaco (South America). *Geomorphology*, 7, 289–303.
- Kullander, S. O. (2003). Family Cichlidae. In R. E. Reis, S. O. Kullander, & C. J. Ferraris (Eds.). *Check list of the freshwater fishes of South and Central America* (pp. 605-654). Edipucrs: Porto Alegre.
- Latrubesse, E. M., Cozzuol, M. A., da Silva-Caminha, S. A. F., Rigsby, C. A., Absy, M. L., & Jaramillo, C. (2010). The Late Miocene paleogeography of the Amazon Basin and the evolution of the Amazon River system. *Earth Science Reviews*, 99, 99-124.
- Latrubesse, E. M. & Franzinelli, E. (2005). The late Quaternary evolution of the Negro River, Amazon, Brazil: Implications for island and floodplain formation in large anabranching tropical systems. *Geomorphology*, 70, 372-397.
- Legendre, P. & Legendre, L. F. J. (2012). *Numerical ecology*. Elsevier, Oxford, UK.
- Leite, R. N., & Rogers, D. S. (2013). Revisiting Amazonian phylogeography: insights into diversification hypotheses and novel perspectives. *Organisms Diversity & Evolution*, 13, 639-664.
- Lundberg, J. G., Marshall, L. G., Guerrero, J., Horton, B., Malabarba, M. C. S. L., &

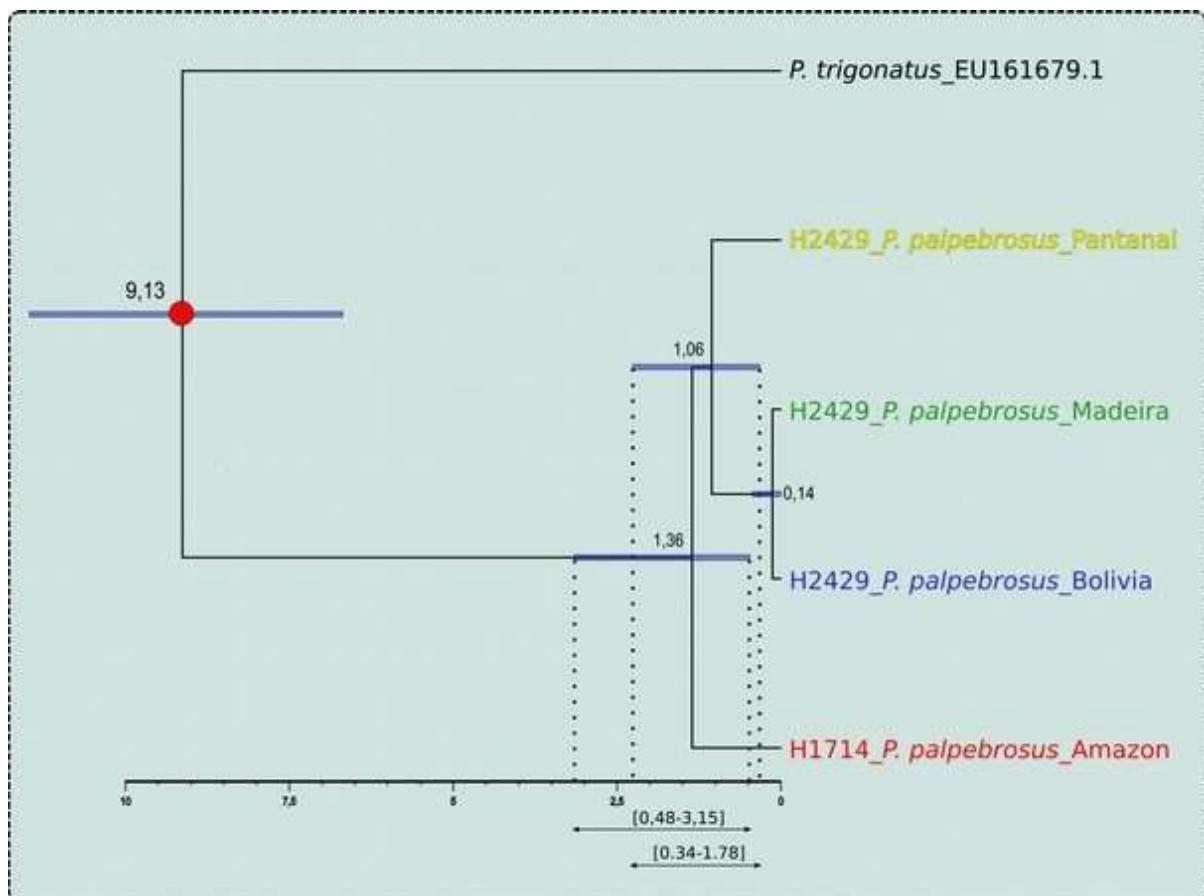
- Wesselingh, F. P. (1998). The stage for Neotropical fish diversification: a history of tropical South American rivers. In L. R. Malabarba, R. E. Reis, R. P. Vari, Z. M. S. Lucena, & C. A. S. Lucena (Eds.), *Phylogeny and Classification of Neotropical Fishes* (pp. 13–48). Edipucrs: Porto Alegre, Brazil.
- Menezes, N. A. (1988). Implications of the distribution patterns of the species of *Oligosarcus* (Teleostei, Characidae) from central and southern South America. *Proceedings of a Workshop on Neotropical Distribution Patterns*, 295-304.
- Muniz, F. L., Campos, Z., Hernández Rangel, S. M., Martínez, J. G., Souza, B. C., De Thoisy, B., Botero-Arias, R., Hrbek, T., & Farias, I. P. (2018). Delimitation of evolutionary units in Cuvier's dwarf caiman, *Paleosuchus palpebrosus* (Cuvier, 1807): insights from conservation of a broadly distributed species. *Conservation Genetics*, 19, 599-610.
- Oaks, J. R. (2011). A time-calibrated species tree of crocodylia reveals a recent radiation of the true crocodiles. *Evolution*, 65, 3285-3297.
- Oliveira, D. P., Carvalho, V. T., & Hrbek, T. (2016). Cryptic diversity in the lizard genus *Plica* (Squamata): Phylogenetic diversity and Amazonian biogeography. *Zoologica Scripta*, 45, 630-641.
- Ortiz, D. A., Lima, A. P., & Werneck, F. P. (2018) Environmental transition zone and rivers shape intraspecific population structure and genetic diversity of an Amazonian rain forest tree frog. *Evolutionary Ecology*, 32, 359–378.
- Peterson, B. K., Weber, J. N., Kay, E. H., Fisher, H. S., & Hoekstra, H. E. (2012). Double digest RADseq: an inexpensive method for de novo SNP discovery and genotyping in model and non-model species. *PLoS ONE*, 7, e37135.
- Pickles, R. S. A., Groombridge, J. J., Zambrana Rojas, V. D., Van Damme, P., Gottelli, D., Kundu, S., Bodmer, R., Ariani, C. V., Iyengar, A., & Jordan, W. C. (2011). Evolutionary history and identification of conservation units in the giant otter, *Pteronura brasiliensis*. *Molecular Phylogenetics and Evolution*, 61, 616–627.
- Pounds, J. A. & Jackson, J. F. (1981). Riverine Barriers to Gene Flow and the Differentiation of Fence Lizard Populations. *Evolution*, 35, 516-528.
- Pyron, R. A. & Wiens, J. J. (2013). Large-scale phylogenetic analyses reveal the causes of high tropical amphibian diversity. *Proceedings of the Royal Society of London Series B: Biological Sciences*, 280, 20131622.
- Rambaut, A. (2014). FigTree v1.4.2, <http://tree.bio.ed.ac.uk/software/figtree/>.
- Rangel, T. F. L. V. B., Diniz-Filho, J. A. F., & Bini, L. M. (2010). SAM: A comprehensive application for Spatial Analysis in Macroecology. *Ecography*, 33, 46-50.

- Räsänen, M. E., Salo, J. S., & Kalliola, R. J. (1987). Fluvial perturbation in the western Amazon basin: regulation by long-term sub-Andean tectonics. *Science*, 238, 1398-1401.
- Ribas, C. C., Aleixo, A., Nogueira, A. C. R., Miyaki, C. Y., & Cracraft, J. (2012). A palaeobiogeographic model for biotic diversification within Amazonia over the past three million years. *Proceedings of the Royal Society of London Series B: Biological Sciences*, 279, 681–689.
- Ribas, C.C., Aleixo, A., Gubili, C., d'Horta, F.M., Brumfield, R.T., & Cracraft, J. (2018) Biogeography and diversification of Rhegmatorhina (Aves: Thamnophilidae): Implications for the evolution of Amazonian landscapes during the Quaternary. *Journal of Biogeography*, 45, 917–928.
- Rodriguez, M. S., Cramer, C. A., Bonatto, S. L., & Reis, R. E. (2008). Taxonomy of *Ixinandria* Isbrücker & Nijssen (Loricariidae: Loricariinae) based on morphological and molecular data. *Neotropical Ichthyology*, 6, 367-378.
- Sambrook, J., Fritsch, E. F., & Maniatis, T. (1989). *Molecular Cloning: A Laboratory Manual*. Cold Springs Harbor Laboratory Press, Cold Springs Harbor, NY.
- Santos, M. C. F., Ruffino, M. L., & Farias, I. P. (2007). High levels of genetic variability and panmixia of the tambaqui *Colossoma macropomum* (Cuvier, 1818) in the main channel of the Amazon River. *Journal of Fish Biology*, 71A, 33-44.
- Schiatti, J., Martins, D., Emilio, T., Souza, P. F., Levis, C., Baccaro, F. B., Pinto, J. L. P. V., Moulatlet, G. M., Stark, S. C., Sarmento, K., de Araújo, R. N. O., Costa, F. R. C., Schöngart, J., Quesada, C. A., Saleska, S. R., Tomasella, J., & Magnusson W. E. (2016). Forest structure along a 600 km transect of natural disturbances and seasonality gradients in central-southern Amazonia. *Journal of Ecology*, 104(5), 1335-1346.
- Schneider, C. H., Gross, M. C., Terencio, M. L., & Porto, J. I. R. (2012). Cryptic diversity in the mtDNA of the ornamental fish *Carnegiella strigata*. *Journal of Fish Biology*, 81, 1210-24.
- Simões, P. I. (2010). *Diversificação do complexo Allobates femoralis (Anura, Dendrobatidae) em florestas da Amazônia brasileira: desvendando padrões atuais e históricos*. Instituto Nacional de Pesquisas da Amazônia, Manaus, Brazil.
- Simões, P. I., Sturaro, M. J., Peloso, P. L. V., & Lima, A. P. (2013). A new diminutive species of *Allobates* Zimmermann and Zimmermann, 1988 (Anura, Aromobatidae) from the northwestern Rio Madeira—Rio Tapajós interfluve, Amazonas, Brazil. *Zootaxa*, 3609, 251-273.
- Sivasundar, A., Bermingham, E., & Ortí, G. (2001). Population structure and biogeography of

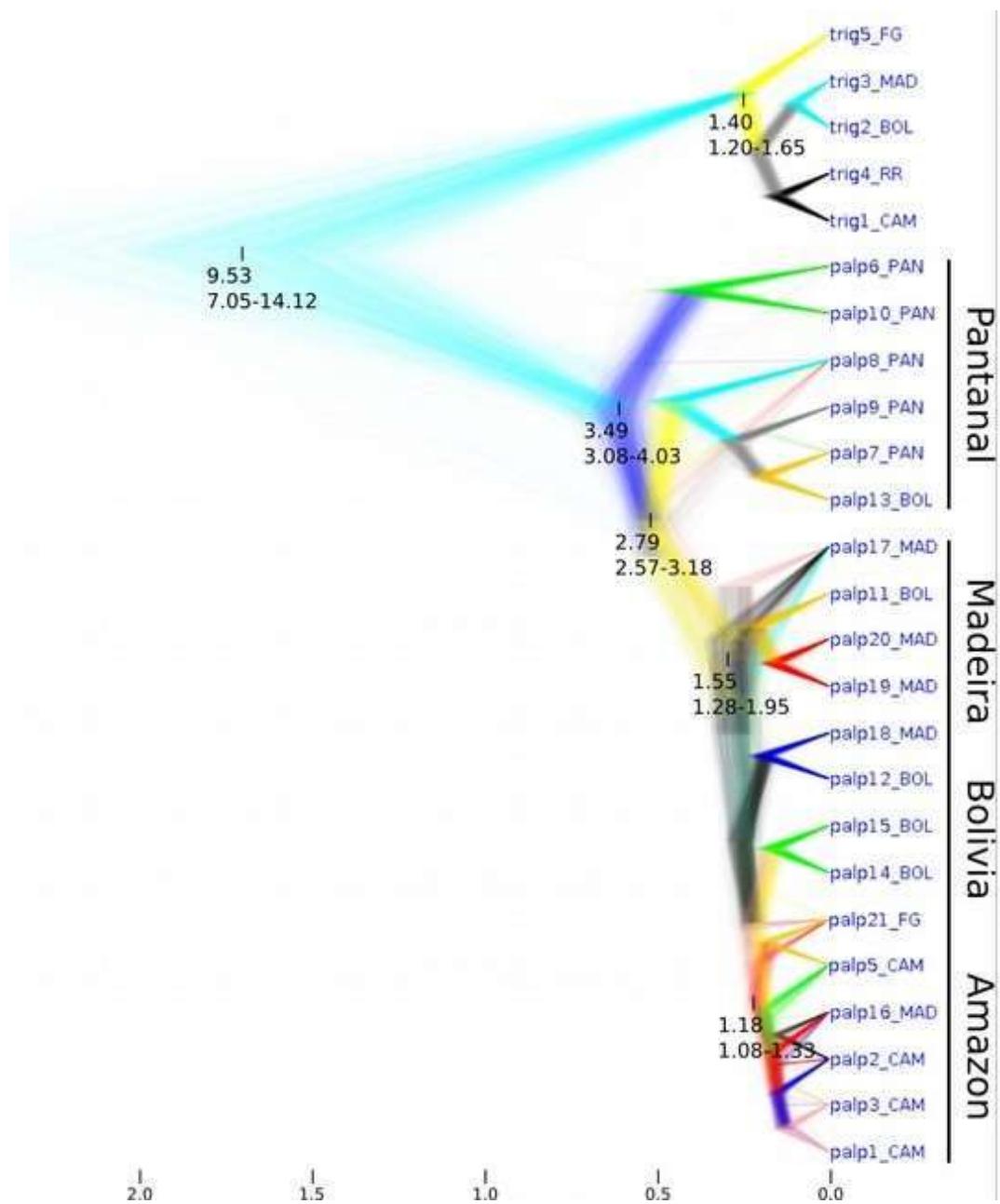
- migratory freshwater fishes (*Prochilodus*: Characiformes) in major South American rivers. *Molecular Ecology*, 10, 407-417.
- Smith, B. T., McCormack, J. E., Cuervo, A. M., Hickerson, M. J., Aleixo, A., Brumfield, R. T. (2014). The drivers of tropical speciation. *Nature*, 515, 406-409.
- Sokal, R. R., Oden, N. L., & Thomson, B. A. (1997). A simulation study of microevolutionary inferences by spatial autocorrelation analysis. *Biological Journal of the Linnean Society*, 60, 73-93.
- Tagliacollo, V. A., Roxo, F. F., Duke-Sylvester, S. M., Oliveira, C., & Albert, J. S. (2015). Biogeographical signature of river capture events in Amazonian lowlands. *Journal of Biogeography*, 42, 2349-2362.
- Ussami, N., Shiraiwa, S., & Dominguez, J. M. L. (1999). Basement reactivation in a sub-Andean foreland flexural bulge: The Pantanal wetland, SW Brazil. *Tectonics*, 18, 25-39.
- Vari, R. P., Ferraris, C. J., & De Pinna, M. C. C. (2005). The Neotropical whale catfishes (Siluriformes: Cetopsidae: Cetopsinae), a revisionary study. *Neotropical Ichthyology*, 3, 127-238.
- Wallace, A. R. (1852). On the monkeys of the Amazon. *Proceedings of the Zoological Society, London*, 20, 107-110.
- Wilkinson, M.J., Marshall, L. G., & Lundberg, J. G. (2006). River behavior on megafans and potential influences on diversification and distribution of aquatic organisms. *Journal of South American Earth Sciences*, 21, 151-172.
- Willis, S. C., Macrander, J., Farias, I. P., & Ortí, G. (2012). Simultaneous delimitation of species and quantification of interspecific hybridization in Amazonian peacock cichlids (genus *Cichla*) using multi-locus data. *BMC Evolutionary Biology*, 12, 96.
- Wimberger, P. H., Reis, R. E., & Thornton, K. R. (1998). Mitochondrial phylogenetics, biogeography, and evolution of parental care and mating systems in *Gymnogeophagus* (Perciformes: Cichlidae). In L. R. Malabarba, R. E. Reis, R. P. Vari, C. A. S. Lucena, & Z. M. S. (Eds.). *Lucena Phylogeny and Classification of Neotropical Fishes* (pp. 509-518). Edipucrs: Porto Alegre.



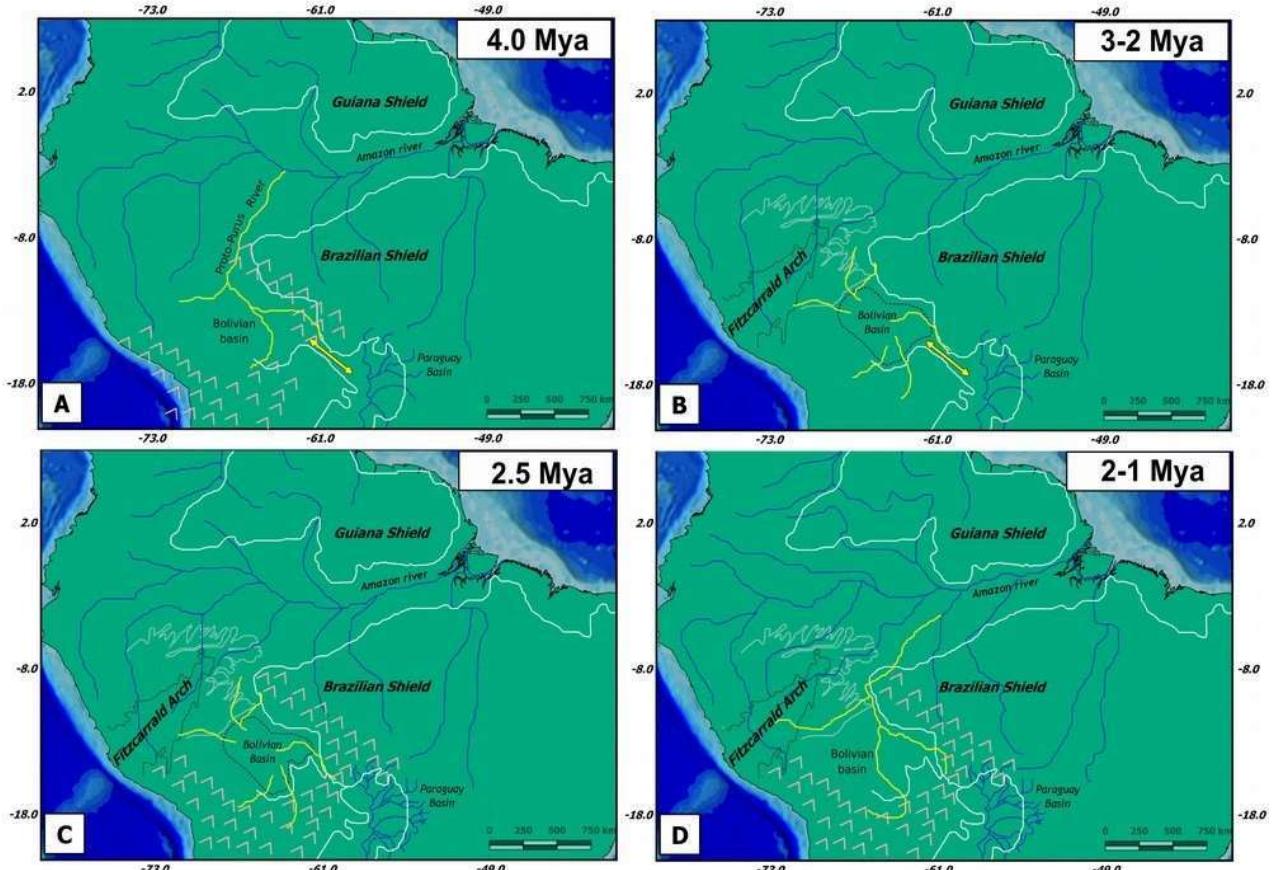
**Figure 1.** Partial Mantel test used to quantify the relative contribution of both current isolation and long-term historical events to explain the observed genetic patterns in the *cyt b* gene of *P. palpebrosus*. The overlap between historical divergence and isolation by distance (IBD) is equal to  $b = (a + b) + (b + c) - (a + b + c)$ , where  $(a + b)$  is the coefficient of determination of the regression using history and  $(b + c)$  is the coefficient of determination of the geographic distance (IBD). The unexplained variation [ $d = 1 - (a + b + c)$ ] was determined using both effects, IBD and historical divergence, as predictors.



**Figure 2.** Time calibrated Bayesian phylogeny of *P. palpebrosus* biological groups based on the analysis of the mitochondrial cyt *b* gene in the program BEAST. The red circle represent secondary calibration point based on Oaks (2011). A sequence of *P. trigonatus* from genbank (EU161679.1) was used as outgroup. Nodes are labeled with the most likely divergence time and bars around nodes represent 95% HPD intervals. Scale is in millions of years ago.



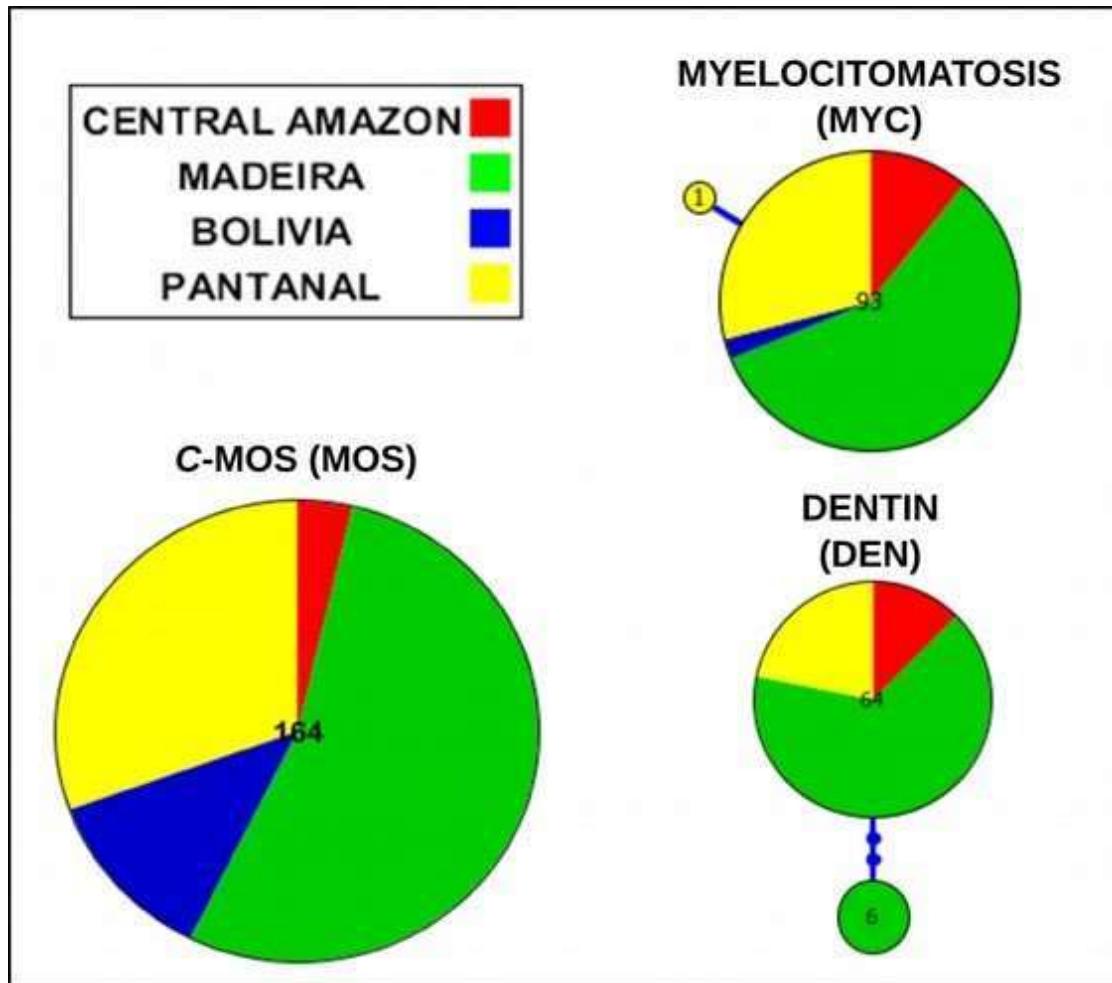
**Figure 3.** Time-calibrated species tree of the genus *Paleosuchus* inferred with SNAPP using 532 unlinked biallelic SNPs. This cladogram represents the posterior distribution of species trees and high color density is indicative of areas in the species trees with high topology agreement. Nodes are labeled with the most likely divergence time and 95% HPD divergence time intervals. Divergence times are in millions of years ago.



**Figure 4.** Geological changes in Amazon and Pantanal landscapes during the last 4 my that influenced in differentiation of the *P. palpebrosus* evolutionary units. (a) The arrangement of drainage system was designed based in Hoorn et al., 2010. Prior to 4 mya there were connection between Bolivian basin and the rest of Amazon basin through the Purus river. In addition, possibly happened events of interchange between Pantanal and Bolivia. (b) The uplift of Fitzcarrald Arch, that finished around 4 Ma, disconnected the populations from Bolivian basin and Amazon (3-2 mya), however Pantanal and Bolivia remained connected. Probably was formed a swamp on Bolivian basin region due their isolation. (c) Total Isolation between Pantanal and Bolivia only occurred after 2.5 Ma, probably when the last event of headwater capture happened between them. (d) After some time, not very well defined, dammed water flowed by easier path, i.e. through Madeira River (presumably 2-1 mya).

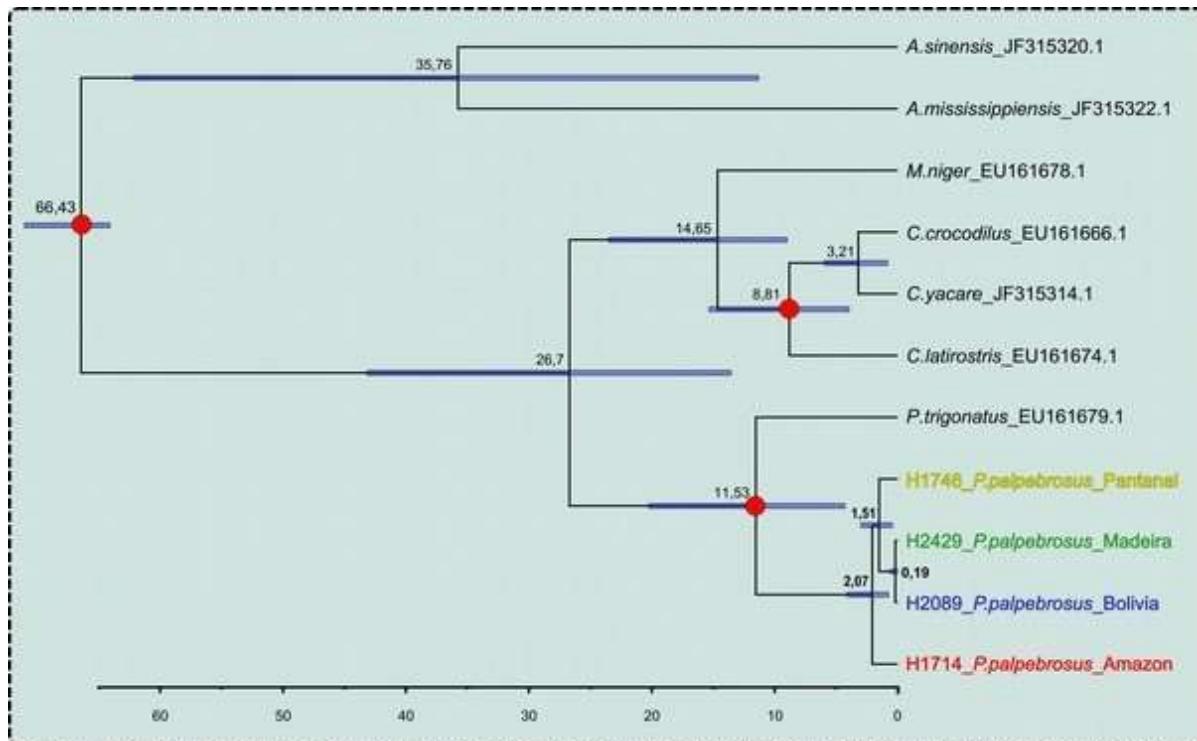
## SUPPORTING INFORMATION

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



2 **Figure S1.** Haplotype networks from genes Myc, C-Mos and DEN. Colors represent the  
3 geographic origin of individuals. Number of gene copies with the same haplotype are given.

4



5 **Figure S2.** Alternative cyt *b* time-calibrated tree using only fossil records. Red circles  
 6 indicate calibrate points we used: (1) Aligatorinae/Caimaninae calibration point at  
 7 between 66-75 mya (Müller & Reisz, 2005); (2) *Caiman* oldest known fossil age at 10 mya,  
 8 modeled with exponential distribution (mean = 4, initial value = 15 and offset = 9); (3) The  
 9 first non-ambiguo *Paleosuchus* fossil at ~12 mya (Salas-Gismondi et al., 2015), modeled with  
 10 exponential distribution (mean = 4, initial value = 15 and offset = 12). Caiman sequences,  
 11 except those of *P. palpebrosus*, were download from genbank.

# CAPÍTULO 3

---

Muniz FL, Campos Z, Polo E, Bittencourt PS, Farias IP, Hrbek T. Filogenômica e reconstrução espaço-temporal das linhagens do complexo de espécies *Paleosuchus palpebrosus* (Alligatoridae: Caimaninae). A ser submetido para o *Journal of Biogeography*.

Artigo Original<sup>1</sup>

Filogenômica e reconstrução espaço-temporal das linhagens do complexo de espécies *Paleosuchus palpebrosus* (Alligatoridae: Caimaninae)

Muniz, Fábio<sup>1,2</sup>; Campos, Zilca<sup>3</sup>; Polo, Érico<sup>2</sup>; Bittencourt, Pedro Senna<sup>2</sup>; Farias, Izeni Pires<sup>2,4</sup>; Hrbek, Tomas<sup>2,4</sup>

<sup>1</sup>*Instituto Nacional de Pesquisas da Amazônia, programa de pós-graduação em Genética, Conservação e Biologia Evolutiva, Manaus, AM, Brazil*

<sup>2</sup>*Laboratório de Evolução e Genética Animal, Departamento de Genética, Instituto de Ciências Biológicas, Universidade Federal do Amazonas, Manaus, AM, Brazil*

<sup>3</sup>*Empresa Brasileira de Pesquisa Agropecuária (Embrapa) Pantanal, Corumbá, MS, Brazil*

<sup>4</sup>*Brigham Young University, Provo, UT*

\*Correspondence: Fábio Muniz, e-mail: fabiolm\_bio@yahoo.com.br

**Palavras-chave:** hibridização, árvore de espécies, área ancestral, jacaré-paguá.

## RESUMO

Estabelecer uma linha prática para delimitar espécies permanece desafiador, principalmente em casos onde ocorre hibridização. Em vertebrados, a hibridização tem sido considerada rara, no entanto, o avanço da tecnologia viabilizou a amostragem genômica de espécies não-modelos, e pesquisas recentes detectaram especiação com hibridização. Estudos prévios sugerem que a história evolutiva de *Paleosuchus palpebrosus* deve ser melhor investigada, uma vez que possivelmente teriam ocorrido múltiplos eventos de isolamento e contato secundário entre as linhagens desse complexo de espécies. Neste estudo, ampliamos a amostragem anterior, reavaliarmos a estrutura populacional e estimamos a origem e rota de dispersão do complexo *P. palpebrosus* usando o gene *cyt b*. Em seguida, utilizando dados genômicos, estimamos as relações filogenéticas entre as linhagens e investigamos os eventos de hibridização ao longo do processo de diversificação do grupo. Por fim, discutimos os efeitos da hibridização na estimativa da árvore de espécies e avaliamos como os resultados se ajustam ao modelo de diversificação previamente proposto. A amostragem foi ampliada ( $N = 357$ ) e análises de estrutura populacional revelaram uma nova linhagem mitocondrial no Escudo Brasileiro. A reconstrução espaço-temporal sugere que o ancestral do complexo *P. palpebrosus* tinha ampla distribuição do Nordeste ao sul da Amazônia. A árvore de espécies com os dados genômicos foi reconstruída usando dois métodos coalescentes e em ambos a linhagem "Amazônia" foi bem suportada, mas as relações filogenéticas entre as demais linhagens permaneceram não resolvidas. Nós também investigamos modelos de divergência com migração ou hibridização pós-divergência e encontramos indícios de hibridização entre as linhagens "Amazônia" e "Madeira". Nossos resultados reforçam o modelo de especiação alopátrica proposto anteriormente, no entanto, será necessário ampliar a amostragem

genômica para investigar a ocorrência de hibridização e testar a hipótese do número de espécies.

## INTRODUÇÃO

Um conceito fundamental em sistemática biológica tem sido o de espécie, alvo de extenso debate na história recente da ciência. O conceito biológico de espécie (Mayr, 1942) foi o paradigma adotado por biólogos durante a segunda metade do século XX, e até pouco tempo influenciou muitas decisões taxonômicas. Mais recentemente, foi proposto o conceito geral de espécie como linhagens evolutivas independentes (de Queiroz, 2007), que foi amplamente aceito, pois conseguiu distinguir filosoficamente o conceito de espécie dos critérios operacionais a serem utilizados para delimitá-las. O debate sobre o conceito foi resolvido, mas o problema de estabelecer uma linha prática para delimitação de espécies permanece, principalmente em casos onde ocorre hibridização ou introgressão.

A hibridização pode prevenir ou promover a especiação, pois assim como a introgressão de genes pode homogeneizar linhagens independentes, diminuindo a divergência genética previamente estabelecida (Runemark et al., 2012), também pode selecionar genes em um novo arcabouço genético, capaz de favorecer o isolamento reprodutivo e a evolução adaptativa de uma linhagem (Butlin & Ritchie, 2009; Abbott et al., 2013; Hedrick et al., 2013; Edwards et al., 2016). A especiação com hibridização é comumente observada em plantas, no entanto, em animais, a hibridização tem sido historicamente considerada rara ou de limitada significância evolutiva (Servedio et al., 2013; Schumer et al., 2014). Porém, o avanço da tecnologia viabilizou a amostragem de genes distribuídos pelo genoma de espécies não-modelos, e em consequência disso, vários estudos encontraram especiação com hibridização em vertebrados na natureza (Brelsford et al., 2011; Hermansen et al., 2011; Stemshorn et al.,

2011; Barbiano et al., 2013; Keller et al., 2013; Monzón et al., 2014; Trier et al., 2014; Lavretsky et al., 2015; Barrera-Guzmán et al., 2018; Gottscho et al., 2017; Maguilla et al., 2018), indicando ser essa uma situação mais comum do que se pensava até pouco tempo atrás.

A revolução tecnológica em curso também repercute no aumento da capacidade de processamento dos computadores, e viabilizou o avanço de técnicas baseadas em coalescência para reconstruir árvores de espécies com dados multiloci. Isso é importante porque a filogenia baseada em um gene pode ser incompatível com a árvore de espécies, em geral, devido ao *Incomplete Lineage Sorting* (ILS). O *Multispecies Coalescent Model* é capaz de considerar as incertezas geradas pelas incompatibilidades entre as árvores de genes e a árvore de espécies (Heled & Drummond, 2010), na tentativa de inferir as reais relações filogenéticas entre os taxóns, mas as incompatibilidades são modeladas como ILS e um dos pressupostos da técnica é ausência de fluxo gênico. Por isso, as relações filogenéticas entre espécies que supostamente hibridizam ou hibridizaram na natureza podem ficar obscurecidas e devem ser avaliadas de maneira complementar a outros métodos analíticos.

Neste estudo, nós investigamos a filogenômica do jacaré-paguá (*Paleosuchus palpebrosus*), uma espécie amplamente distribuída na América do Sul. Muniz et al. (2018; cap. 1 da tese) foram os primeiros a detectar estrutura genética em *P. palpebrosus*, ao proporem a existência de pelo menos três *Evolutionarily Significant Units* (ESUs) no corredor Amazônia-Madeira-Pantanal, que deveriam ser consideradas como espécies candidatas desse complexo de espécies.

Muniz et al. (in prep.; cap. 2 desta tese) propuseram uma hipótese de mudanças sucessivas na paisagem que melhor explicariam os processos de diversificação das linhagens recém-descobertas, assumindo um modelo de especiação alopátrica. Neste estudo foi

encontrado incongruência entre a árvore filogenética reconstruídas com o gene *cyt b* e aquela reconstruída com os marcadores SNPs, de modo que as relações filogenéticas entre as espécies candidatas ainda não puderam ser completamente resolvidas. Foi encontrada monofilia dos grupos com o marcador mitocondrial, mas não com o marcador genômico, o que pode ter sido causado por vários processos evolutivos, dentre os quais os principais são: ILS e hibridização.

Os resultados encontrados em ambos os trabalhos (Muniz *et al.*, 2018; Muniz *et al.*, in prep) sugerem que podem ter ocorrido múltiplos eventos de isolamento e contato secundário entre as linhagens e que existem mecanismos de isolamento reprodutivo entre "Amazonia" e "Madeira". Como já visto, a ocorrência de hibridização pode influenciar na tomada de decisões taxonômicas, então é imprescindível que a história evolutiva do complexo *P. palpebrosus* seja desvendada antes que análises de delimitação de espécies sejam aplicadas.

A seguir destacamos os indícios percebidos nos estudos anteriores que motivaram a elaboração deste: (1) o fato de um indivíduo com haplótipo mitocondrial da linhagem "Bolívia" ter apresentado frequência dos loci nucleares compatível com a linhagem "Pantanal" (ver Muniz *et al.*, 2018), sugere que houve introgressão do genoma do "Pantanal" em "Bolivia" devido a migração dos machos, já que o genoma mitocondrial de "Bolivia" foi mantido; (2) A proporção de alelos compartilhados entre "Bolivia" e "Pantanal" diminui gradativamente à medida que aumenta a distância para os limites atuais entre as bacias Amazônica e do Alto Paraguai, indicando que há uma zona de contato secundário que se estende por toda a drenagem do Rio Guaporé; (3) A monofilia recíproca encontrada com o marcador mitocondrial e a ausência de mistura com os marcadores nucleares, entre as linhagens adjacentes "Amazônia" e "Madeira", sugerem que existe um mecanismo de

isolamento reprodutivo entre elas, uma vez que não há barreira física aparente que os impeçam de trocar genes.

Neste estudo, testamos a ocorrência de possíveis linhagens não identificadas anteriormente, dado que a amostragem com o marcador mitocondrial foi ampliada em relação aos trabalhos anteriores, para incluir localidades nas bacias dos rios Paraná, São Francisco, Parnaíba, entre outros (ver Material e Métodos). Nossos objetivos foram: (1) investigar demografia histórica, estimando a origem e a rota de dispersão das linhagens mitocondriais; (2) reconstruir a árvore de espécies utilizando diferentes métodos coalescentes, a fim de resolver as relações entre as espécies candidatas; (3) estimar o melhor modelo de migração entre as linhagens, descrevendo como o fluxo gênico teria influenciado o processo de especiação das espécies candidatas do complexo *P. palpebrosus*; (4) e avaliar o ajuste destes processos ao modelo biogeográfico previamente proposto (capítulo 2 desta tese).

## MATERIAL E MÉTODOS

### Coleta dos dados genéticos

Nós utilizamos todas as 206 amostras previamente sequenciadas para o citocromo *b* (cyt *b*) utilizadas em Muniz et al. (2018). Sequenciamos 151 amostras adicionais provenientes de 34 localidades em várias bacias hidrográficas situadas nos biomas Cerrado e Caatinga (ver Figura 1). Ao todo, foram obtidas 357 sequências do gene cyt *b* de indivíduos amostrados em 82 localidades (Figura 1). As amostras de tecido adicionais foram coletadas sob a licença brasileira #13048-1/IBAMA e foram depositadas na Coleção de Tecidos de Genética Animal (CTGA) do Laboratório de Evolução e Genética Animal (LEGAL) na Universidade Federal do Amazonas (UFAM), Manaus, Brasil.

Além da amostragem com o marcador mitocondrial nós utilizamos diferentes filtragens de loci com os dados genômicos obtidos a partir de um subset de 20 indivíduos pertencentes ao complexo *P. palpebrosus*, sendo cinco de cada linhagem mitocondrial previamente identificada ("Amazônia", "Madeira", "Bolívia" e "Pantanal"), além de cinco indivíduos de *P. trigonatus* usados como grupo externo.

Para obtenção das sequências adicionais do gene *cyt b* o DNA genômico foi isolado usando o método de extração com CTAB 2 % (Doyle & Doyle, 1987) com uma adição de 15 mg/mL de Proteinase K. O gene *cyt b* foi amplificado usando o protocolo descrito em Hrbek et al. (2008) e as sequências foram obtidas por meio de sequenciamento usando o método de Sanger no sequenciador automático ABI 3500xl (Applied Biosystems). As sequências obtidas foram editadas, montadas e alinhadas no software Geneious 6.1.8 (Drummond et al., 2014).

Nós também produzimos uma representação reduzida do genoma usando o protocolo de sequenciamento de *double digest RAD sequencing* (ddRADseq) (Peterson et al., 2012) e obtivemos as sequências no Ion Torrent PGM utilizando o chip 318 Ion PGM (Life Technologies™). Os detalhes sobre como foram feitas as reduções do genoma são dados em Muniz et al. (2018) e o protocolo completo de *Next-generation sequencing* (NGS) utilizado neste trabalho está disponível no GitHub (<https://github.com/legalLab>).

As leituras obtidas com a corrida de NGS foram primeiramente processadas no *pipeline* pyRAD v3.0.6 (Eaton, 2014) aplicando uma cobertura mínima de 6X e removendo leituras com erros no barcode (sequência identificadora de cada indivíduo). Leituras com mais de quatro nucleotídeos com baixa qualidade foram eliminadas (índice de qualidade Phred abaixo de 20), assim como alelos com frequência mínima abaixo de 0,01. A filtragem inicial dos dados genômicos no pyRAD resultou em 1248 loci que passaram por novas etapas de

filtragem até compor os diferentes conjuntos de dados (*datasets*) utilizados nas análises posteriores, conforme os critérios apresentados a seguir (ver Tabela 1).

### Análise dos dados mitocondriais

O conjunto completo de dados mitocondriais (357 sequências) foi analisado quanto a estrutura populacional por meio de Inferência Bayesiana (IB) no programa BAPS v6.0 (Corander et al., 2008). Foi utilizada a comparação direta das probabilidades posteriores de diferentes número de grupos (K) utilizando o logarítmico de verossimilhança para escolher o K mais provável. Foi efetuada uma análise de *admixture* em nível individual com K variando de 1 a 10 e com 10 corridas independentes para cada valor de K.

Uma árvore de máxima verossimilhança foi reconstruída no programa RAxML v8.1.1 (Stamatakis, 2014) utilizando o modelo evolutivo GTR+G e sua topologia foi utilizada para reconstruir uma rede de haplótipos no programa HAPLOVIEWER (Salzburger et al., 2011). Adicionalmente, geramos uma árvore Bayesiana no programa MrBayes v3.2.2 (Ronquist et al., 2012), com o intuito de testar monofilia e investigar sua posição filogenética da linhagem "Cerrado" em relação às demais linhagens (Figura S1).

A história demográfica das linhagens identificadas com o *cyt b* foi investigada por meio da análise *Bayesian Skyline Plot* (BSP) (Drummond et al., 2005) implementada no programa BEAST v2.4.8 (Bouckaert et al., 2014). Essa análise assume que as populações são panmíticas e utiliza o padrão coalescente inferido para ajustar um modelo demográfico adequado ao conjunto dos dados de sequência disponível (Drummond et al., 2005), e por isso, foram realizadas para cada linhagem separadamente. Foi utilizado o relógio molecular estrito (*strict clock*) e o tempo foi particionado em seis intervalos em cada estimativa usando o BSP. A linhagem "Madeira" não apresentou informação suficiente para realizar o BSP, então os

modelos *Constant Size* e *Exponential growth* foram confrontados usando o método de comparação de modelos AICM (Baele et al., 2012) no programa Tracer v1.6 (Rambaut et al., 2013). Para cada corrida do BSP nós utilizamos uma cadeia com 100 milhões de passos, com árvores sendo gravadas a cada 100 mil passos. A convergência dos parâmetros foi checada no programa Tracer v1.6 (Rambaut et al., 2013). A fim de converter a unidade do parâmetro  $\tau$  para tempo em anos utilizamos a taxa de mutação do mtDNA de crocodilianos de  $3,95 \times 10^{-9}$  mutações por sítio por ano estimada por Eo & DeWoody (2010).

A distribuição do ancestral comum e sua subsequente expansão por parte da América do Sul foi inferida usando o método de difusão Bayesiana de árvore de espécies (Nylander et al.. 2014) usando o marcador mitocondrial. Primeiramente uma árvore de espécies datada foi reconstruída no programa STARBEAST v1.8.4 (Drummond et al., 2012) utilizando o tempo de divergência entre *P. palpebrosus* e *P. trigonatus* (média = 9.53 ma; 95% HPD = 7.54–11.31 ma) como ponto de calibração (Oaks et al., 2011). Posteriormente, nós utilizamos a distribuição posterior de árvores da árvore de espécies para levar em conta as incertezas da inferência filogenética e estimamos a difusão espaço-temporal das espécies usando o modelo de difusão Browniano (Lemey et al., 2010). Nessa análise, foi utilizado um *prior* coalescente considerando o tamanho populacional constante e um modelo de *Relaxed Random Walk* (RRW) com distribuição log-normal relaxada, bem como o modelo de substituição TrN+I indicado pelo jMODELTEST. O tamanho da cadeia foi 10 milhões de passos com posterior descarte de 10% dos primeiros passos como *burn-in*. O programa Tracer v1.6 foi utilizado para checar a convergência dos parâmetros.

A análise de Difusão de Árvores de Espécies requer que sejam fornecidas as distribuições geográficas das espécies-alvo em forma de polígonos no formato Keyhole

Markup Language (KML), os quais foram gerados no Google Earth (<http://earth.google.com>) com base na distribuição dos pontos amostrais de nosso estudo (ver Figura 1). O recurso “timeslice” do programa Spread v1.0 (Bielejec et al., 2011) foi usado para estimar as áreas de distribuição ancestral, extraíndo 80% HPD da distribuição de probabilidade posterior combinada, e então foi produzido um arquivo KML para ser visualizado no Google Earth.

A área ancestral do complexo *P. palpebrosus* também foi estimada no pacote BioGeoBEARS (Matzke, 2014) do programa R (R Development Core Team 2011). Além de inferir a área ancestral, essa análise permite investigar se a diversificação das linhagens se deu por vicariância, dispersão ou evento fundador. Para isso, foram consideradas cinco possíveis áreas ancestrais: Escudo das Guianas (Egui), Planície Amazônica (PAm), Sul da Amazônia (SAM), Cerrado (Cer) e Pantanal (Pant) (Figura 6). O número máximo de áreas ancestrais foi estabelecido como sendo igual ao número total de áreas definidas, cinco.

## Análise dos dados genômicos

Na tentativa de resolver as relações filogenéticas entre as linhagens, os dados genômicos foram utilizados para reconstruir árvores de espécies aplicando dois métodos: o *Multispecies Species Coalescent* (MSC) implementado no programa STARBEAST2 (Bouckaert et al., 2014) e o método coalescente que se baseia em quartetos de topologia de árvores gênicas, implementado no programa ASTRAL-III (Zhang et al., 2018).

O STARBEAST2 é capaz de co-estimar a árvore de espécies e as árvores de genes e leva em consideração as incertezas geradas pelas incompatibilidades entre as árvores de genes (Heled & Drummond, 2010). No entanto, o programa modela essas incompatibilidades exclusivamente como *Incomplete Lineage Sorting*, um efeito direto da retenção de

polimorfismo ancestral entre pares de populações, assumindo ausência de fluxo gênico. Essa análise, porém, se torna inviável para muitos bancos de dados genômicos, devido ao número de parâmetros a serem estimados e ao grande esforço computacional exigido.

Alternativamente, o programa ASTRAL-III busca pela árvore que compartilha o maior número de quartetos de topologia com as árvores gênicas de entrada (Zhang et al., 2018) estimadas previamente usando outro método de reconstrução filogenética. O método implementado no ASTRAL-III é mais rápido e viável de ser aplicado com dados genômicos grandes, além de ser baseado em coalescência e também considerar o efeito do ILS (Mirarab et al., 2014; Zhang et al., 2018).

Para a análise no STARBEAST2, fizemos uma filtragem dos loci com o objetivo de maximizar a informação filogenética contida em um conjunto de dados reduzido. Então, inicialmente, filtramos somente os loci que foram amostrados em pelo menos um indivíduo de cada linhagem e que apresentaram pelo menos um sítio parcimoniosamente informativo (PIS - sítios que agrupam pelo menos dois indivíduos). Dentre estes, filtramos os loci que produziram árvores gênicas com suporte maior que 75% em pelo menos um nó, após análise no RAxML v8.1.1 (Stamatakis, 2014). Ao todo foram retidos 111 loci compondo o *dataset 1*. O particionamento ideal do *dataset 1* foi estimado no programa PartitionFinder v2.1.1 (Lanfear et al., 2012) e os loci pertencentes a uma mesma partição tiveram seus modelos de evolução molecular ligados no STARBEAST2. Os indivíduos foram agrupados de acordo com sua distribuição nas quatro linhagens predefinidas ("Amazonas", "Madeira", "Bolívia" e "Pantanal").

A estratégia utilizando o *dataset 1* não permitiu resolver as relações filogenéticas entre "Madeira", "Bolívia" e "Pantanal" (ver resultados). Então, adotamos uma estratégia

complementar, na qual subdividimos os indivíduos das linhagens "Madeira" e "Bolívia" em dois grupos cada, de acordo com a proximidade geográfica entre eles, produzindo um total de seis grupos ao invés de quatro (ver Figura 6). Em seguida nós filtramos os loci que foram amostrados em pelo menos um indivíduo de cada grupo e que apresentaram pelo menos um PIS para grupos (sítios que unem pelo menos dois grupos), totalizando 334 loci. Após essa filtragem, nós estimamos o particionamento ideal de modelos evolutivos no PartitionFinder v2.1.1 (Lanfear et al., 2012) e concatenamos os loci que apresentaram topologias iguais (estimadas com o RAxML) dentro de uma mesma partição, reduzindo o conjunto de dados para 135 sequências concatenadas que compõem o *dataset 2*.

Para todas as corridas no programa STARBEAST2 foram aplicados o modelo de especiação de Yule como *Tree prior* e o relógio molecular estrito (*Strict clock*) para todos os loci. As corridas foram realizadas na plataforma CIPRES (Miller et al., 2010; <http://www.phylo.org/index.php>) utilizando 2 bilhões de passos e árvores sendo gravadas a cada 100 mil passos, resultando em uma posterior de 20.000 árvores. A convergência dos parâmetros foi checada no programa Tracer v1.6 para ter certeza de que os *Effective Sample Sizes* (ESS) foram > 200 (Rambaut et al., 2013). A árvore de máxima credibilidade dos clados foi sumarizada a partir do conjunto de árvores posteriores usando o TreeAnnotator v1.8.0 (Drummond & Rambaut, 2007) e editada no FigTree v1.4.2.

Para análise no ASTRAL-III foi utilizado o *dataset 3*, que possui o maior número de loci (989 loci), uma vez que foram retidos os loci que estavam presente em pelo menos um indivíduo de cada linhagem. As árvores gênicas para cada locus foram reconstruídas separadamente no programa RAxML v8.1.1 (Stamatakis, 2014), sendo que a árvore de máxima verossimilhança para cada locus foi escolhida dentre 20 geradas e o número de

*bootstraps* foi determinado por autoMRE. O suporte da árvore de espécies do ASTRAL-III foi avaliado anotando-se a filogenia com as probabilidades posteriores.

A causa do baixo suporte de nós encontrados na árvore de espécies do ASTRAL-III foi investigada usando-se o programa Quartet Sampling (Pease et al., 2018) a partir da matriz concatenada dos dados. Esse programa utiliza métricas de suporte baseada em quartetos para descrever se o suporte dos nós está sendo influenciado por terminais com posição incerta (*rogue taxa*), por falta de informação ou por informações conflitantes (Pease et al., 2018).

Uma vez que os métodos que estimam árvore de espécies assumem ausência de fluxo gênico, nós utilizamos o *Generalized Phylogenetic Coalescent Sampler*, G-PhoCS v1.2.3 (Gronau et al., 2011) para investigar a ocorrência de hibridização entre as linhagens atuais e/ou ancestrais. Esse programa também foi utilizado para estimar parâmetros demográficos populacionais, como: tamanho efetivo populacional ( $\theta$ ), taxas de migrações ( $m$ ) e tempos de divergência ( $\tau$ ), para quatro linhagens do complexo *P. palpebrosus* investigadas com os dados genômicos. O fluxo gênico foi modelado usando diferentes bandas de migração, que são equivalentes a taxas de migração constantes entre duas linhagens ao longo de todo o período de coexistência delas. A presença de fluxo gênico pode afetar diretamente a estimativa desses parâmetros, por isso, espera-se que análises que consideram isolamento com migração forneçam estimativas mais próximas do real. Além disso, o G-PhoCS possibilita integrar todas as possíveis fases dos dados diploides, eliminando a necessidade de esforço computacional prévio para inferir haplótipos (Gronau et al., 2011), que é uma potencial fonte de erro (Garrick et al., 2010).

Para tornar as corridas computacionalmente possíveis nós reduzimos o número de indivíduos, selecionando seis representantes das quatro linhagens, sendo: um indivíduo de

"Amazônia" (CTGAH\_0884), um de "Pantanal" (CTGAH\_1741), dois indivíduos de "Madeira" (CTGAH\_2173 e CTGAH\_2145) e dois de "Bolívia" (CTGAH\_2310 e CTGAH\_2427). "Madeira" e "Bolívia" apresentam indícios de introgessão do genoma (ver resultados) e por isso selecionamos mais indivíduos destas linhagens. Em seguida, foram testadas cinco topologias possíveis e, para cada uma delas, foram gerados modelos de migração de modo a testar todas as combinações de bandas de migração possíveis entre linhagens adjacentes (Figura 2). Então, nós comparamos os 224 modelos avaliados no G-PhoCS utilizando o critério de AICM (Baele et al., 2012) e escolhemos os modelos mais prováveis. Por fim, nós estimamos os parâmetros demográficos com base na topologia e modelo de migração mais ajustados utilizando todos os cinco indivíduos de cada linhagem.

Os *priors* das distribuições de probabilidade para todos os modelos testados foram modelados com distribuição gamma. Para  $\tau$  e  $\theta$  nós usamos um  $\alpha = 1$ ,  $\beta = 10.000$  (exceto para os  $\theta$  ancestrais, onde usamos  $\beta = 20.000$ ) e para  $m$  nós usamos  $\alpha = 0.002$ ,  $\beta = 0.00001$ . Todos os "finetune parameters" foram ativados no modo automático. Para cada modelo testado nas análises com seis indivíduos, foram feitas 10 réplicas contendo 100 mil passos cada. Os primeiros 10 mil passos de cada réplica foram descartados como *burn in* e então as corridas foram combinadas. Para as análises com todos os 20 indivíduos foram feitas 3 réplicas contendo 2 milhões de passos cada. Os primeiros 200 mil passos de cada réplica foram descartados como *burn in* e então as corridas foram combinadas. O programa Tracer v1.6 foi usado pra checar a convergência dos parâmetros.

De acordo com o sugerido por Gronou et al. (2011), aplicamos a equação  $\theta = 4Ne\mu$  para estimar o tamanho efetivo populacional em número de indivíduos diploides ( $N$ ). Também foi utilizada a equação  $\tau = T\mu/g$  para converter o  $\tau$  em tempo de divergência em anos  $T$ , sendo

g o tempo de geração médio da espécie, que assumimos como sendo 20 anos. Para isso, foi considerada a taxa de mutação neutra para crocodilianos de  $\mu = 7,9 \times 10^{-9}$  substituições/sítio/geração, estimada a partir da divergência entre o genoma completo de *Alligator mississippiensis* e de *Crocodylus porosus* (Green et al., 2014). Para converter as taxas de migração  $m$  em taxa de migração por geração nós usamos a equação  $M_{sx} = m_{sx}\theta_x/4$ .

## RESULTADOS

### Estruturação genética e demografia histórica com o gene *cyt b*

A análise de estrutura populacional realizada no BAPS indicou que o valor de  $K = 5$  foi o mais provável. Com isso foi detectado uma nova linhagem no bioma Cerrado e no bioma Caatinga, no Escudo Brasileiro (Figura 1). Além disso, podemos constatar que a linhagem previamente detectada nas cabeceiras do Rio Paraguai não estão restritas a essa região, e se estendem por toda a bacia do Rio Paraná (a leste) e cabeceiras dos Rios Teles Pires e Juruena, formadores do Rio Tapajós.

A rede de haplótipos indica que a recém-descoberta linhagem "Cerrado" tem uma proximidade genealógica com a linhagem "Pantanal" (Figura 3), embora um indivíduo amostrado na bacia do Rio Juruena apresente haplótipo mais relacionado aos da linhagem "Bolívia". Em virtude de haver pelo menos 16 passos mutacionais separando "Pantanal+Cerrado" de "Bolívia+Madeira" podemos inferir que esse indivíduo é descendente de um migrante entre as bacias do Rio Guaporé e Juruena. Também é possível observar que somente nas bacias do Alto Rio Xingu e Alto Rio Araguaia ocorrem indivíduos de ambos os grupos "Pantanal" e "Cerrado", com predominância de indivíduos de "Cerrado", sendo essa uma possível zona de contato entre as linhagens.

O alinhamento das sequências de *cyt b* (1097 pb) no programa Geneious e a tradução dos códons em aminoácidos permitiu verificar que o último dos sítios diagnósticos da linhagem "Amazônia", identificado na *Population Aggregation Analisys* (PAA) realizada no capítulo 1, produziu um códon de parada prematuro. Com isso, o *cyt b* de todos os indivíduos da linhagem "Amazônia" apresentam dois aminoácidos a menos que as demais linhagens, o que caracteriza uma autopoapomorfia dessa linhagem.

As reconstruções demográficas estimadas com *Bayesian Skyline Plot* usando o gene *cyt b* não detectaram variação significativa no tamanho populacional recente de nenhuma das linhagens do complexo *P. palpebrosus* ao longo dos últimos 200 mil anos (Figura 4 A-E).

As relações filogenéticas entre as linhagens, suas áreas ancestrais e posterior difusão pelas principais bacias hidrográficas na América do Sul são mostradas na Figura 5 A-D. A estimativa da área de distribuição ancestral usando a análise de Difusão de Árvores de Espécies indicou que o ancestral de *P. palpebrosus* seria amplamente distribuído no sul da Amazônia e em boa parte do Escudo Brasileiro (Figura 5A). A primeira diversificação dentro do complexo teria ocorrido entre a "Amazônia" e as demais linhagens, quando houve uma expansão da distribuição no sentido norte e sul da bacia Amazônica (Figura 5B). Em seguida a linhagem do Escudo Brasileiro teria se diversificado no ancestral de "Madeira-Bolívia" e no ancestral de "Pantanl-Cerrado" (Figura 5C), quando se expandiu para o sul do Escudo Brasileiro, em direção à bacia do Alto Paraguai. Por fim, o Pantanal e a região leste do Escudo Brasileiro foram colonizados e "Bolívia" e "Madeira" se diversificaram (Figura 5D).

A reconstrução da área ancestral usando o BioGeoBEARS sugeriu que o modelo DIVALIKE melhor se ajusta aos dados de *cyt b* de *Paleosuchus* (Tabela 2). O DIVALIKE indicou que o ancestral do complexo *P. palpebrosus* foi amplamente distribuído, semelhante

ao encontrado com a análise de Difusão de Árvores de Espécies, mas com a pequena diferença de que esse ancestral já estaria distribuído no norte da Amazônia (Figura 6). Os eventos de diversificação teriam ocorrido por alopatria, com exceção da linhagem "Pantanal" que teria sido colonizada recentemente por dispersão de indivíduos provenientes do Escudo Brasileiro (Figura 6). Adicionalmente foi possível inferir a origem de *P. trigonatus* no Escudo das Guianas. O ancestral teria se expandido por toda bacia Amazônica e posteriormente sofrido diversificação por alopatria entre o Escudo das Guianas e o resto da Amazônia (Figura 6).

### **Filogenômica e hibridização no complexo *P. palpebrosus***

A árvore de espécies reconstruída no STARBEAST2 com o *dataset* 1 (Figura 7) recuperou com alto suporte a relação da linhagem "Amazônia" como grupo irmão de "Madeira+Bolívia+Pantanal", em concordância com a topologia do *cyt b*. Não foi possível definir , porém, se "Bolívia" é grupo irmão de "Madeira" ou de "Pantanal" (Figura 7). As topologias alternativas são expressas em forma de árvore de densidade com diferentes cores, azul e vermelho, e foram igualmente prováveis (Figura 7). Em seguida nós adotamos uma estratégia complementar utilizando o *dataset* 2 e recuperamos uma topologia bem suportada, com "Madeira" sendo grupo irmão de "Bolívia+Pantanal". A linhagem "Bolívia" foi parafilética em relação a "Pantanal" (Figura 7).

A árvore de espécies estimada com o programa ASTRAL-III apresentou nós pouco suportados e relações filogenéticas entre os grupos não muito bem definidas. "Amazônia" e "Pantanal" foram monofiléticos e bem suportados, enquanto "Bolívia" foi monofilético, mas com baixo suporte no nó basal ( $pp = 0,64$ ), sendo grupo irmão de "Pantanal" ( $pp = 0,92$ )

(Figura 8). Os indivíduos do "Madeira", no entanto, estão distribuídos pela árvore com valores de baixo suporte, de modo que provavelmente estariam diminuindo o suporte dos ramos adjacentes. Então, nós investigamos a causa do baixo suporte de alguns nós cruciais usando o Quartet Sampling (Figura S2). É importante ressaltar que nenhum dos nós foram pouco suportados devido à falta de informação, com valores de QI altos (quarteto de informatividade). No entanto, encontramos um indivíduo da linhagem "Madeira" (CTGAH\_2145) com valor de QF (quarteto de fidelidade) substancialmente menor que os demais (Figura S2), podendo ser um provável "*rogue taxa*". Avaliando o suporte do nó que agrupa "Pantanal" e "Bolívia" identificamos baixo valor de QC (0,24, quarteto de concordância) e alto valor de QD (0,71, quarteto diferencial) indicando baixo suporte por conflito de informação e que existe uma topologia concorrente àquela apresentada.

A hibridização entre as linhagens atuais e/ou ancestrais pode ter sido a causa das incertezas encontradas nas relações filogenéticas. Então, nós investigamos 224 hipóteses de demografia histórica no programa G-PhoCS e escolhemos o melhor modelo utilizando o critério de AICM. Os dois melhores modelos de migração ( $\Delta\text{AICM} < 2$ ) pertencem às topologias A (Modelo 1) e B (Modelo 2) e contam histórias alternativas (Figura 9). Na topologia A, "Amazônia" é grupo irmão de "Madeira+Bolívia+Pantanal". De acordo com o modelo 1 houve hibridização persistente entre "Amazônia" e as linhagens adjacentes em três bandas de migração diferentes, além de ter ocorrido fluxo gênico pós-divergência entre "Madeira" e "Bolívia" (Figura 9). Na topologia B, "Pantanal" é grupo irmão de "Amazonia+Madeira+Bolívia". De acordo com o modelo 2 a hibridização persistente ocorreu entre "Pantanal" e as linhagens adjacentes também em três bandas de migração (Figura 9), mas sem fluxo gênico entre "Madeira" e "Bolívia".

Os parâmetros demográficos: tamanho efetivo populacional ( $Ne$ ), tempo de divergência ( $T$ ) e taxa de migração ( $m$ ); foram estimados para os dois melhores modelos utilizando todos os 20 indivíduos de *P. palpebrosus*, e os resultados são mostrados na Tabela 3. Destacamos a ocorrência de taxas de migração assimétricas, com algumas bandas de migração contendo menos que um migrante por geração.

## DISCUSSÃO

Estudos prévios demonstraram que *Paleosuchus palpebrosus*, uma espécie com ampla distribuição em grande parte da América do Sul, é na verdade, um complexo de espécies que deveria ser melhor investigado (Muniz et al., 2018; Muniz et al., in prep, cap. 2 da tese). Nós ampliamos a amostragem realizada nos estudos anteriores e descobrimos a existência de uma linhagem evolutiva. Essa linhagem está distribuída em boa parte do Escudo Brasileiro, ocupando principalmente o bioma Cerrado e as bacias hidrográficas do Rio São Francisco, Rio Parnaíba e drenagens adjacentes que deságuam no Atlântico, bacia dos Rios Tocantins-Araguaia e cabeceiras do Rio Xingu.

Um resultado relevante envolvendo o gene *cyt b* foi a constatação de que a linhagem "Amazônia" (ver capítulo 1) apresenta um códon de parada prematuro que elimina dois aminoácidos que estão presentes nas demais linhagens do complexo. O *cyt b* é um gene codificador de uma proteína que participa na cadeia transportadora de elétrons da mitocôndria. Uma mutação que resulta na perda de aminoácidos em uma proteína e cuja frequência foi fixada em toda a população com tamanho efetivo populacional grande (> 350.000, Muniz et al., 2018), indica a atuação de forças seletivas por um período prolongado e reforça ainda mais que a linhagem "Amazônia" é uma espécie diferente das demais

linhagens do complexo *P. palpebrosus*. Roos et al. (2007) identificou que três genes mitocondriais ( NADH4, NADH5 e Cyt b) de *P. palpebrosus* são menores que os de *P. trigonatus* devido a ocorrência de um códon de parada prematuro. No entanto, essa comparação foi feita com um indivíduo da linhagem "Amazônia", e pelo menos para o gene cyt b, não há diferenças nos tamanhos do gene de *P. trigonatus* em relação às demais linhagens ("Madeira", "Bolívia", "Pantanal" e "Cerrado") do complexo *P. palpebrosus*.

Muniz et al. (2018) sugeriram que é importante avaliar se a linhagem "Pantanal" ocorre em outras bacias além da bacia do alto Paraguai, pois caso contrário, isso teria importantes implicações para a conservação dessa linhagem, uma vez que ela estaria restrita à uma área sob forte impacto antropogênico. Neste trabalho, foi possível detectar que a linhagem "Pantanal" não está restrita à bacia do alto Paraguai, ocupando também a bacia do Rio Paraná, à leste, e as cabeceiras dos rios Juruena e Teles Pires, ao norte (Figura 1). Assim, essa linhagem inspira menor preocupação do ponto de vista da conservação do que se imaginava.

Algumas localidades nas cabeceiras do Rio Xingu e nas cabeceiras do Rio Araguaia apresentaram haplótipos provenientes de ambas linhagens, "Pantanal" e "Cerrado", embora haja predominância de haplótipos pertencentes ao grupo "Cerrado" (Figura 1). A rede de haplótipos do cyt b sugere uma relação genealógica próxima de "Pantanal" e "Cerrado" e o compartilhamento de haplótipos entre bacias pode estar se mantendo simplesmente por retenção de polimorfismo ancestral, ou alternativamente, indicar a existência de uma zona de contato entre elas.

De fato, é provável que eventos de capturas de cabeceiras tenham sido cruciais para entender a diversificação entre "Pantanal" e "Cerrado". Existe uma relação estreita entre a

fauna aquática da bacia do Alto Paraguai e as cabeceiras dos rios amazônicos que nascem no Escudo Brasileiro (Hubert & Renno, 2006; Carvalho & Alberts, 2011). A presença de espécies de peixes compartilhadas indica que esta é uma importante rota de dispersão entre essas bacias (Carvalho & Alberts, 2011). O evento de compressão do cinturão Andino no final do Plioceno (~2,5 Ma) deu origem à atual bacia do Pantanal (Assine, 2004). Este evento teria promovido capturas de cabeceiras pela bacia do alto Paraguai tanto de Bolívia quanto de alguns tributários do alto Paraná e alto Tocantins-Araguaia (Menezes et al., 2008). Mesmo eventos de capturas mais recentes podem ter ocorrido, estabelecendo novos limites entre as bacias Amazônica e alto Paraguai (Wilkinson et al., 2006).

### **Reconstrução das áreas ancestrais e rota de dispersão**

As reconstruções biogeográficas de espécies que são continuamente distribuídas através da paisagem são difíceis, pois requerem o particionamento das espécies em áreas geograficamente discretas (Ronquist & Sanmartín, 2011). O método de difusão Bayesiana de árvores de espécies tem a vantagem de não precisar de áreas discretas pré-definidas para fazer uma inferência da área ancestral (Nylander et al., 2014), porém não possibilita inferir de maneira explícita a causa da diversificação das espécies (vicariância, dispersão ou evento fundador) como no BioGeoBEARS (Matzke, 2014). Desse modo, ambos os métodos de reconstrução espaço-temporal podem ser utilizados de forma complementar para montar um cenário biogeográfico mais completo.

Ambas as análises reconstruíram uma área ancestral ampla para *P. palpebrosus*, porém o BioGeoBEARS estimou que o ancestral de *P. palpebrosus* ocorreu no norte da Amazônia enquanto a Difusão de Árvores de Espécies estimou uma distribuição que não

inclui essa região. Apesar dessa diferença, os métodos de reconstrução ancestral concordam em relação aos eventos de diversificação alopátrica subsequentes e que a linhagem "Pantanal" se formou recentemente a partir de um evento fundador da linhagem ancestral que ocupava o Escudo Brasileiro.

De um modo geral, nossas reconstruções espaço-temporais não contradizem o modelo biogeográfico proposto no capítulo 2, e ainda fornecem informações adicionais que contribuem para desvendar a rota de dispersão das linhagens. Em ambos os modelos a linhagem ancestral de *P. palpebrosus* teria ocupado o sul da Amazônia e o Escudo Brasileiro antes de colonizar a bacia do alto Paraguai, evidenciando uma rota de dispersão que precisa ser melhor investigada. Outra consequência disso é que no intervalo de tempo em que a subbacia Boliviana endorreica teria se formado (ver capítulo 2) *P. palpebrosus* ainda não havia colonizado a bacia do alto Paraguai. Portanto, ainda que tenham ocorrido eventos de capturas de cabeceiras entre a subbacia Boliviana e a bacia do alto Paraguai, isso não resultou em fluxo gênico ancestral entre as linhagens.

É importante ressaltar que a nossas reconstruções espaço-temporais foram feitas com dados mitocondriais, e que uma amostragem genômica que inclua a linhagem "Cerrado" pode ajudar a esclarecer melhor a rota de dispersão das linhagens do complexo.

### **Filogenômica de *P. palpebrosus***

As árvores de espécie reconstruídas com os dados genômicos no STARBEAST2 recuperaram a linhagem "Amazônia" como grupo irmão das demais linhagens do complexo *P. palpebrosus*, suportando a hipótese de diversificação norte-sul na bacia Amazônica. As relações filogenéticas entre "Pantanal", "Madeira" e "Bolivia", foram resolvidas com a

utilização do *dataset* 2, com "Madeira" sendo grupo irmão de "Bolívia+Pantanal".

A árvore de espécies do ASTRAL-III também não foi capaz de resolver as relações filogenéticas entre essas três linhagens, mas permitiu observar que alguns indivíduos da linhagem "Madeira" possuem posição incerta na árvore filogenética. As incertezas nas relações filogenéticas podem ter sido geradas por (1) informação insuficiente, (2) diversificação rápida das linhagens e consequente *Incomplete Lineage Sorting*, (3) ou fluxo gênico pós-divergência (hibridização). Após descartar que as incertezas nas relações filogenéticas foram causadas pela presença de "*rogue taxa*" ou por falta de informação, nós investigamos modelos de divergência com migração no programa G-PhoCS e detectamos que os melhores modelos suportam hibridização entre linhagens.

Para cada banda de migração foram estimadas as taxas de migração em ambas os sentidos. Desse modo, observamos um fluxo gênico assimétrico, geralmente no sentido de "Madeira+Bolívia". O número de migrantes, para algumas bandas de migração, foram menores que um indivíduo por geração (ver Tabela 3), ou seja, uma taxa de migração baixa para marcadores neutros (Felsenstein, 1976) em comparação com os efeitos da deriva genética. Com isso, é provável que tenha ocorrido uma introgressão persistente de genes de "Amazônia" (topologia A) ou "Pantanal" (topologia B) em "Madeira+Bolívia". Em ambos os cenários, as relações filogenéticas da árvore de espécies seriam impactadas pela introgressão recorrente, produzindo histórias conflitantes que diminuem o suporte dos ramos.

Os melhores modelos de migração foram complementares, pois sustentam introgressão persistente de "Amazônia" ou "Pantanal" para as linhagens adjacentes. Embora as topologias alternativas sejam igualmente prováveis nas comparações utilizando o critério de AICM, nós podemos afirmar que a topologia A (ver Figura 9) é mais coerente, devido sua

congruência com os resultados genômicos encontrados no STARBEAST2 e ASTRAL-III (Figuras 7 e 8) e com as relações filogenéticas encontradas utilizando o marcador mitocondrial (Figura S1), que é livre do efeito da hibridização (Brown et al., 1982; Meyer, 1993). Adicionalmente, a migração persistente entre "Amazônia" e as linhagens adjacentes, sobretudo entre "Amazônia" e o ancestral de "Madeira+Bolívia" (ver Tabela 3) favorece o modelo 2 e pode ter contribuído para que esse modelo tenha sido sugerido como história alternativa igualmente provável.

Não obstante essa hipótese ser uma explicação plausível para a diversificação de *P. palpebrosus* e compatível com o modelo biogeográfico proposto no capítulo 2, existe uma hipótese alternativa ainda não avaliada, de que a linhagem "Madeira" teria surgido como produto da hibridização entre "Amazônia" e "Bolívia". Essa hipótese é complexa e não pôde ser avaliada com as ferramentas analíticas utilizadas nesse trabalho. Entretanto, essas hipóteses alternativas devem ser testadas de maneira mais apropriada em um estudo futuro.

Este estudo fornece contribuições importantes para entender a biogeografia e a dinâmica evolutiva do complexo de espécies *P. palpebrosus*. Algumas perguntas permanecem não respondidas, como: quantas e quais espécies são válidas no complexo de espécies *P. palpebrosus*? Para investigá-las com maior confiabilidade, no entanto, sabemos que será necessário utilizar métodos analíticos capazes de diferenciar o efeito do *Incomplete Lineage Sorting* e da hibridização.

**Tabela 1.** Sumarização das filtragens realizadas com o banco de dados genômicos. Para cada *dataset* são mostrados: a presença e ausência dos critérios de cada filtragem, incluindo os Sítios Parcimoniosamente Informativos (PIS), o número de indivíduos analisados, o número de loci retidos e a análise em que cada *dataset* foi utilizado. O símbolo (+) indica que a filtragem foi aplicada para o *dataset* e o símbolo (-) indica que a filtragem não foi aplicada.

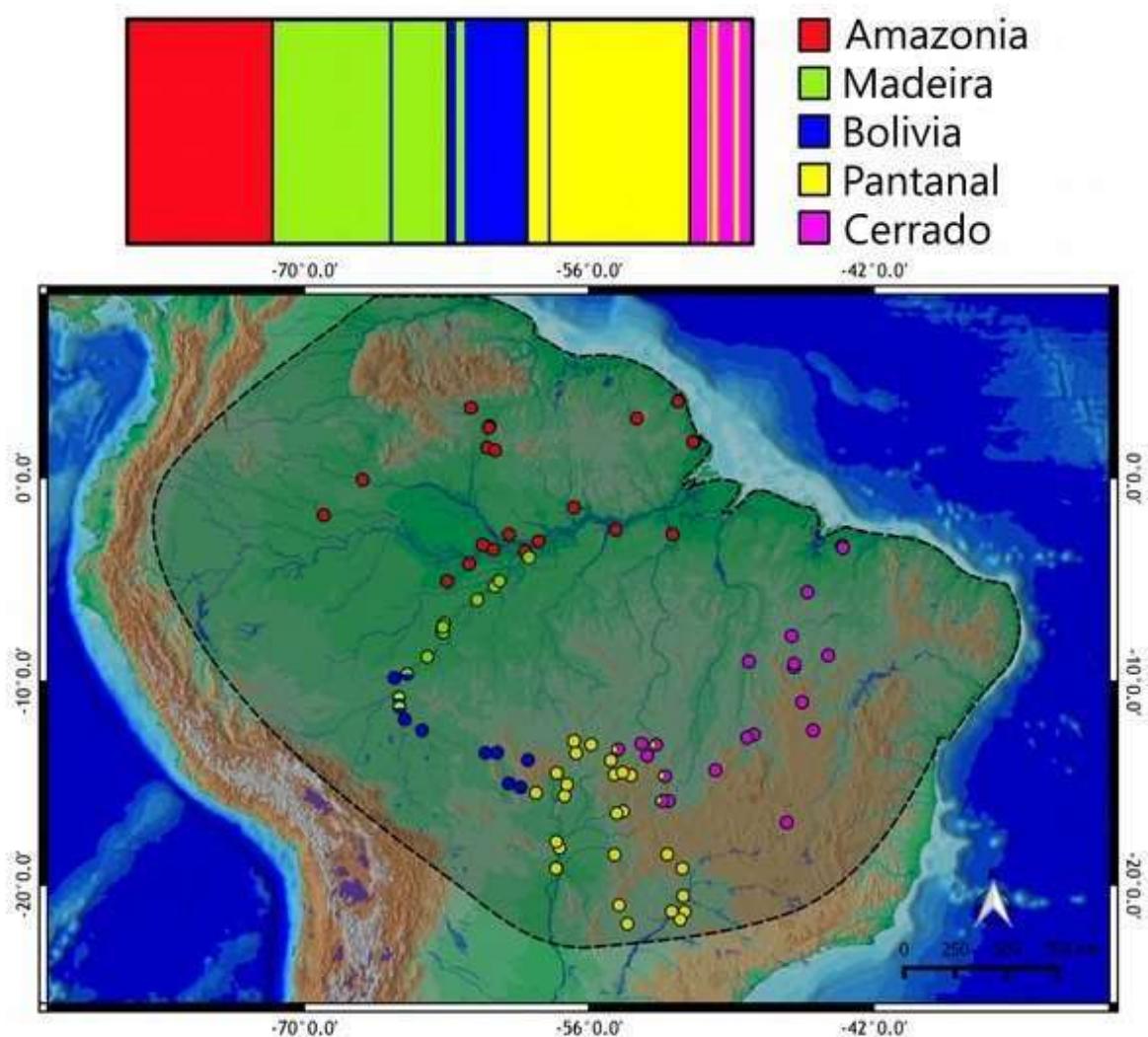
Filtragem de loci	Ao menos 1 indiv. por linhagem	Ao menos um PIS	Ao menos 1 nó bem suportado (> 75%)	Particionamento por modelo evolutivo	Particionamento por topologia	Nº Indiv.	Nº loci	ANÁLISE
PyRAD	-	-	-	-	-	25	1.248	-
<i>Dataset 1</i>	+	+	+	-	-	20	111	STARBEAST2
<i>Dataset 2</i>	+	+	-	+	+	20	334	STARBEAST2
<i>Dataset 3</i>	+	-	-	-	-	25	989	ASTRAL-III
<i>Dataset 4</i>	+	+	-	-	-	6	95	G-PhoCS

**Tabela 2.** Comparação entre os valores de *log Likelihood* para cada modelo do BioGeoBEARS (Matzke, 2013) estimados com base nos dados de *cyt b*. As linhagens foram assinaladas a cinco possíveis áreas ancestrais (ver Figura 6). Foram testados seis modelos, cuja acurácia foram assessadas usando o *Akaike Information Criterion* (AIC). Note que o modelo DIVALIKE possui o menor valor de AIC e por isso foi escolhido como o modelo mais ajustado aos dados.

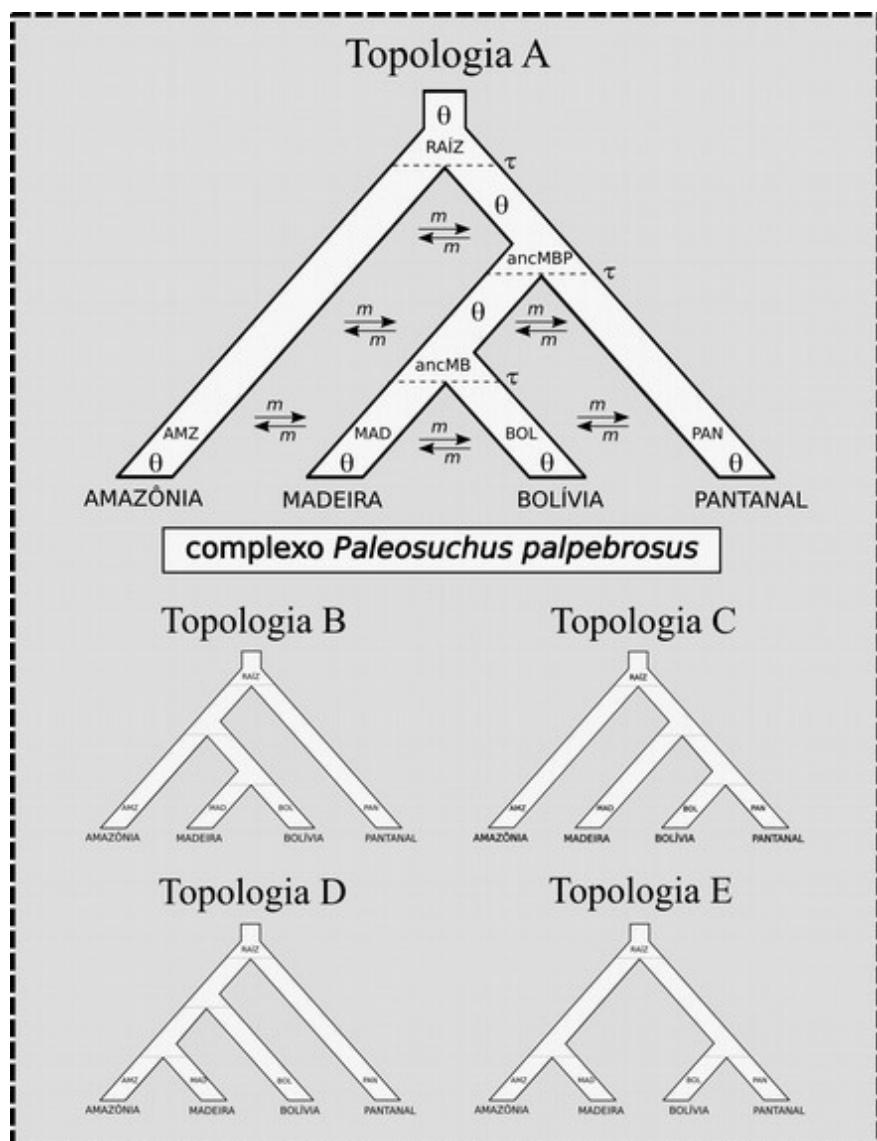
Modelo	log likelihood	dispersão ( <i>d</i> )	extinção ( <i>e</i> )	Evento fundador ( <i>j</i> )	AIC
DEC	-16,07	0,21	0,19	0,00	39,15
DEC+J	-16,06	0,21	0,19	0,01	46,13
DIVALIKE	-14,05	0,17	0,14	0,00	35,10
DIVALIKE+J	-14,05	0,18	0,14	0,00	42,10
BAYAREALIKE	-26,62	0,42	0,65	0,00	60,25
BAYAREALIKE+J	-18,68	0,09	0,18	0,21	51,37

**Tabela 3.** Parâmetros demográficos estimados com base nos melhores modelos de migração estimados no program G-PhoCS (Gronau et al., 2011). Os modelos e topologias correspondem aos apresentados na figura 9. As linhagens foram codificadas em letras: "Amazônia" (A), "Madeira" (M), "Bolívia" (B) e "Pantanal" (P); e os nós ancestrais foram codificados com a combinação dessas letras (ex. MB = ancestral de "Madeira" e "Bolívia"). Os valores médios estimados para o tamanho efetivo populacional ( $N_e$  em mil indivíduos) e para o tempo de divergência ( $T$  em milhões de anos) são mostrados para os modelos 1 e 2. Dentro de parênteses são mostrados os valores mínimos e máximos de cada estimativa. Para cada banda de migração foram estimadas as Taxas de Migração ( $M$ ; número médio de indivíduos migrantes por geração) em ambos os sentidos e as setas indicam a direção do fluxo gênico.

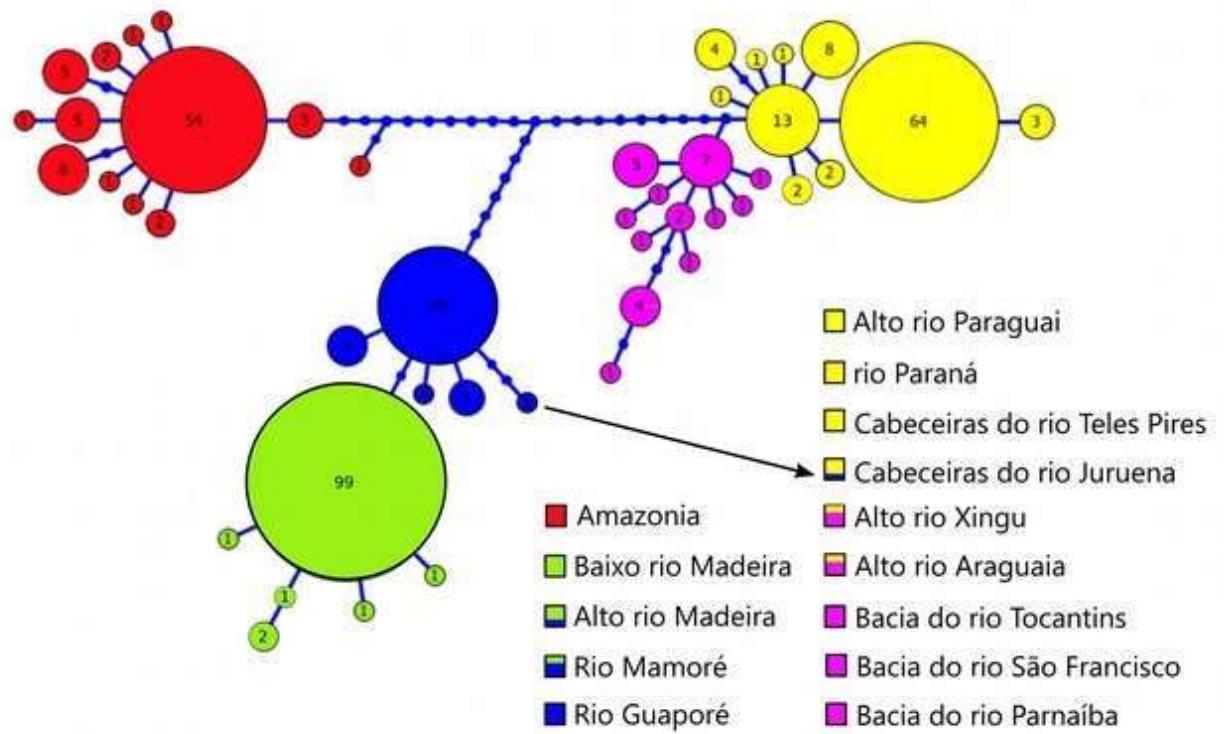
Modelo 1/Topologia A				
	$N_e$ mil indiv. (95% HDP)	$T$ Ma (95% HDP)	Bandas de migração	$M$ indiv/geração
<b>Amaz (A)</b>	11,2 (0,0-30,0)	-	<b>A → M</b>	1,74
<b>Bol (B)</b>	9,6 (0,0-26,8)	-	<b>M → A</b>	0,67
<b>Mad (M)</b>	8,6 (0,0-24,6)	-	<b>M → B</b>	0,28
<b>Pan (P)</b>	10,5 (0,0-28,6)	-	<b>B → M</b>	0,28
<b>BM</b>	118,3 (2,1-281,7)	2,9 (0,54-5,66)	<b>A → BM</b>	96,8
<b>BMP</b>	121,7 (2,6-291,2)	3,2 (0,87-6,06)	<b>BM → A</b>	9,46
<b>Raíz</b>	3,0E3 (2,4E3-3,6E3)	3,9 (1,26-6,89)	<b>A → BMP</b>	41,01
			<b>BMP → A</b>	9,36
Modelo 2/Topologia B				
	$N_e$ mil indiv. (95% HDP)	$T$ Ma (95% HDP)	Bandas de migração	$M$ indiv/geração
<b>Amaz (A)</b>	11,0 (2,3-21,3)	-	<b>B → P</b>	0,95
<b>Bol (B)</b>	28,4 (5,1-57,5)	-	<b>P → B</b>	1,69
<b>Mad (M)</b>	32,5 (5,6-64,2)	-	<b>BM → P</b>	0,14
<b>Pan (P)</b>	4,7 (0,0-13,7)	-	<b>P → BM</b>	4,69
<b>BM</b>	145,4 (3,8-327,3)	1,0 (0,20-2,06)	<b>P → ABM</b>	147,23
<b>ABM</b>	179,8 (8,1-432,6)	1,5 (0,38-2,76)	<b>ABM → P</b>	4,01
<b>Raíz</b>	4,2E3 (3,7E3-4,8E3)	2,2 (0,62-4,41)		



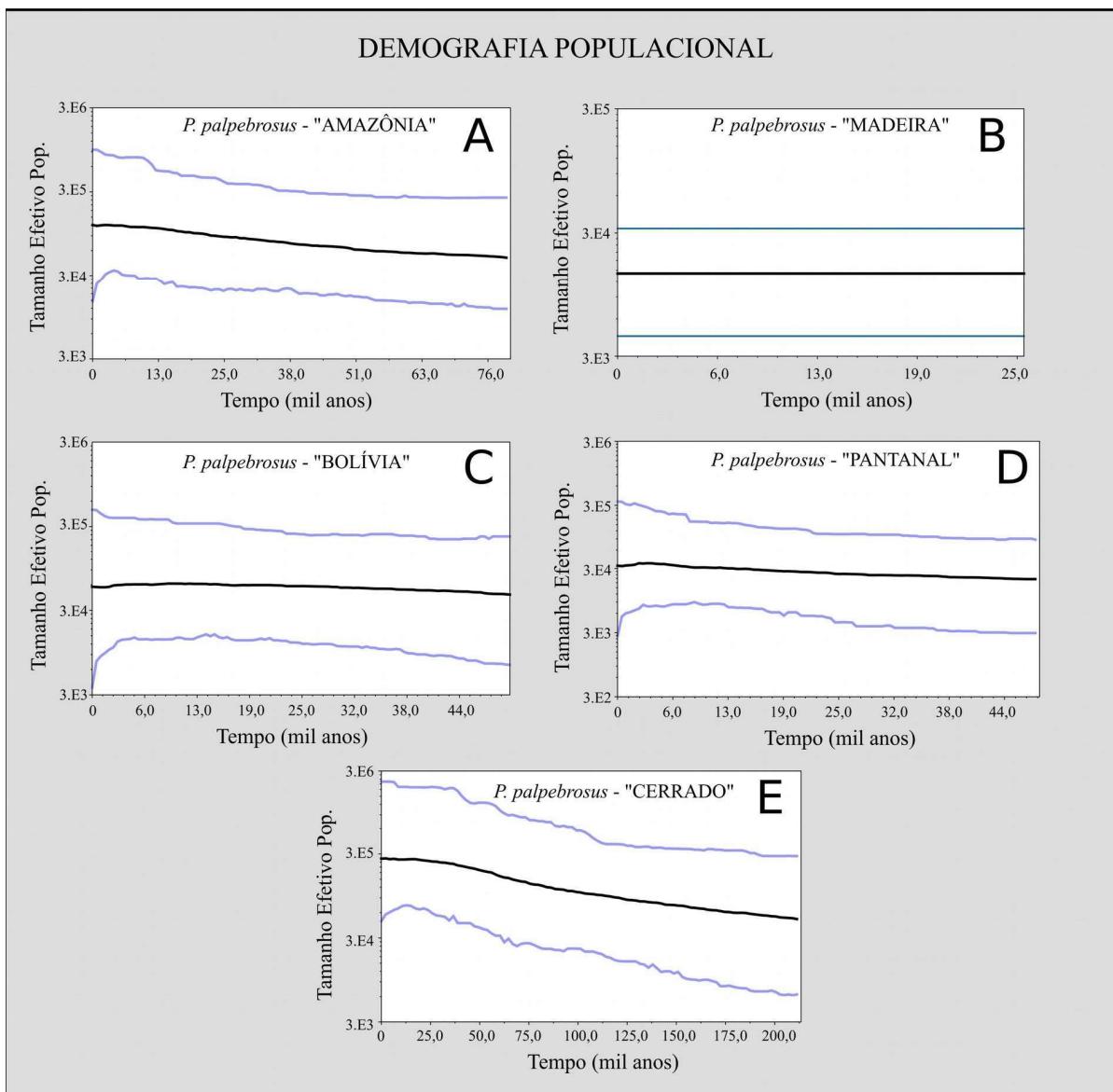
**Figura 1.** Distribuição geográfica das localidades amostradas, incluindo a amostragem feita por Muniz et al. (2018), e gráfico do BAPS mostrando o valor de K mais provável,  $K = 5$ . Os pontos amostrais são coloridos conforme o resultado no BAPS e os círculos com duas cores indicam as localidades onde foram encontrados indivíduos atribuídos à grupos diferentes. A correspondência entre as cores e os grupos populacionais são mostrados no canto superior direito.



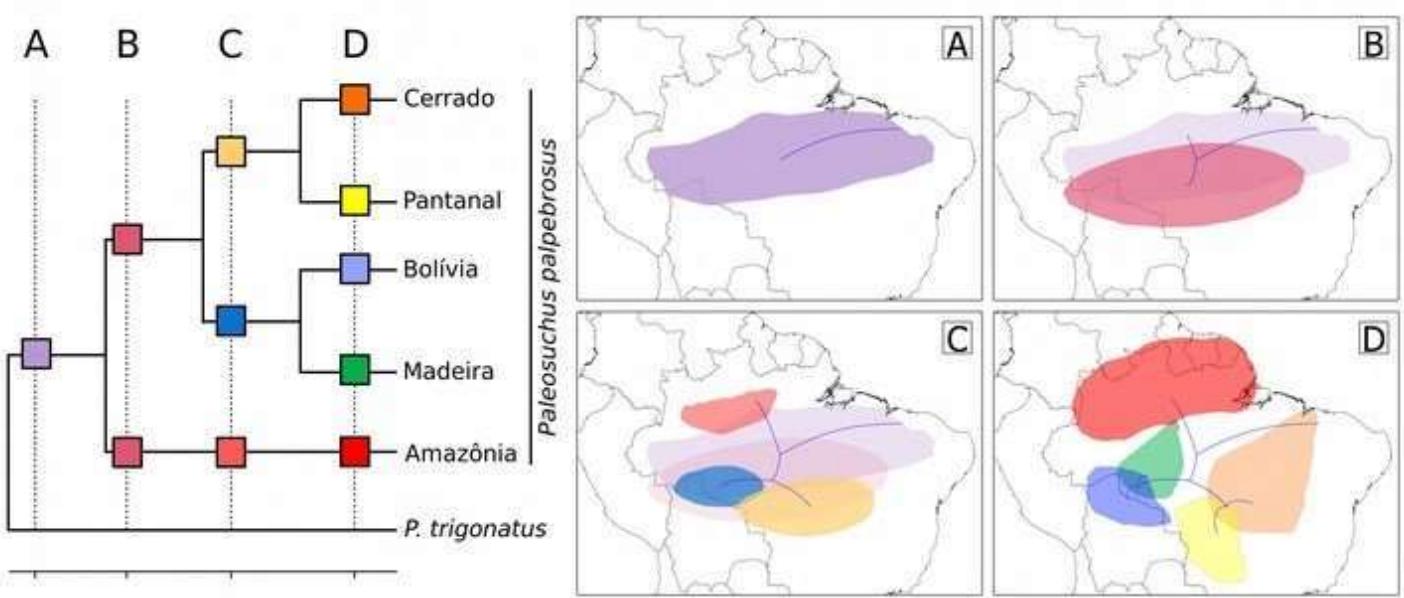
**Figura 2.** Parâmetros estimados e modelos de isolamento com migração testados usando o programa G-PhoCS (Gronau et al., 2011). As linhagens do complexo *P. palpebrosus* estão como terminais da árvore e os ancestrais estão indicados nos nós (ancMB = ancestral de "Madeira" e "Bolívia"; ancMBP = ancestral de "Madeira+Bolívia" e "Pantanal"). Parâmetros dos modelos demográficos incluem tamanho efetivo populacional ( $\theta$ ) para todos os ramos na árvore, tempos de divergência ( $\tau$ ) para todos os nós internos e taxas de migração ( $m$ ) para todas as bandas de migração avaliadas. Foram testadas cinco topologias (A-E) e, para cada uma delas, todas as combinações possíveis de bandas de migração entre linhagens adjacentes. As bandas de migração foram bidirecionais e para cada topologia o modelo sem fluxo gênico também foi testado.



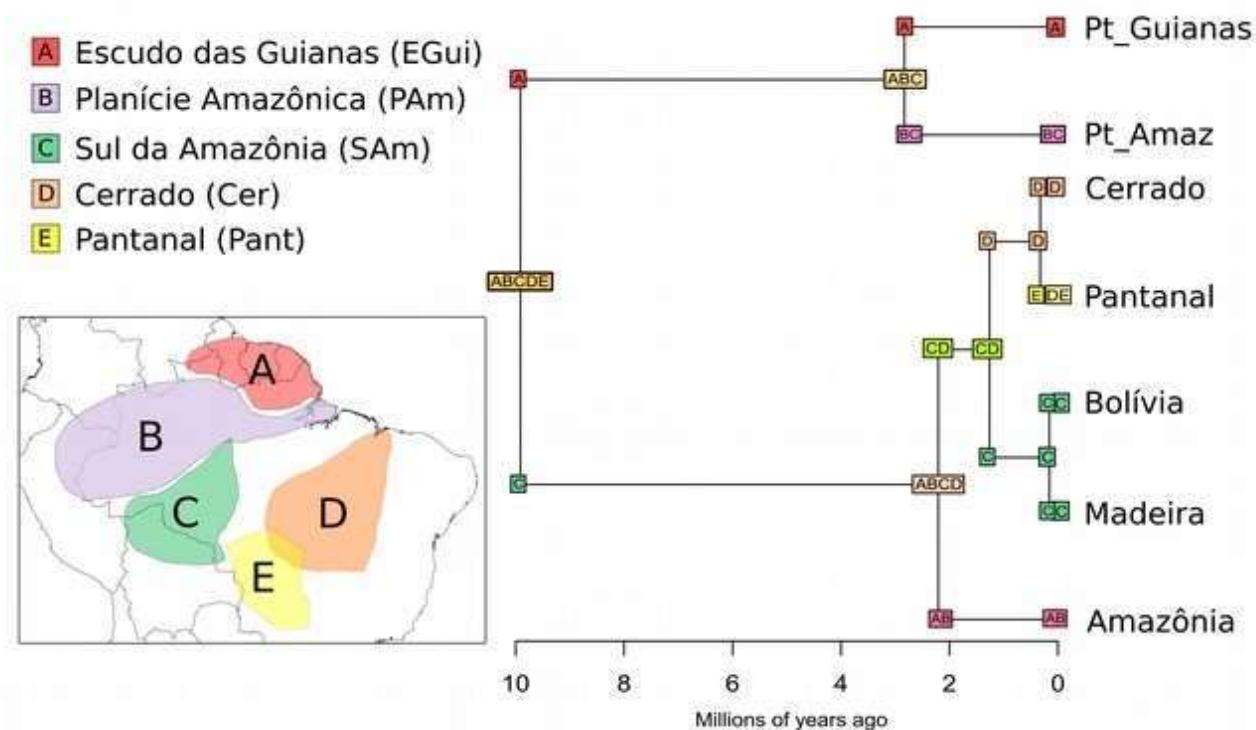
**Figura 3.** Rede de haplótipos mostrando a relação genealógica entre as linhagens de *Paleosuchus palpebrosus*. As cores correspondem aos grupos detectados na análise do BAPS (mostrados na Figura 1). Os quadrados representam as bacias amostradas e aqueles com duas cores correspondem às bacias que continham indivíduos com haplótipos de mais de um grupo. Alguns rios importantes não foram citados na legenda, pois a ênfase foi dada à amostragem inédita realizada no Escudo Brasileiro.



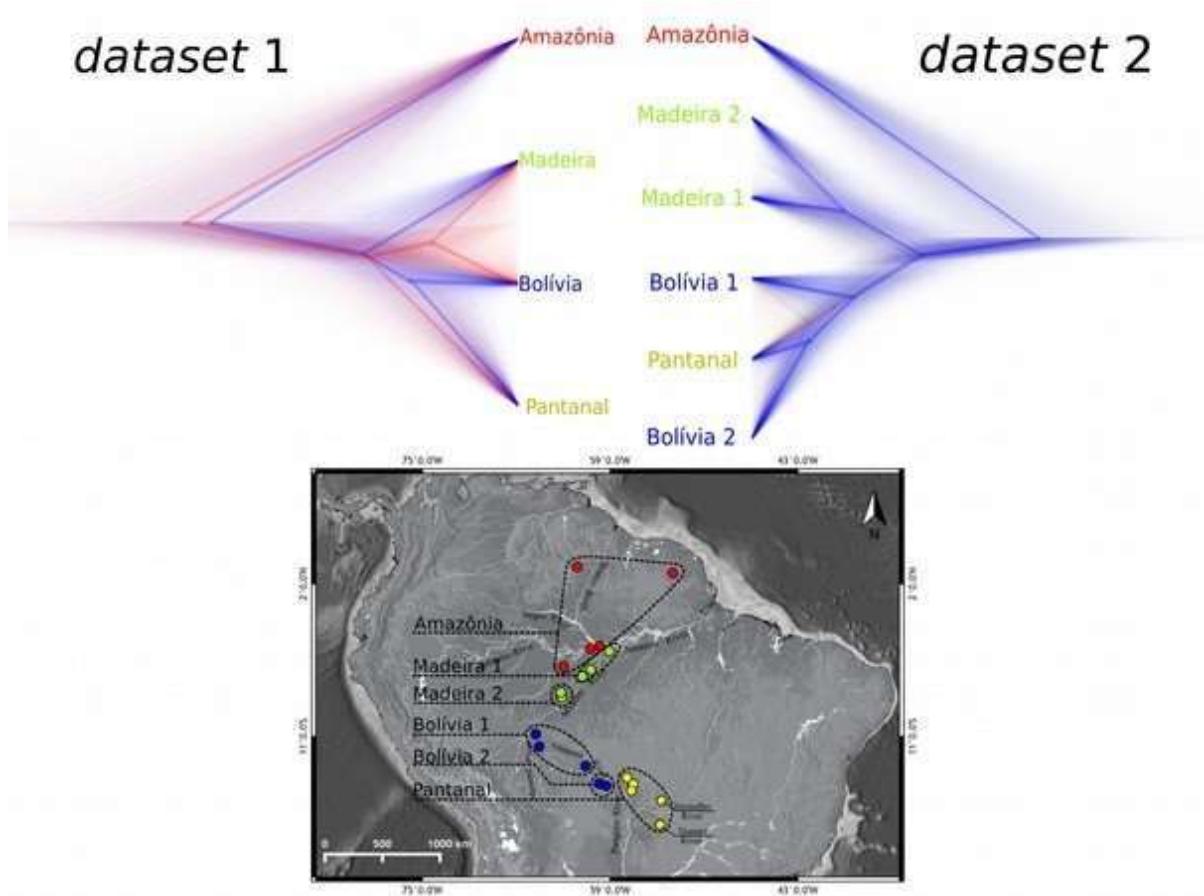
**Figura 4.** Demografia histórica de cada uma das linhagens do complexo *P. palpebrosus* estimada com base em sequências do gene mitocondrial *cyt b*. As estimativas foram feitas com *Bayesian Skyline Plots* (A, C-E), exceto para "Madeira" (B) cujo modelo de população constante foi melhor que o exponencial. No eixo Y é expresso o tamanho efetivo populacional em escala logarítmica e no eixo X o tempo na escala de mil anos, considerando a taxa de mutação do mtDNA de  $3,9 \cdot 10^{-9}$  substituições/sítio/ano (Eo & DeWoody, 2010).



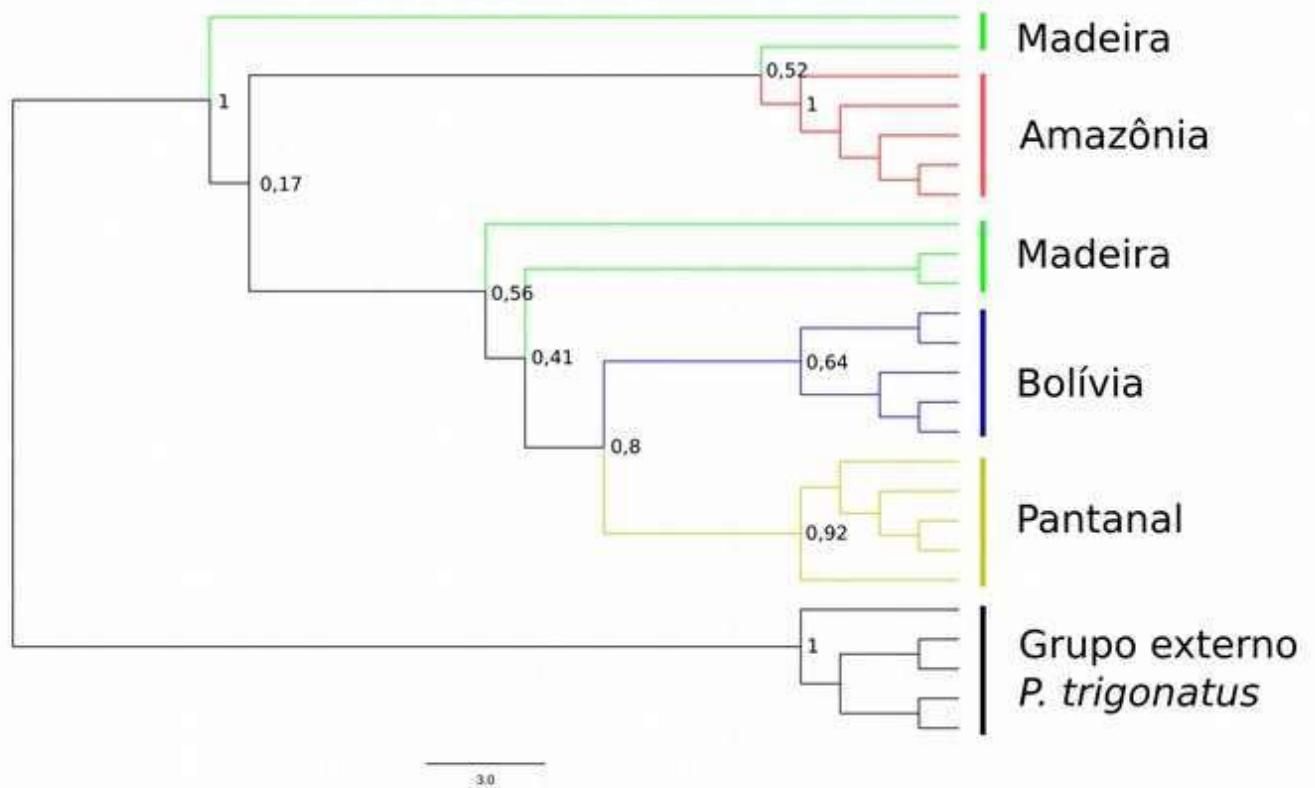
**Figura 5.** Difusão de Árvores de Espécies no gênero *Paleosuchus*. À esquerda é mostrada a árvore de espécies contendo quadrados coloridos nos ramos que são correspondentes aos cenários A-D. Os cenários mostram as áreas estimadas das populações ancestrais (80% HPD - highest-posterior density) em cinco intervalos de tempo, com cores correspondentes aos quadrados na árvore de espécies. No quadro (D) são mostradas as distribuições atuais que foram utilizadas como *input* para a análise de Difusão de Árvores de Espécies. Essas distribuições foram delimitadas com base na amostragem deste trabalho. Os mapas foram feitos no programa QGIS v.2.18.



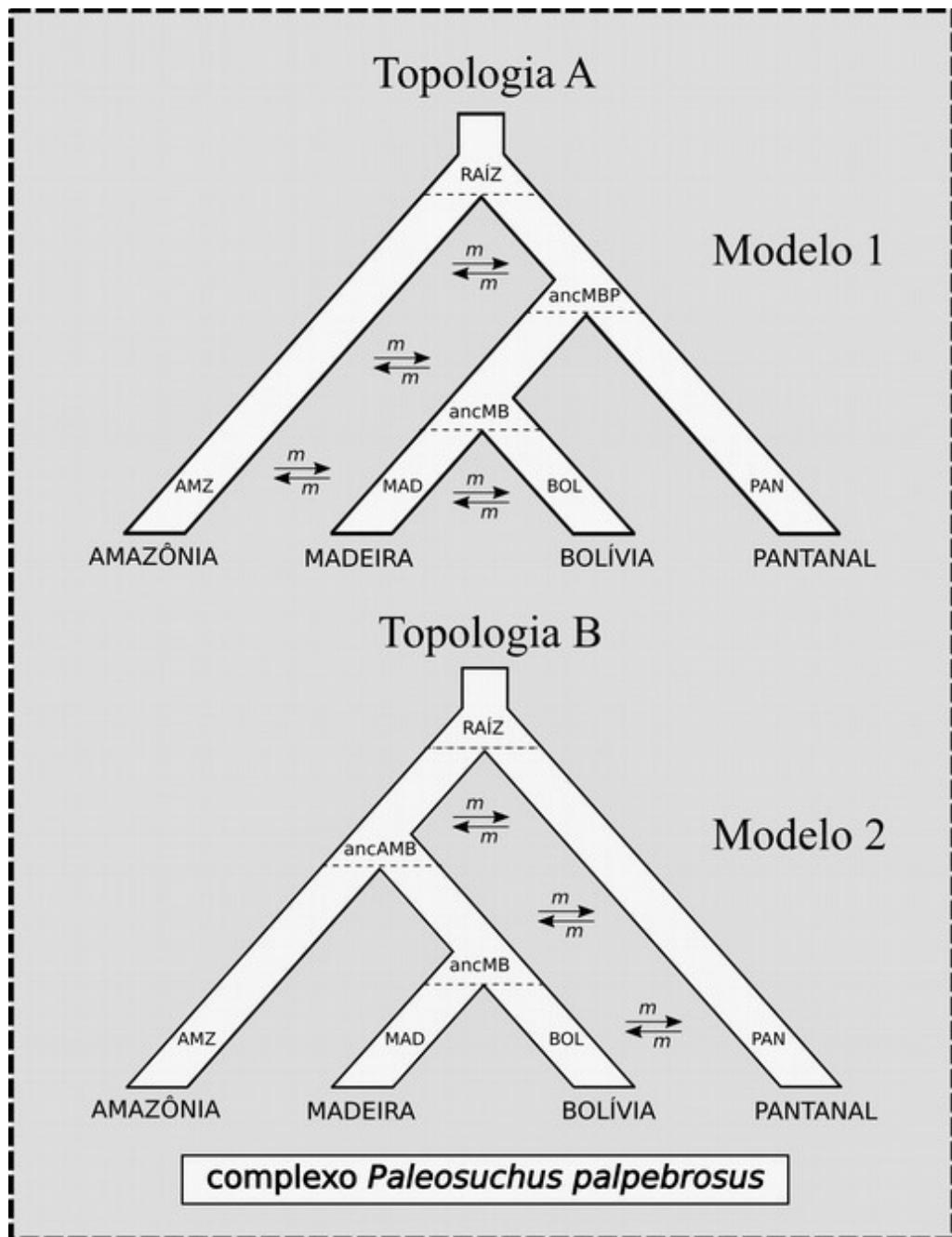
**Figura 6.** Reconstrução de áreas ancestrais do gênero *Paleosuchus* realizadas no BioGeoBEARS (Matzke, 2013). No mapa (à esquerda) são delimitadas as áreas geográficas utilizadas como referência para a análise, com as letras de A a E sendo associadas a cada uma das áreas. Uma árvore Bayesiana calibrada com tempo (à direita) foi reconstruída de acordo com o modelo DIVALIKE, que foi apontado como melhor modelo utilizando o critério de AIC (ver Tabela 2). As áreas ancestrais são mostradas em cada nó da árvore, sendo possível discriminar se a diversificação das linhagens se deu por vicariância, dispersão ou evento fundador.



**Figura 7.** Árvores de espécies reconstruídas no programa STARBEAST2 (Bouckaert et al., 2014) utilizando dois conjuntos de dados diferentes, o *dataset 1* com 111 loci e o *dataset 2* com 334 loci (ver tabela 1). Os claudogramas representam a distribuição posterior de árvores de espécies. A maior densidade de áreas da árvore indica maior concordância topológica e as cores dos ramos azul e vermelha expressam topologias alternativas. As árvores consenso são mostradas com linhas mais fortes.



**Figura 8.** Árvore de espécies reconstruída no programa ASTRAL-III (Zhang et al., 2018). As árvores gênicas de 989 loci obtidos por ddRADseq (*dataset 3*, ver Tabela 1) foram reconstruídas separadamente no programa RaxML usando o modelo de evolução nucleotídica GTR+G e então processadas pelo algoritmo do programa ASTRAL-III. A probabilidade posterior é indicada nos nós. A árvore de espécies no ASTRAL-III foi enraizada com *Paleosuchus trigonatus* e os ramos dos indivíduos pertencentes às espécies candidatas do complexo *P. palpebrosus* estão coloridos conforme sua linhagem de origem.



**Figura 9.** Melhores topologias e modelos de migração estimados no programa G-PhoCS (Gronau et al., 2011) usando o critério de AICM (Baele et al., 2012). Os parâmetros estimados são apresentados na Tabela 3.

## REFERÊNCIAS

- Abbott, R., Albach, D., Ansell, S., et al. (2013) Hybridization and speciation. *Journal of Evolutionary Biology*, **26**, 229–246.
- Assine, M.L., Mantesso Neto, V., Bartorelli, A., Carneiro, C.D.R., & Brito Neves, B.B. (2004) A bacia sedimentar do Pantanal Mato-Grossense. *Geologia do Continente Sul-Americano: evolução da obra de Fernando Flávio Marques de Almeida* (ed. by V. Mantesso-Neto, A. Bartorelli, C.D.R. Carneiro, and B.B. Brito-Neves), pp. 61–74. Beca, São Paulo.
- Baele, G., Lemey, P., Bedford, T., Rambaut, A., Suchard, M.A., & Alekseyenko, A. V. (2012) Improving the accuracy of demographic and molecular clock model comparison while accommodating phylogenetic uncertainty. *Molecular Biology and Evolution*, **29**, 2157–2167.
- Barbiano, L.A. da, Gompert, Z., Aspbury, A.S., Gabor, C.R., & Nice, C.C. (2013) Population genomics reveals a possible history of backcrossing and recombination in the gynogenetic fish Poecilia formosa. *Proceedings of the National Academy of Sciences*, **110**, 13797–13802.
- Barrera-Guzmán, A.O., Aleixo, A., Shawkey, M.D., & Weir, J.T. (2018) Hybrid speciation leads to novel male secondary sexual ornamentation of an Amazonian bird. *Proceedings of the National Academy of Sciences*, **115**, E218–E225.
- Bielejec, F., Rambaut, A., Suchard, M.A., & Lemey, P. (2011) SPREAD: Spatial phylogenetic reconstruction of evolutionary dynamics. *Bioinformatics*, **27**, 2910–2912.
- Bouckaert, R., Heled, J., Kühnert, D., Vaughan, T., Wu, C.H., Xie, D., Suchard, M.A., Rambaut, A., & Drummond, A.J. (2014) BEAST 2: A Software Platform for Bayesian Evolutionary Analysis. *PLoS Computational Biology*, **10**, e1003537.
- Brelsford, A., Milá, B., & Irwin, D.E. (2011) Hybrid origin of Audubon's warbler. *Molecular Ecology*, **20**, 2380–2389.
- Brown, W.M., Prager, E.M., Wang, A., & Wilson, A.C. (1982) Mitochondrial DNA sequences of primates: Tempo and mode of evolution. *Journal of Molecular Evolution*, **18**, 225–239.
- Butlin, R.K. & Ritchie, M.G. (2009) Genetics of speciation. *Heredity*, **102**, 1–3.
- Carvalho, T.P. & Albert, J.S. (2011) The Amazon-Paraguay Divide. *Historical Biogeography of Neotropical Freshwater Fishes* (ed. by J.S. Albert and R.E. Reis), pp. 192–202. University of California Press, Los Angeles.

- Corander, J., Marttinen, P., Sirén, J., & Tang, J. (2008) Enhanced Bayesian modelling in BAPS software for learning genetic structures of populations. *BMC Bioinformatics*, **9**, .
- Darriba, D., Taboada, G.L., Doallo, R., & Posada, D. (2012) JModelTest 2: More models, new heuristics and parallel computing. *Nature Methods*, **9**, 772.
- Doyle, J.J. & Doyle, J.L. (1987) A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochem Bull Bot Soc Am*, **19**, 11–15.
- Drummond, A.J., Ashton, B., Buxton, S., Cheung, M., Cooper, A., Duran, C., Field, M., Heled, J., Kearse, M., Markowitz, S., Moir, R., Stones-Havas, S., Sturrock, S., Thierer, T., & Wilson, A.C. (2014) Geneious v6.1.8. .
- Drummond, A.J. & Rambaut, A. (2007) BEAST: Bayesian evolutionary analysis by sampling trees. *BMC evolutionary biology*, **7**, 214.
- Drummond, A.J., Rambaut, A., Shapiro, B., & Pybus, O.G. (2005) Bayesian coalescent inference of past population dynamics from molecular sequences. *Molecular Biology and Evolution*, **22**, 1185–1192.
- Drummond, A.J., Suchard, M. a, Xie, D., & Rambaut, A. (2012) Bayesian phylogenetics with BEAUTi and the BEAST 1.7. *Molecular biology and evolution*, **29**, 1969–73.
- Eaton, D.A.R. (2014) PyRAD: Assembly of de novo RADseq loci for phylogenetic analyses. *Bioinformatics*, **30**, 1844–1849.
- Edwards, S. V., Potter, S., Schmitt, C.J., Bragg, J.G., & Moritz, C. (2016) Reticulation, divergence, and the phylogeography–phylogenetics continuum. *Proceedings of the National Academy of Sciences*, **113**, 8025–8032.
- Eo, S.H. & DeWoody, J.A. (2010) Evolutionary rates of mitochondrial genomes correspond to diversification rates and to contemporary species richness in birds and reptiles. *Proceedings of the Royal Society B: Biological Sciences*, **277**, 3587–3592.
- Felsenstein, J. (1976) The theoretical population genetics of variable selection and migration. *Annual Review of Genetics*, **10**, 253–280.
- Garrick, R.C., Sunnucks, P., & Dyer, R.J. (2010) Nuclear gene phylogeography using PHASE: Dealing with unresolved genotypes, lost alleles, and systematic bias in parameter estimation. *BMC Evolutionary Biology*, **10**, 118.
- Gottsch, A.D., Wood, D.A., Vandergast, A.G., Lemos-Espinal, J., Gatesy, J., & Reeder, T.W. (2017) Lineage diversification of fringe-toed lizards (Phrynosomatidae: *Uma notata* complex) in the Colorado Desert: Delimiting species in the presence of gene flow. *Molecular Phylogenetics and Evolution*, **106**, 103–117.

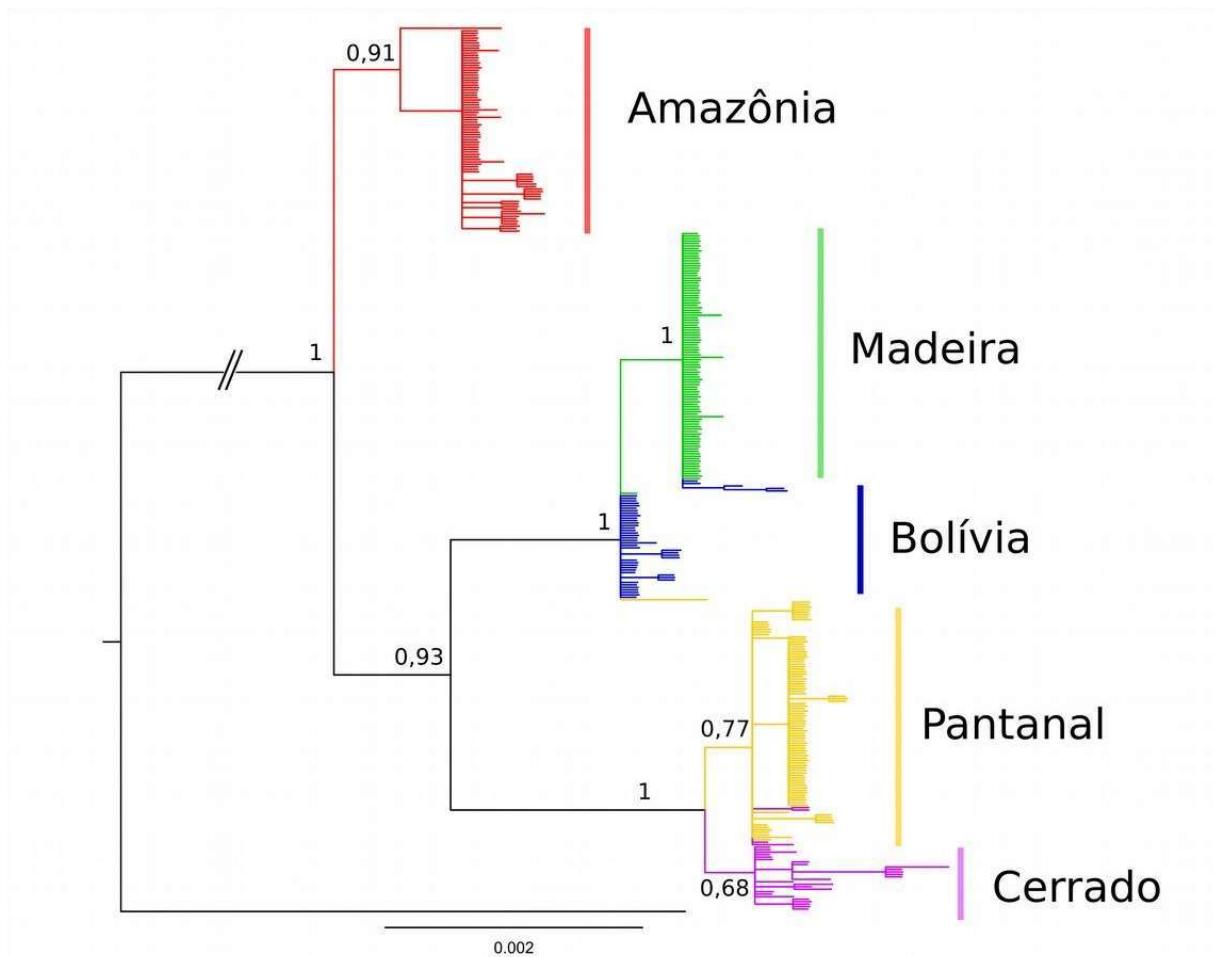
- Green, R.E., Braun, E.L., Armstrong, J., et al. (2014) Three crocodilian genomes reveal ancestral patterns of evolution among archosaurs. *Science*, **346**, 10.1126/science.1254449.
- Hedrick, P.W. (2013) Adaptive introgression in animals: Examples and comparison to new mutation and standing variation as sources of adaptive variation. *Molecular Ecology*, **22**, 4606–4618.
- Heled, J. & Drummond, A.J. (2010) Bayesian Inference of Species Trees from Multilocus Data. *Molecular Biology and Evolution*, **27**, 570–580.
- Hermansen, J.S., Sæther, S.A., Elgvin, T.O., Borge, T., Hjelle, E., & Sætre, G.P. (2011) Hybrid speciation in sparrows I: Phenotypic intermediacy, genetic admixture and barriers to gene flow. *Molecular Ecology*, **20**, 3812–3822.
- Hrbek, T., Vasconcelos, W.R., Rebêlo, G.H., & Farias, I.P. (2008) Phylogenetic relationships of South American alligatorids and the Caiman of Madeira River. *Journal of Experimental Zoology*, **309A**, 588–599.
- Hubert, N. & Renno, J.-F. (2006) Historical biogeography of South American freshwater fishes. *Journal of Biogeography*, **33**, 1414–1436.
- Keller, I., Wagner, C.E., Greuter, L., Mwaiko, S., Selz, O.M., Sivasundar, A., Wittwer, S., & Seehausen, O. (2013) Population genomic signatures of divergent adaptation, gene flow and hybrid speciation in the rapid radiation of Lake Victoria cichlid fishes. *Molecular Ecology*, **22**, 2848–2863.
- Lanfear, R., Calcott, B., Ho, S.Y.W., & Guindon, S. (2012) PartitionFinder: Combined selection of partitioning schemes and substitution models for phylogenetic analyses. *Molecular Biology and Evolution*, **29**, 1695–1701.
- Lavretsky, P., Engilis, A., Eadie, J.M., & Peters, J.L. (2015) Genetic admixture supports an ancient hybrid origin of the endangered Hawaiian duck. *Journal of Evolutionary Biology*, **28**, 1005–1015.
- Lemey, P., Rambaut, A., Welch, J.J., & Suchard, M.A. (2010) Phylogeography takes a relaxed random walk in continuous space and time. *Molecular Biology and Evolution*, **27**, 1877–1885.
- Maguilla, E., Escudero, M., Hipp, A.L., & Luceño, M. (2017) Allopatric speciation despite historical gene flow: Divergence and hybridization in Carex furva and C. lucennoiberica (Cyperaceae) inferred from plastid and nuclear RAD-seq data. *Molecular Ecology*, **26**, 5646–5662.
- Mayr, E. (1942) Systematics and the origin of species from the viewpoint of a zoologist.

*Systematics and the origin of species from the viewpoint of a zoologist.*

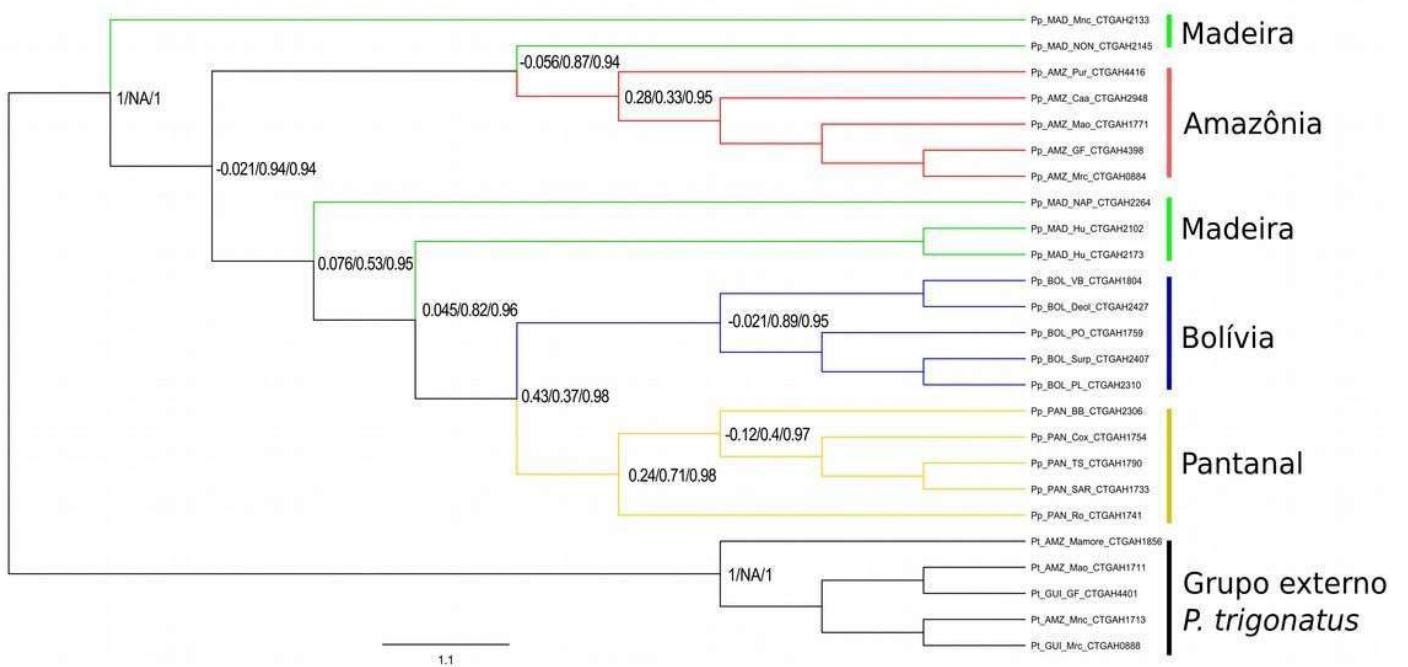
- Menezes, N. a, Ribeiro, A.C., Weitzman, S., & Torres, R. a (2008) Biogeography of Glandulocaudinae ( Teleostei : Characiformes : Characidae ). *Zootaxa*, **48**, 33–48.
- Meyer, A. (1993) Evolution of mitochondrial DNA in fishes. *Molecular biology frontiers, biochemistry and molecular biology of fishes* (ed. by W. Hochachka & T.P. Mommesen), Vol. 2, pp. 1-38. Elsevier Science Publishers, Amsterdam.
- Miller, M.A., Pfeiffer, W., & Schwartz, T. (2010) Creating the CIPRES Science Gateway for inference of large phylogenetic trees. *2010 Gateway Computing Environments Workshop, GCE 2010*, .
- Mirarab, S., Reaz, R., Bayzid, M.S., Zimmermann, T., S. Swenson, M., & Warnow, T. (2014) ASTRAL: Genome-scale coalescent-based species tree estimation. *Bioinformatics*, **30**, 541–548.
- Monzón, J., Kays, R., & Dykhuizen, D.E. (2014) Assessment of coyote-wolf-dog admixture using ancestry-informative diagnostic SNPs. *Molecular Ecology*, **23**, 182–197.
- Muniz, F.L., Campos, Z., Hernández Rangel, S.M., Martínez, J.G., Souza, B.C., De Thoisy, B., Botero-Arias, R., Hrbek, T., & Farias, I.P. (2018) Delimitation of evolutionary units in Cuvier's dwarf caiman, *Paleosuchus palpebrosus* (Cuvier, 1807): insights from conservation of a broadly distributed species. *Conservation Genetics*, **19**, 599–610.
- Nylinder, S., Lemey, P., De Bruyn, M., Suchard, M.A., Pfeil, B.E., Walsh, N., & Anderberg, A.A. (2014) On the biogeography of centipeda: A species-tree diffusion approach. *Systematic Biology*, **63**, 178–191.
- Oaks, J.R. (2011) A time-calibrated species tree of crocodylia reveals a recent radiation of the true crocodiles. *Evolution*, **65**, 3285–3297.
- Pease, J.B., Brown, J.W., Walker, J.F., Hinchliff, C.E., & Smith, S.A. (2018) Quartet Sampling distinguishes lack of support from conflicting support in the green plant tree of life. *American Journal of Botany*, **105**, 385–403.
- Peterson, B.K., Weber, J.N., Kay, E.H., Fisher, H.S., & Hoekstra, H.E. (2012) Double digest RADseq: An inexpensive method for de novo SNP discovery and genotyping in model and non-model species. *PLoS ONE*, **7**, .
- De Queiroz, K. (2007) Species concepts and species delimitation. *Systematic biology*, **56**, 879–886.
- Rambaut, A., Drummond, A.J., & Suchard, M. (2013) Tracer v1. 6—MCMC trace analysis package. *Institute of Evolutionary Biology, University of Edinburgh, UK*.

- Ronquist, F., Teslenko, M., van der Mark, P., Ayres, D.L., Darling, A., Hohna, S. et al (2012) MrBayes 3.2: efficient bayesian phylogenetic inference and model choice across a large model space. *Syst Biol* 61:539–554.
- Ronquist, F. & Sanmartín, I. (2011) Phylogenetic Methods in Biogeography. *Annual Review of Ecology, Evolution, and Systematics*, **42**, 441–464.
- Roos, J., Aggarwal, R.K., & Janke, A. (2007) Extended mitogenomic phylogenetic analyses yield new insight into crocodylian evolution and their survival of the Cretaceous-Tertiary boundary. *Molecular Phylogenetics and Evolution*, **45**, 663–673.
- Runemark, A., Hey, J., Hansson, B., & Svensson, E.I. (2012) Vicariance divergence and gene flow among islet populations of an endemic lizard. *Molecular Ecology*, **21**, 117–129.
- Salzburger, W., Ewing, G.B., & Von Haeseler, A. (2011) The performance of phylogenetic algorithms in estimating haplotype genealogies with migration. *Molecular Ecology*, **20**, 1952–1963.
- Schumer, M., Rosenthal, G.G., & Andolfatto, P. (2014) How common is homoploid hybrid speciation? *Evolution*, **68**, 1553–1560.
- Servedio, M.R., Hermisson, J., & Van Doorn, G.S. (2013) Hybridization may rarely promote speciation. *Journal of Evolutionary Biology*, **26**, 282–285.
- Stamatakis, A. (2014) RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. *Bioinformatics*, **30**, 1312–1313.
- Stemshorn, K.C., Reed, F.A., Nolte, A.W., & Tautz, D. (2011) Rapid formation of distinct hybrid lineages after secondary contact of two fish species (*Cottus* sp.). *Molecular Ecology*, **20**, 1475–1491.
- Trier, C.N., Hermansen, J.S., Sætre, G.P., & Bailey, R.I. (2014) Evidence for Mito-Nuclear and Sex-Linked Reproductive Barriers between the Hybrid Italian Sparrow and Its Parent Species. *PLoS Genetics*, **10**, e1004075.
- Wilkinson, M.J., Marshall, L.G., & Lundberg, J.G. (2006) River behavior on megafans and potential influences on diversification and distribution of aquatic organisms. *Journal of South American Earth Sciences*, **21**, 151–172.
- Zhang, C., Rabiee, M., Sayyari, E., & Mirarab, S. (2018) ASTRAL-III: Polynomial time species tree reconstruction from partially resolved gene trees. *BMC Bioinformatics*, **19**, 15–30.

## MATERIAL SUPPLEMENTAR



**Figura S1.** Árvore filogenética reconstruída com base no *cyt b* ( $N = 357$ ) por meio de Inferência Bayesiana (IB) realizada no programa MrBayes v3.2.2 (Ronquist et al., 2012). Cada terminal na árvore representa um indivíduo e as cores dos ramos indicam a que linhagem o indivíduo deveria pertencer dada sua origem geográfica. Para esta análise foram usadas quatro cadeias independentes e os *priors* utilizados em cada análise foram mantidos como padrão. A IB foi realizada usando 1 milhão de passos, com amostragem a cada 1 mil passos resultando em uma posterior de 1 mil árvores. Foram descartadas os primeiros 25% das árvores amostradas. As topologias foram sumarizadas e visualizadas no FigTree v1.4.2 (Rambaut 2014).



**Figura S2.** Análise de suporte dos nós da árvore de espécies reconstruída no programa ASTRAL-III (Zhang et al., 2018) feita com o programa Quartet Sampling (Pease et al., 2018). Para cada nó relevante na árvore são fornecidos três valores de suporte separados por barras. O primeiro valor é o QC (quarteto de concordância) que indica com que frequência o quarteto concordante é inferido em detrimento dos quartetos discordantes. O segundo valor é o QD (quarteto diferencial) que indica o balanço da frequência das topologias discordantes. Ele também pode indicar se existe uma topologia discordante tão frequente quanto a topologia concordante. O terceiro valor é o QI (quarteto de informatividade) que indica qual a proporção das réplicas foram informativas. Todos esses valores são calculados para cada nó. Para melhor entendimento sobre a interpretação dos valores ver Pease et al. (2018).

## SÍNTSE GERAL

Neste estudo nós investigamos a diversidade críptica em *Paleosuchus palpebrosus* e produzimos importantes contribuições para a conservação, biogeografia e biologia evolutiva desse complexo de espécies. Nós coletamos amostras em grande parte da distribuição de *P. palpebrosus* e utilizamos uma combinação de marcadores moleculares, mitocondrial e genômico, para fazer inferências evolutivas e desvendar os processos históricos ocorridos durante a diversificação do grupo.

No capítulo 1, nós delimitamos Unidades Evolutivas Significantes utilizando os critérios estabelecidos pela abordagem AEC (*Adaptive Evolutionary Conservation*). Muitos desses critérios se confundem com os utilizados para delimitar espécies, e sugerimos que *P. palpebrosus* é na verdade um complexo de espécies. Nós avaliamos a diversidade genética das linhagens recém descobertas e comparamos com a estimada em outras espécies/linhagens de crocodilianos. Além disso, discutimos as implicações para a conservação de *P. palpebrosus*, sugerindo que cada linhagem deve ser avaliada em separado, independentemente de serem reconhecidas ou não taxonomicamente.

No capítulo 2, nós utilizamos um teste explícito e descartamos a hipótese de que o isolamento por distância sozinho teria gerado o padrão observado de diversidade genética, aceitando a hipótese histórica de rearranjo de drenagem como promotor da diversificação do complexo *P. palpebrosus* no corredor Paraguai-Madeira-Amazônia. Com base em nossos resultados, nós propomos cenários hipotéticos de evolução da paisagem para explicar a diversificação de *P. palpebrosus*. Também discutimos a utilidade desse modelo biogeográfico para a fauna aquática da região, mostrando que a diversificação de algumas espécies de

vertebrados aquáticos poderiam ser explicadas à luz do modelo proposto.

No capítulo 3, nós ampliamos a amostragem com mtDNA e detectamos uma linhagem adicional no Escudo Brasileiro. Nós também estimamos a árvore de espécies utilizando dois métodos coalescentes, testamos modelos de hibridização vs *Incomplete Lineage Sorting* e reconstruímos área do ancestral comum e a rota de dispersão das linhagens. Por fim, avaliamos o ajuste destes processos ao modelo biogeográfico previamente proposto.

De um modo geral, nosso trabalho contribuiu não só para o entendimento da biologia evolutiva de uma espécie supostamente com ampla distribuição, mas também para a biogeografia de espécies aquáticas que ocorrem ao longo do corredor Amazonas-Madeira-Pantanal. Algumas questões permanecem não resolvidas, tais como: quantas e quais linhagens devem ser reconhecidas taxonomicamente e se houveram eventos de hibridização que estariam dificultando a resolução das relações filogenéticas. Para isso, nova amostragem genômica será necessária, bem como o uso de ferramentas analíticas que possibilitem testar explicitamente modelos de especiação híbrida.

## REFERÊNCIAS BIBLIOGRÁFICAS

- Albert, J.S., & Reis, R.E. (2011) *Historical Biogeography of Neotropical Freshwater Fishes*. University of California Press.
- Allendorf, F.W., Hohenlohe, P.A., & Luikart, G. (2010) Genomics and the future of conservation genetics. *Nature Publishing Group*, **11**, 697–709.
- Allendorf, F.W. & Luikart, G. (2007) *Conservation and the Genetics of Populations*. Blackwell Publishing.
- Allendorf, F.W., Luikart, G.H., & Aitken, S.N. (2012) *Conservation and the Genetics of Populations*. John Wiley & Sons.
- Avise, J.C. (1994) *Molecular Markers, Natural History and Evolution*. Chapman & Hall, New York.
- Avise, J.C. (2000) *Phylogeography: the history and formation of species*. Harvard university press, Cambridge, MA.
- Avise, J.C. (2009) Phylogeography: Retrospect and prospect. *Journal of Biogeography*, **36**, 3–15.
- Avise, J.C., Arnold, J., Ball, R.M., Bermingham, E., Lamb, T., Neigel, J.E., Reeb, C.A., & Saunders, N.C. (1987) Intraspecific Phylogeography: The Mitochondrial DNA Bridge Between Population Genetics and Systematics. *Annual Review of Ecology and Systematics*, **18**, 489–522.
- Bloor, P., Ibáñez, C., & Viloria-Lagares, T.A. (2015) Mitochondrial DNA analysis reveals hidden genetic diversity in captive populations of the threatened American crocodile (*Crocodylus acutus*) in Colombia. *Ecology and evolution*, **5**, 130–40.
- Brown, W.M., Prager, E.M., Wang, A., & Wilson, A.C. (1982) Mitochondrial DNA sequences of primates: Tempo and mode of evolution. *Proceedings of the National Academy of Sciences, USA*, **76**, 1976–1971.
- Campos, Z., Coutinho, M., & Abercrombie, C. (1995) Size structure and sex ratio of dwarf caiman in the Serra Amolar, Pantanal, Brazil. *Herpetological Journal*, **5**, 321–322.
- Campos, Z., Marioni, B., Farias, I., Verdade, L.M., Bassetti, L., Coutinho, M.E., Mendonça, S.H.S.T. De, Vieira, T.Q., & Magnusson, W.E. (2013) Avaliação do risco de extinção do jacaré-paguá *Paleosuchus palpebrosus* (Cuvier, 1807) no Brasil. *Biodiversidade Brasileira*, **3**, 40–47.
- Campos, Z., Muniz, F., Farias, I.P., & Hrbek, T. (2015) Conservation status of the Dwarf

caiman *Paleosuchus palpebrosus* in the region of the Araguaia-Tocantins Basin, Brazil. *Crocodile Specialist Group Newsletter*, 6–8.

Campos, Z., Sanaiotti, T., & Magnusson, W. (2010) Maximum size of dwarf caiman, *Paleosuchus palpebrosus* (Cuvier, 1807), in the Amazon and habitats surrounding the Pantanal, Brazil. *Amphibia-Reptilia*, **31**, 439–442.

Crandall, K.A., Bininda-Emonds, O.R.P., Mace, G.M., & Wayne, R.K. (2000) Considering evolutionay processes in conservation biology. *Trends in Ecology and Evolution*, **15** (17), 290-295.

Dormann, C.F., Schweiger, O., Augenstein, I., Bailey, D., Billeter, R., De Blust, G., Defilippi, R., Frenzel, M., Hendrickx, F., Herzog, F., Klotz, S., Liira, J., Maelfait, J.P., Schmidt, T., Speelmans, M., Van Wingerden, W.K.R.E., & Zobel, M. (2007) Effects of landscape structure and land-use intensity on similarity of plant and animal communities. *Global Ecology and Biogeography*, **16**, 774–787.

Eaton, M.J. (2010) Dwarf Crocodile *Osteolaemus tetraspis*. In S.C. Manolis & C. Stevenson (Eds.) *Crocodiles. Status Survey and Conservation Action Plan* (p. 127–132). Crocodile Specialist Group, Darwin.

Eaton, M.J., Martin, A., Thorbjarnarson, J., & Amato, G. (2009) Species-level diversification of African dwarf crocodiles (Genus *Osteolaemus*): A geographic and phylogenetic perspective. *Molecular Phylogenetics and Evolution*, **50**, 496–506.

Ewers, R.M. & Didham, R.K. (2006) Confounding factors in the detection of species responses to habitat fragmentation. *Biological Reviews of the Cambridge Philosophical Society*, **81**, 117–142.

Franke, F.A., Schmidt, F., Borgwardt, C., Bernhard, D., Bleidorn, C., Engelmann, W.E., & Schlegel, M. (2013) Genetic differentiation of the African dwarf crocodile *Osteolaemus tetraspis* Cope, 1861 (Crocodylia: Crocodylidae) and consequences for European zoos. *Organisms Diversity & Evolution*, **13**, 255–266.

Frankham, R., Ballou, J.D., & Briscoe, D.A. (2010) *Introduction to conservation genetics*. Cambridge University Press, Cambridge, UK.

Fraser, D.J., & Bernatchez, L. (2001) Adaptive evolutionary conservation: towards a unified concept for defining conservation units. *Molecular Ecology*, **10**, 2741–2752.

Funk, W.C., McKay, J.K., Hohenlohe, P.A., & Allendorf, F.W. (2012) Harnessing genomics for delineating conservation units. *Trends in Ecology and Evolution*, **27**, 489–496.

Godshalk, R. (2006) *Phylogeography and conservation genetics of the yacare caiman (Caiman yacare) of South America*. University of Florida, Gainesville, Florida, USA.

- Gravena, W., Farias, I.P., Da Silva, M.N.F., Da Silva, V.M.F., & Hrbek, T. (2014) Looking to the past and the future: Were the Madeira River rapids a geographical barrier to the boto (Cetacea: Iniidae)? *Conservation Genetics*, **15**, 619–629.
- Gravena, W., da Silva, V.M.F., da Silva, M.N.F., Farias, I.P., & Hrbek, T. (2015) Living between rapids: genetic structure and hybridization in botoes (Cetacea: Iniidae: Inia spp.) of the Madeira River, Brazil. *Biological Journal of the Linnean Society*, **114**, 764–777.
- Grigg, G., & Kirshner, D. (2015) *Biology and Evolution of Crocodylians*. Cornell University Press.
- Haig, S.M. (1998) Molecular contributions to conservation. *Ecology*, **79**, 413–425.
- Hekkala, E., Shirley, M.H., Amato, G., Austin, J.D., Charter, S., Thorbjarnarson, J., Vliet, K.A., Houck, M.L., Desalle, R., & Blum, M.J. (2011) An ancient icon reveals new mysteries: mummy DNA resurrects a cryptic species within the Nile crocodile. *Molecular ecology*, **20**, 4199–4215.
- Henle, K., Davies, K.F., Kleyer, M., Margules, C., & Settele, J. (2004) Predictors of Species Sensitivity to Fragmentation. *Biodiversity and Conservation*, **13**, 207–251.
- Hrbek, T., Da Silva, V.M.F., Dutra, N., Gravena, W., Martin, A.R., & Farias, I.P. (2014) A new species of river dolphin from Brazil or: How little do we know our biodiversity. *PLoS ONE*, **9(1)**, e83623.
- Hrbek, T., Vasconcelos, W.R., Rebelo, G., & Farias, I.P. (2008) Phylogenetic relationships of South American alligatorids and the Caiman of Madeira River. *Journal of Experimental Zoology Part A: Ecological Genetics and Physiology*, **309**, 588–599.
- Hubert, N., Duponchelle, F., Nuñez, J., Garcia-Davila, C., Paugy, D., & Renno, J.F. (2007) Phylogeography of the piranha genera *Serrasalmus* and *Pygocentrus*: Implications for the diversification of the Neotropical ichthyofauna. *Molecular Ecology*, **16**, 2115–2136.
- Hubert, N., & Renno, J.F. (2006) Historical biogeography of South American freshwater fishes. *Journal of Biogeography*, **33**, 1414–1436.
- Hudson, R.R., & Turelli, M. (2003) Stochasticity overrules the “three-times rule”: genetic drift, genetic draft, and coalescence times for nuclear loci versus mitochondrial DNA. *Evolution*, **57**, 182–190.
- Hughes, C.E., Pennington, R.T., & Antonelli, A. (2013) Neotropical Plant Evolution: Assembling the Big Picture. *Botanical Journal of the Linnean Society*, **171**, 1–18.
- Leite, R.N., & Rogers, D.S. (2013) Revisiting Amazonian phylogeography: Insights into diversification hypotheses and novel perspectives. *Organisms Diversity and Evolution*, **13**, 639–664.

- Li, W.H. (1997) *Molecular evolution*. Sinauer Associates. Sunderland, MA.
- Luikart, G.H., England, P., Tallmon, D.A., Jordan, S., & Taberlet, P. (2003) The power and promise of population genomics: from genotyping to genome-typing. *Nature Rev. Genet.*, **4**, 981–994.
- McAliley, L.R., Willis, R.E., Ray, D.A., White, P.S., Brochu, C.A., & Densmore, L.D. (2006) Are crocodiles really monophyletic? Evidence for subdivisions from sequence and morphological data. *Molecular Phylogenetics and Evolution*, **39**, 16–32.
- Meyer, A. (1993) Evolution of mitochondrial DNA in fishes. In: Hochachka, W., & Mommsen T.P. (Eds). *Molecular biology frontiers, biochemistry and molecular biology of fishes*, (Vol. 2, p. 1-38). Elsevier Science Publishers, Amsterdam.
- Moritz, C. (1994) Defining ‘Evolutionarily Significant Units’ for conservation. *Trends in Ecology & Evolution*, **9**, 373–375.
- Moritz, C., Lavery, S., & Slade, R. (1995) Using allele frequency and phylogeny to define units for conservation and management. 249–262.
- Moritz, C., Patton, J.L., Schneider, C.J., & Smith, T.B. (2000) Diversification of rainforest faunas: An integrated molecular approach. *Annual Review of Ecology and Systematics*, **31**, 533–563.
- Muniz, F.L. (2012) *Filogeografia e genética de populações de jacaré-paguá (*Paleosuchus palpebrosus*) ao longo do rio Madeira e bacia do rio Paraguai (Pantanal)*. Instituto Nacional de Pesquisas da Amazônia, Manaus, Brasil.
- Nichols, R. (2001) Gene trees and species trees are not the same. *Trends in Ecology & Evolution*, **16**, 358–364.
- Öckinger, E., Schweiger, O., Crist, T.O., Debinski, D.M., Krauss, J., Kuussaari, M., Petersen, J.D., Pöyry, J., Settele, J., Summerville, K.S., & Bommarco, R. (2010) Life-history traits predict species responses to habitat area and isolation: A cross-continental synthesis. *Ecology Letters*, **13**, 969–979.
- Nedbal, M.A., & Flynn, J.J. (1998) Do the combined effects of the asymmetric process of replication and DNA damage from oxygen radicals produces a mutation rate signature in the mitochondrial genome. *Molecular Biology*, **15**, 219-223.
- Pickles, R.S.A., Groombridge, J.J., Zambrana Rojas, V.D., Van Damme, P., Gottelli, D., Kundu, S., Bodmer, R., Ariani, C.V., Iyengar, A., & Jordan, W.C. (2011) Evolutionary history and identification of conservation units in the giant otter, *Pteronura brasiliensis*. *Molecular Phylogenetics and Evolution*, **61**, 616–627.

- Prugh, L.R., Hodges, K.E., Sinclair, A.R.E., & Brashares, J.S. (2008) Effect of habitat area and isolation on fragmented animal populations. *Proceedings of the National Academy of Sciences*, **105**, 20770–20775.
- de Queiroz, K. (2007) Species concepts and species delimitation. *Systematic biology*, **56**, 879–886.
- Roe, K.J. & Lydeard, C. (1998) Species Delineation and the Identification of Evolutionarily Significant Units : Lessons from the Freshwater Mussel Genus *Potamilus* (Bivalvia : Unionidae). *Journal of Shellfish Research*, **17**, 1359–1363.
- Shirley, M.H., Villanova, V.L., Vliet, K. a, & Austin, J.D. (2014a) Genetic barcoding facilitates captive and wild management of three cryptic African crocodile species complexes. *Animal Conservation*, **18**, 322–330.
- Shirley, M.H., Vliet, K. a, Carr, A.N., & Austin, J.D. (2014b) Rigorous approaches to species delimitation have significant implications for African crocodilian systematics and conservation. *Proceedings. Biological sciences / The Royal Society*, **281**, 20132483.
- Smolensky, N.L. (2015) Co-occurring cryptic species pose challenges for conservation: a case study of the African dwarf crocodile (*Osteolaemus* spp.) in Cameroon. *Oryx*, **49**, 584–590.
- Vogler, A.P. & Desalle, R.O.B. (1994) Diagnosing Units of Conservation Management. *Conservation Biology*, **8**, 354–363.
- Waples, R.S. (1991) Pacific salmon, *Oncorhynchus* spp., and the definition of “species” under the Endangered Species Act. *Marine Fisheries Review*, **53**, 11–22.
- Waples, R.S. (1995) Evolutionarily significant units and the conservation of biological diversity under the Endangered Species Act.

## **APÊNDICE A**

Campos Z, Muniz FL, Farias IP, Hrbek T (2015b) Conservation status of the dwarf caiman *Paleosuchus palpebrosus* in the region of the Araguaia-Tocantins basin, Brazil. Crocodile Spec Group News 34:4–8.

# **CROCODILE SPECIALIST GROUP NEWSLETTER**

VOLUME 34 No. 3 • JULY 2015 - SEPTEMBER 2015



IUCN • Species Survival Commission

reorganise it, ensuring the sustainability of a fine institution. I remember his wife "Pipi", who survives him, and is as gracious as Harry often appeared not to be, telling me with concern that she had never seen him work so hard.

His retirement from the Crocodile Specialist Group in 2004 saw a decline in his active participation in crocodilian conservation matters, but he remained active, travelled often, and seemed to enjoy life immensely. He had mellowed - but just a little. His last trip to crocodile country in the Northern Territory was in 2008, and his last outback camping adventure into the Simpson Desert was in 2012 - at 90 years of age! If there is a single legacy from Harry Messel that stands out, it is his personal conviction that "The Pursuit of Excellence" is a laudable goal in life - not just in science and conservation.

Professor Grahame Webb, CSG Chairman [modified slightly version to that provided to the Species Survival Commission of the IUCN].

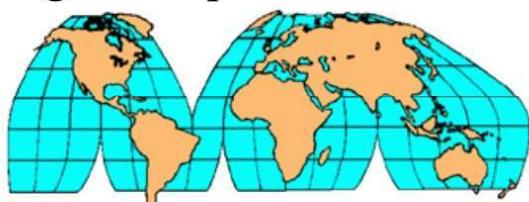
### CSG Student Research Assistance Scheme

The CSG Student Research Assistance Scheme (SRAS; <http://www.iucncsg.org/pages/General-Information.html>) provided funding to two students in the July-September 2015 quarter.

1. Abel Ricardo Pineda Avendaño (Colombia): Population ecology of *Crocodylus acutus* and *Caiman crocodilus fuscus* in Urra Reservoir, Cordoba, Colombia.
2. Andre Yves (Brazil): Population structure of Broad-snouted caiman in Rio Doce State Park, Brazil.

Tom Dacey, CSG Executive Officer, <[csg@wmi.com.au](mailto:csg@wmi.com.au)>

## Regional Reports



### Latin America and the Caribbean

#### Brazil

CONSERVATION STATUS OF THE DWARF CAIMAN *PALEOSUCHUS PALPEBROSUS* IN THE REGION OF THE ARAGUAIA-TOCANTINS BASIN, BRAZIL. The Dwarf caiman (*Paleosuchus palpebrosus*) occurs in 10 South American countries (Medem 1981), including Brazil, where the species is widely distributed except in the southern part of the country (Magnusson 1989). In the latest conservation assessment by the Crocodile Specialist Group, Magnusson and Campos (2010) reported threats to

*P. palpebrosus* populations and their habitats. Campos *et al.* (2013) recommended the establishment of permanent aquatic reserves for the protection of the species in Brazil.

Based on IUCN criteria for endangered species, *P. palpebrosus* falls within the category of "Low Concern," mainly due to its large area of distribution. However, *P. palpebrosus* is a highly secretive species that lives in environments such as springs and headwaters of fast-running rivers, wetlands, flooded forest and swampy plains between hills (Medem 1981; Magnusson 1989; Campos *et al.* 1995), which are environments under strong anthropogenic pressure. Until about 20 years ago, very little was known about the species, except for the pioneering work of Medem (1981) in Colombia and Magnusson (1989, 1992) in the Brazilian Amazon. This lack of information had been pointed out as a problem for the conservation of the species (Thorbjarnason 1992).

Efforts have focused on increasing the body of scientific knowledge about the species, not only in the region of the Pantanal wetland but also in those of Guapore-Mamore-Madeira and the Central Amazon (Campos *et al.* 1995; Campos and Sanaiotti 2006; Botero-Arias 2009; Campos *et al.* 2010, 2011, 2015; Campos and Magnusson 2013). Nevertheless, their unique environments continue to be under threat, their habitats being modified and/or destroyed by human activities such as urbanization, road construction, hydroelectric plants, deforestation, pollution, mining, and subsistence and accidental hunting (Campos and Mourão 2006). Muniz (2012) warned that the fragmentation of *P. palpebrosus* populations in the Pantanal and the Amazon, resulting from habitat destruction, directly affects the species' genetic variability.

The Cerrado biome is considered one of the world's hotspots that is under serious threat of disappearing, and the last remaining 20% of this biome is under direct threat of expansion of agribusiness involving soybeans, cereals and other cash crops (Myers *et al.* 2000). Few studies of the Dwarf caiman have been carried out in the Cerrado biome (Vilhaça 2004; Carvalho Jr and Batista 2013). We investigated the threats to the conservation of Dwarf caiman along river banks, swampy plains between hills, and small tributaries of the rivers and secondary tributaries of the Araguaia-Tocantins Basin which flow through the Cerrado.

In October 2014 we traveled almost 5000 km to the aquatic environments of the Dwarf caiman in the Araguaia-Tocantins Basin (14°54'S, 51°5'W), central Brazil, to document the conservation status of their environments. This area is one of the regions studied by Fábio Muniz during the course of his doctorate at INPA/UFAM, with Tomas Hrbek and Izeki Farias as mentors, and Zilca Campos as co-supervisor. During this field trip, we carried out surveys in the evenings to identify the presence of the species in the Garças, Mortes, Araguaia and Tocantins Rivers and their smaller tributaries.

During this survey, we found that most of the Cerrado vegetation has been replaced with cotton or soybean plantations, depending on the time of year, which stretch all the



Figure 1. (left) Pastures in permanent preservation area in the Garças River; (centre) Housing in permanent preservation area in the Garças River; (right) Beaches formed due to deforestation of the Araguaia River.

way down to the banks of small streams, in the riparian forests in the rivers. The typical plain formations of the Cerrado, which are important areas of springs and swamps, have been completely destroyed and transformed into watering holes for cattle, with rarely a swampy plain left intact. The gallery forests of the Araguaia, Garças, and Mortes Rivers have been cut down to make way for pastureland for cattle and human settlements (Fig. 1). The destruction of gallery forests has caused the erosion of soil, which is carried down to riverbeds, forming beaches that the locals use for leisure and recreation (Fig. 1). We found no Dwarf caiman along these stretches of river altered by anthropic activities and by intense traffic of people and boats, and the species was limited to stretches of river with preserved gallery forests.

Crocodilians are being hunted in this region, and we found a dead Black caiman (*Melanosuchus niger*) and a dead Spectacled caiman (*Caiman crocodilus*) at the Araguaia River (11°43'S, 50°43'W). The situation observed on the Tocantins River and its tributary streams was similar to that of the Araguaia River. However, the most striking change is the construction of dams for the formation of reservoirs for the region's hydroelectric plants (Fig. 2). The permanent flooding of areas of gallery forests due to river damming has brought the nesting areas of caimans close to roads and cities, making these previously protected and remote areas easily accessible along the roads.



Figure 2. Permanent Preservation Areas flooded by hydroelectric dam on the Tocantins River.

The threats are similar to those found in the surroundings

of the Pantanal (Campos and Mourão 2006), but with the aggravating factor that the process of human occupation in the Araguaia-Tocantins Basin is continuous, intense and growing. In Brazil, this central region of the Dwarf caiman distribution is considered an agricultural region and much of the native vegetation has already been transformed into monoculture, and its aquatic environments have been fragmented. The roads are duplicated and asphalted in order to link cities and transport the crops produced in the region. Today there is also a railway for transporting crops from the region for export through Brazil's ports. Brazil's new Forest Code (Law No.12651/2012) for the region establishes the protection of 20% of a rural properties, and the restoration of permanent preservation area (PPA) in 30-m wide protective strips along up to 10 m wide. However, small streams and wetlands are not protected. The Dwarf caiman still appears to persist in preserved and uninhabited stretches of rivers, but these stretches are dwindling in the region of the Araguaia-Tocantins Basin, and if nothing is done, the area of distribution of this species may be restricted solely to the region's Conservation Units.

#### Acknowledgments

We thank the José Augusto and Denis Tilcara by help in field and Embrapa Pantanal. This research was supported by grants from National Counsel of Technological and Scientific Development (CNPq) SISBIOTA/FAPEAM to IPF, and CNPq/Universal 482662/2013-1 to TH. This work forms a portion of FM's PhD thesis at the Genetics, Conservation and Evolutionary Biology program of INPA/UFAM, with a scholarship from FAPEAM.

#### Literature Cited

- Campos, Z., Coutinho, M. and Abercrombie, C. (1995). Size structure and sex ratio of dwarf caiman in the Serra Amolar, Pantanal, Brazil. *Herpetological Journal* 5(4): 321-322.
- Campos, Z. and Sanaiotti, T. (2006). *Paleosuchus palpebrosus* (Dwarf caiman) nesting. *Herpetological Review* 37: 81.
- Campos, Z. and Mourão, G. (2006). Conservation status of the dwarf caiman, *Paleosuchus palpebrosus*, in the region surrounding Pantanal. *Crocodile Specialist Group Newsletter* 25(4): 9-10.

- Campos, Z., Sanaiotti, T. and Magnusson, W.E. (2010). Maximum size of dwarf caiman, *Paleosuchus palpebrosus* (Cuvier, 1807), in the Amazon and habitats surrounding the Pantanal, Brazil. *Amphibia-Reptilia* 31: 439-442.
- Campos, Z., Muniz, F. and Magnusson, W. (2012). Dead *Paleosuchus* on roads in Brazil. *Crocodile Specialist Group* 31(4): 12-13.
- Campos, Z., Sanaiotti, T., Muniz, F., Farias, I. and Magnusson, W.E. (2012). Parental care in the dwarf caiman, *Paleosuchus palpebrosus* Cuvier, 1807 (Reptilia: Crocodilia: Alligatoridae). *Journal of Natural History* 46: 2979-2984.
- Campos, Z. and Magnusson, W.E. (2013). Thermal evidence of dwarf caiman, *Paleosuchus palpebrosus*, in a hillside stream: Evidence for an unusual thermal niche among crocodilians. *Journal of Thermal Biology* 38: 20-23.
- Campos, Z.; Magnusson, W.E. and Marques, V. (2013). Growth rates of *Paleosuchus palpebrosus* at the southern limit of its range. *Herpetologica* 69(4): 405-410.
- Campos, Z., Marioni, B., Farias, I., Verdade, L.M., Bassetti, L., Coutinho, M.E., Mendonça, S.H.S., Vieira, T.Q. and Magnusson, W.E. (2013). Avaliação de risco de extinção do jacaré-paguá, *Paleosuchus palpebrosus* (Cuvier, 1807), no Brasil. *Biodiversidade Brasileira* 3(1): 40-47.
- Campos, Z., Sanaiotti, T., Marques, V. and Magnusson, W. E. (2015). Geographic variation in clutch size and reproductive season of the dwarf caiman, *Paleosuchus palpebrosus*, in Brazil. *Journal of Herpetology* 49(1): 95-98.
- Carvalho Jr., E.A.R. and Batista, V.B.G.V. (2013). Distribution and abundance of *Caiman latirostris* and *Paleosuchus palpebrosus* at Grande Sertão Veredas National Park, Central Brazil. *Herpetological Conservation and Biology* 8(3): 771-777.
- Magnusson, W.E. (1992). *Paleosuchus palpebrosus*. Catalogue of American Amphibians and Reptiles 554.1: 554.2.
- Magnusson, W.E. (1989). *Paleosuchus*. Pp. 101-109 in *Crocodiles. Their Ecology, Management and Conservation. A special publication of the IUCN-SSC Crocodile Specialist Group*. IUCN: Gland, Switzerland.
- Magnusson, W. E. and Campos, Z. (2010). Cuvier's smooth-fronted caiman *Paleosuchus palpebrosus*. Pp. 40-42 in *Crocodiles. Status Survey and Conservation Action Plan*. 3rd edition, ed. by S.C. Manolis and C. Stevenson. Crocodile Specialist Group: Darwin, Australia.
- Myers, N., Mittermeier, R.A., Mittermeier, C.G., Fonseca, G.A.B. and Kent, J. (2000). Biodiversity hotspots for conservation priorities. *Nature* 403: 853-858.
- Medem, F. (1981). Los Crocodylia de sur América. Vol. 2. Los Crocodylia de Colômbia. Ed. Carrera 7<sup>a</sup> Ltda.: Bogotá.
- Muniz, F.L. (2012). Filogeografia e genética de populações de jacaré-paguá (*Paleosuchus palpebrosus*) ao longo do rio Madeira e bacia do rio Paraguai (Pantanal). Dissertação (Mestrado em Genética, Conservação e Biologia Evolutiva). Instituto Nacional de Pesquisa da Amazônia/Universidade Federal do Amazonas. 61 p.
- Thorbjarnason, J. (1992). *Crocodiles: An Action Plan for Their Conservation*, ed. by H. Messel, F.W. King and J.P. Ross. IUCN: Gland, Switzerland.
- Vilhaça, A.M. (2004). Uso de habitat por *Caiman crocodilus* and *Paleosuchus palpebrosus* no reservatório de UHE de Lajeado, Tocantins. Tese de mestrado. 59 p.
- Zilca Campos<sup>1</sup>, Fábio Muniz<sup>2,3</sup>, Izeni Pires Farias<sup>3</sup> and Tomas Hrbek<sup>3</sup>; <sup>1</sup>*Embrapa Pantanal, CP 109 Corumbá, MS 79320-900 Brazil*; <sup>2</sup>*Instituto Nacional de Pesquisa da Amazônia (INPA), CP 478 Manaus, AM, Brazil*; <sup>3</sup>*Universidade Federal do Amazonas (UFAM), Av. General Rodrigo Ramos, 3000 Manaus, AM, Brazil*.

## South Asia and Iran

### India

CROCODILE SURVEYS IN CORBETT NATIONAL PARK 2015. Surveys in areas in Corbett National Park (CNP) of the Corbett Tiger Reserve (CTR) were conducted in March 2015 as a part of the on-going Crocodilian and Freshwater Turtle Research and Conservation Project being implemented by Subir Mario Chowdhury and Dr. Alison Leslie. Surveys in CNP include the use of trail cameras, shoreline surveys by boat, and stationary counts from vantage points (see Fig. 1).



Figure 1. Vantage point locations on the Ramganga River in Corbett National Park, Corbett Tiger Reserve.

The surprise of the season was the recording of 17 adult Gharial (inclusive of 2 adult males) on the Ramganga River between Gairal and Ghetia Rao, based on vantage point observations at High Bank and Champion's Pool. Previous surveys of this stretch of the Ramganga River recorded 3 adults (inclusive

## APÊNDICE B

Muniz F, Bittencourt PS, Farias IP, Hrbek T, Campos Z (2015) New records on occurrence of *Paleosuchus* in the Branco river basin, Roraima state, Brazil. Crocodile Specialist Group Newsletter 34: 8–10.

# CROCODILE SPECIALIST GROUP NEWSLETTER

VOLUME 34 No. 4 • OCTOBER 2015 - DECEMBER 2015



IUCN • Species Survival Commission

## **CSG Student Research Assistance Scheme**

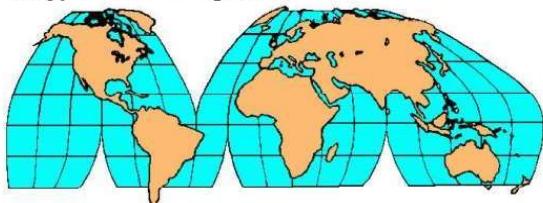
The CSG Student Research Assistance Scheme (SRAS; <http://www.iucncsg.org/pages/General-Information.html>) provided funding to 7 students in the October-December 2015 quarter, bringing the total approvals for the year to 15.

1. Fabio Muñiz (Brazil): Phylogeography and population genetics of Cuvier's Dwarf caiman (*Paleosuchus palpebrosus*) in the Amazon: next-generation sequencing approach.
2. Sebastian Brackhane (Germany): Implementation of a community-based monitoring system (CBMS) for *Crocodylus porosus* in Timor Leste.
3. Hernan Ciocan (Mexico): Population dynamics of wild and reintroduced *Caiman latirostris* and evaluation of their monitoring methods.
4. Liliana Berenice Garcia Reyes (Mexico): Determination and quantification of heavy metals in *Crocodylus acutus* and *Caiman crocodilus*, Tonala, Chiapas, Mexico.
5. Andrea Argumedo León (Mexico): Population status of *Caiman crocodilus* for its conservation: a genetic focus.
6. Maria Laura Romito (Argentina): Oxidative damage of DNA in *Caiman latirostris* exposed to pesticides under semi-natural conditions.
7. Miriam Boucher (USA): Investigating bioacoustics and behaviour of American crocodiles in Belize.

It is now 7 years since the CSG Student Research Assistance Scheme commenced in January 2009. During this period 94 applications have been approved from 29 countries. (Argentina 15, Australia 5, Benin 1, Bolivia 1, Brazil 11, Burkina Faso 1, Canada 1, China 1, Colombia 6, Costa Rica 3, Cuba 1, Ecuador 1, Ethiopia 1, Germany 3, India 1, Iran 1, Malaysia 1, Mexico 7, Nepal 1, Netherlands 1, Panama 1, Peru 1, Philippines 1, South Africa 7, Thailand 1, Uganda 2, United Kingdom 1, USA 14, Venezuela 3). To date reports have been received on 70 projects, with 24 still ongoing. Full details on all grant proposals can be found on the CSG website at: [www.iucncsg.org](http://www.iucncsg.org) (under Grants).

Tom Dacey, CSG Executive Officer, <[csg@wmi.com.au](mailto:csg@wmi.com.au)>

## **Regional Reports**



## **Latin America and the Caribbean**

### **Brazil**

**FIELD COURSE AND WORKSHOP “ECOLOGIA DE TRANSICAO CERRADO-FLORESTA AMAZONICA”.** On 27 July to 28 August 2015 a practical-theoretical course held by PROCAD (031/2013) and conducted by the Universidade de Brasilia (UNB), Universidade do Estado de Mato Grosso (UNEMAT) and Universidade Federal de Tocantins (UFT), in Nova Xavantina and Gaúcha do Norte (Mato Grosso, Brazil). The aims of the course were to increase the interaction between the three universities (UNB, UNEMAT, UFT), and develop studies for the conservation of this very important transitional ecosystem (Brazilian savannah/Amazon forest).

The activities were divided in: 2 weeks in Nova Xavantina, with theoretical and practical courses on statistics, reptile physiology and climatic change; and other 3 weeks in Gaúcha do Norte, with different courses about identification and characterization of plants, insects behaviour, fish morphology, turtles nesting, surveys and physiological studies of crocodilians and lizards. All students had the opportunity to participate during theoretical and practical sessions. The different elements of the course were conducted by: Dr. Rinaldi Colli, Dr. Leza, Dra. Malvasio, Dr. Brandao, Dr. Pellegrin, Dra. Simoncini. Invited professors were Dr. Donald Miles (USA) and Dr. Barry Sinervo (USA). The field course was directed to undergraduates, graduates and professionals from three universities of different areas, and was attended by approximately 70 people.

The field work on crocodilians was conducted with night surveys for species identification, capture, evaluation of health, and to collect important data for ecological and conservation studies. Also the students read publications of the Crocodile Specialist Group about conservation status of two species identified (*Caiman crocodilus* and *Paleosuchus palpebrosus*) and different aspects of crocodilian ecology. Then the students designed individual projects to evaluate the thermoregulatory behaviour of caiman, and the abundance and distribution in different habitats for the two species. Those activities were supervised by Dra. Malvasio and Dra. Simoncini. All the methodologies and results were discussed in the field course and the students are writing their manuscript for publication. Many of the students were interested to develop studies on this important transition ecosystem (Brazilian savannah/Amazon forest) with caimans and collaborating with CSG action plans.

Melina Simoncini, *Proyecto Yacaré-CICyTP (CONICET)*, Argentina.

**NEW RECORDS ON OCCURRENCE OF PALEOSUCHUS IN THE BRANCO RIVER BASIN, RORAIMA STATE, BRAZIL.** Most living crocodilians are endemic to a single zoogeographic region (Martin 2008). In general, the distribution of crocodilians can be explained by a succession

of paleogeographic events (Brochu 2003). Recent studies have demonstrated the importance of these events in the diversification of cryptic species of *Osteolaemus* (Eaton *et al.* 2009; Franke *et al.* 2013; Shirley *et al.* 2014a,b) and of *Paleosuchus palpebrosus* (Muniz 2012). Both these genera comprise small species that live in headwater streams in forest environments and are among the most terrestrial of living crocodilians (Eaton 2010; Magnusson and Campos 2010a,b). These and other shared traits appear to render these species more susceptible to diversification due to events of fragmentation and isolation of populations, probably associated with paleogeographic barriers.

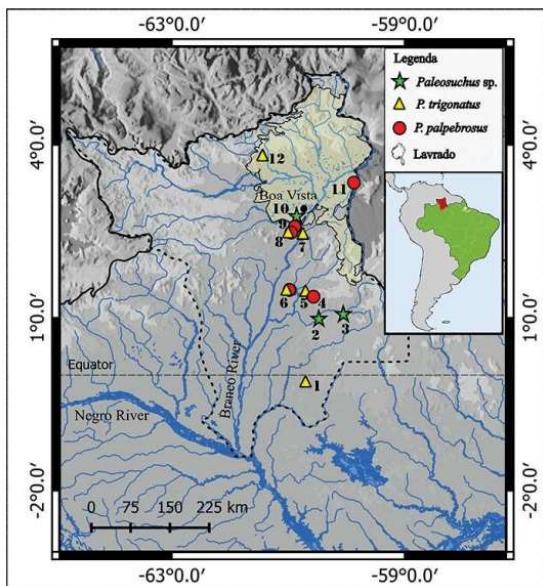


Figure 1. Locations where *Paleosuchus trigonatus* and *P. palpebrosus* specimens were recorded in the state of Roraima. Stars represent individuals not identified to species level. Locations are: (1) Branquinho River, Vila Jundiá; (2) Rorainópolis; (3) São Luiz do Anauá; (4) one *P. palpebrosus* specimen run over on Highway BR 174; (5) Itã River; (6) Viruá National Park; (7) Villa Serra Grande II; (8) buriti palm groves on the right bank of the Mucajá River, “Igarapé da Muda” and “Igarapé Trairão” streams; (9) buriti palm groves of the left bank of the Mucajá River, “Igarapé Tacacá” and “Igarapé Santa Rita” streams; (10) Água Boa River; (11) buriti palm grove along the “Igarapé da Vaca” stream; (12) Amajari River.

The history of the formation of the Branco River basin in Brazil is fairly well known. The headwaters of the Branco River flowed north towards the Atlantic, forming the proto-Berbice basin, up to the end of the Pleistocene (Schaefer and Dalrymple 1995; Lujan and Armbruster 2011). These headwaters were located in a mountain range called a “paleogeographic divide”, which separated the proto-Berbice basin from the Amazon basin. This “paleogeographic divide” is believed to have been eroded during the Pleistocene,

resulting in the redirection of the Branco River southwards. Today, the Branco River is part of the Amazon Basin, the main tributary of the Negro River, and is located on the Guiana Shield, a megadiverse region in which four to five areas of endemism are located (Cracraft 1985; Naka 2011; Naka *et al.* 2012).

Four species of crocodilians occur in the Branco River basin: *Caiman crocodilus* (Spectacled caiman), *Melanosuchus niger* (Black caiman), *P. palpebrosus* (Cuvier’s Dwarf caiman) and *P. trigonatus* (Schneider’s Smooth-fronted caiman). The two *Paleosuchus* species, both of which are small and shy, occur in low densities (Magnusson and Campos 2010a) and require survey methodologies different from those used for the other species (Magnusson and Lima 1991; Campos *et al.* 1995). Earlier studies reported the occurrence of *P. palpebrosus* in the Mucajá River and its tributaries, of *P. trigonatus* in Viruá National Park, and of both species in the Maracá Ecological Station (Rebêlo *et al.* 1997; Gordo *et al.* 2009; Souza 2010).

The purpose of this note is to report new points of occurrence of *Paleosuchus* spp. to the north and south of the proto-Berbice/Amazon paleogeographic divide, in the Branco River basin, and to identify potential threats to the species in the state of Roraima, Brazil. Subsequent studies are planned to examine the effect of paleogeographic events in the Branco River basin on *Paleosuchus* using population genetic tools.

Nighttime surveys of *Paleosuchus* spp. were made by canoe and on foot in March, August and November 2015 along rivers, streams and buriti palm groves in areas of forests and savannas (known locally as “lavrado”). Caiman that were spotted were georeferenced and, when captured, their snout-vent length (SVL, in cm) was measured. A piece of caudal scale tissue was removed and stored in 95% alcohol and deposited in Animal Genetic Tissue Collection (CTGA) of the Federal University of Amazonas (UFAM). Tissue samples of *Paleosuchus* spp. will be used in population genetic analyses by Fabio Muniz in his doctoral studies and by Pedro Senna in his masters studies in the Graduate Program of Genetics, Conservation and Evolutionary Biology at the National Institute for Amazon Research (INPA).

We spotted 52 *Paleosuchus* spp. individuals at 12 locations in the state of Roraima, 22 of which were *P. trigonatus* and 26 were *P. palpebrosus* - four individuals were not identified to species level. Of these, 33 individuals were captured (16 *P. trigonatus* and 17 *P. palpebrosus*). The densities were calculated using only individuals with a SVL of >40 cm. *Paleosuchus trigonatus* were found in 6 locations at an average density of 0.66 ind./km and *P. palpebrosus* were found in 5 locations at an average density of 0.41 ind./km.

New records of *P. palpebrosus* in the state of Roraima were made in Viruá National Park and in a buriti palm grove of the “Igarapé da Vaca,” a tributary of the Tacutu River. The species was also recorded in tributaries of the Mucajá River, and in buriti palm groves along the “Igarapé da Muda,” “Trairão,” “Santa Rita” and “Tacacá” streams. New records of *P. trigonatus* were made in the Branquinho River near

Vila Jundiá, in the Itã River in Vila Serra Grande II, in the Amajari River, and in a buriti palm grove along the “Igarapé da Muda,” a tributary of the Mucajáí River.

The species were found to co-exist in two creeks: one *P. trigonatus* hatchling (SVL= 22.0 cm) and three *P. palpebrosus* hatchlings (SVL= 22.0, 22.6 and 22.9 cm) in the “Igarapé da Muda,” and one *P. trigonatus* (SVL= 95.0 cm) and one *P. palpebrosus* (SVL= 44 cm) in a stream near the entrance to Viruá National Park, all captured during the same night (Fig. 1).

In the state of Roraima, *P. trigonatus* was found in rivers with flowing waters and rocky substrate, such as the Branquinho, Itã and Amajari Rivers; in small montane forest streams, such as Serra Grande II; and, in a buriti palm grove located in a savanna and ombrophilous forest ecotone. *Paleosuchus palpebrosus* was typically found in buriti palm groves located in open areas of savanna and “campinarana” vegetation and in forested areas. The species was also recorded in large rivers such as the Apiaú, Mucajáí and Uraricoera, in points closest to headwater (Rebelo *et al.* 1997; Souza 2010).

Two new records were made in situations that indicate human impact, burned gallery forest and roadkill. In the first instance, a *P. palpebrosus* individual was found in a pool in the “Igarapé da Vaca” stream in burned buriti palm vegetation (03°19' N, 59°51' W). Burning and deforestation of buriti palm groves are reportedly common practices in areas of savanna and can pose a direct threat to *P. palpebrosus*, mainly due to habitat loss. In the second instance, a dead *P. palpebrosus* was found run over on highway BR 174 (01°21' N, 60°36' W; Fig. 1), near a stream in a buriti palm forest. We also recorded a dead *Caiman crocodilus* run over on a stretch of highway BR 174 in November, close to where the *P. palpebrosus* was found (01°25' N, 60°43' W). Being run over has been reported as one of the threats to *Paleosuchus* in Brazil (Campos *et al.* 2012). The roads in the region of Roraima pose a threat to *Paleosuchus* particularly because they pass through a variety of aquatic environments as streams, ponds seasonal flood and areas of buriti palm groves. The individuals can move through the land and cross the highway in order to reach another water body, running the risk to be run over.

Both species are subject to poaching in the region as well. We documented the death of two *P. trigonatus* individuals caused by residents. According to reports, poaching is common both in Indian reserves and in communities along the roads. In general, the conservation status of *Paleosuchus* can be considered of “low concern” due to low human density and the fact that Roraima has one of the last preserved wetland forests in the world (Hammond 2005; Naka *et al.* 2012). However, we identified some areas where the species are more vulnerable, such as savannahs, known as “lavrado”, and in urbanized areas close to Boa Vista and around Rorainópolis, where human occupation and deforestation grow apace (Diniz and Lacerda 2015).

Population genetic studies of these *Paleosuchus* spp. specimens are being conducted to investigate the influence of

the historical processes that occurred in the state of Roraima on the genetic diversity of the species. Is there a genetic structure associated with the paleogeographic events that gave rise to the current Branco River? What is the genetic diversity of these populations? and is there a cause for concern in terms of conservation? These are the questions that underpin our future research.

#### Acknowledgments

We are indebted to Priscila Azarak, Beatriz Lisboa, Bruno de Souza Campos and the staff of Viruá National Park for their practical support, and INPA (National Institute for Amazon Research), UFAM (Federal University of Amazonas) and Embrapa Pantanal for their financial support. The master's and doctoral scholarships were granted by FAPESAM (Amazonas State Research Foundation) and the capture permit was issued by SISBIO/Brazil (no. 49641-2).

#### Literature Cited

- Brochu, C. (2003). Phylogenetic approaches toward crocodylian history. Annual Review of Earth and Planetary Sciences 31(1): 357-397.
- Campos, Z., Coutinho, M. and Abercrombie, C. (1995). Size structure and sex ratio of dwarf caiman in the Serra Amolar, Pantanal, Brazil. Herpetological Journal 5(4): 321-322.
- Campos, Z., Muniz, F. and Magnusson, W. (2012). Dead *Paleosuchus* on roads in Brazil. Crocodile Specialist Group Newsletter 31(4): 12-14.
- Cracraft, J. (1985). Historical biogeography and patterns of differentiation within the South American avifauna: areas of endemism. Ornithological Monographs 36: 49-84.
- Diniz, A.M.A. and Lacerda, E.G. (2015). The colonization of Roraima State, Brazil: an analysis of its major migration flows (1970 to 2010). Espace populations sociétés 3: 1-16.
- Eaton, M.J. (2010). Dwarf crocodile *Osteolaemus tetraspis*. Pp. 127-132 in Crocodiles: Status Survey and Conservation Action Plan, Third Edition, ed. by S.C. Manolis and C. Stevenson. Crocodile Specialist Group: Darwin, Australia.
- Eaton, M.J., Martin, A., Thorbjarnarson, J. and Amato, G. (2009). Species-level diversification of African dwarf crocodiles (Genus *Osteolaemus*): a geographic and phylogenetic perspective. Molecular Phylogenetics and Evolution 50: 496-506.
- Franke, F.A., Schmidt, F., Borgwardt, C., Bernhard, D., Bleidorn, C., Engelmann, W.E. and Schlegel, M. (2013). Genetic differentiation of the African dwarf crocodile *Osteolaemus tetraspis* Cope, 1861 (Crocodylia: Crocodylidae) and consequences for European zoos. Organisms Diversity and Evolution 13: 255-266.
- Gordo, M., Carvalho, V.T., Oliveira, M.E., Dubyna, F.A.,

- Lemos, M., Bernhard, R., Bernardes, V.C.D., Nascente, L.B. and Seixas, M. (2009). Diagnóstico ambiental do Parque Nacional do Viruá: relatório temático de herpetologia. Roraima, Brazil, 43p.
- Hammond, D.S. (2005). Tropical Forests of the Guiana Shield: Ancient Forests in a Modern World. CABI Publishing: Cambridge. 541 p.
- Lujan, N.K. and Armbruster, J.W. (2011). The Guiana Shield. Pp. 211-224 in Historical Biogeography of Neotropical Freshwater Fishes, First edition, ed. By J. Albert and R. Reis. University of California Press: California, USA.
- Magnusson, W.E. and Campos, Z. (2010a). Cuvier's Smooth-fronted Caiman *Paleosuchus palpebrosus*. Pp. 40-42 in Crocodiles: Status Survey and Conservation Action Plan, Third Edition, ed. by S.C. Manolis and C. Stevenson. Crocodile Specialist Group: Darwin, Australia.
- Magnusson, W.E. and Campos, Z. (2010b). Schneider's Smooth-fronted Caiman *Paleosuchus trigonatus*. Pp. 43-45 in Crocodiles: Status Survey and Conservation Action Plan, Third Edition, ed. by S.C. Manolis and C. Stevenson. Crocodile Specialist Group: Darwin, Australia.
- Magnusson, W.E. and Lima, A.P. (1991). The Ecology of a Cryptic Predator, *Paleosuchus trigonatus*, in a Tropical Rainforest. Journal of Herpetology 25(1): 41-48.
- Martin, S. (2008). Global diversity of crocodiles (Crocodylia, Reptilia) in freshwater. Hydrobiologia 595: 587-591.
- Muniz, F.L. (2012). Filogeografia e genética de populações de jacaré-paguá (*Paleosuchus palpebrosus*) ao longo do rio Madeira e bacia do rio Paraguai (Pantanal). Dissertação (Mestrado em Genética, Conservação e Biologia Evolutiva). Instituto Nacional de Pesquisas da Amazônia/Universidade Federal do Amazonas. 61p.
- Naka, L.N. (2011). Avian distribution patterns in the Guiana Shield: implications for the delimitation of Amazonian areas of endemism. Journal of Biogeography 38: 681-696.
- Naka, L.N., Bechtoldt, C.L., Henriques, L.M.P. and Brumfield, R.T. (2012). The role of physical barriers in the location of avian suture zones in the Guiana Shield, northern Amazonia. The American Naturalist 179: 115-132.
- Rebêlo, G.H., Brazaitis, P., Yamashita, C. and Souza, B.C. (1997). Similaridade entre localidades e associações entre três espécies de jacarés em Roraima. Pp. 558-563 in Homem, Ambiente e Ecologia no Estado de Roraima, First edition, ed. by R.I. Barbosa, E.J.G. Ferreira and E.G. Castellón. Instituto Nacional de Pesquisas da Amazônia: Roraima, Brazil.
- Schaefer, C. and Dalrymple, J. (1995). Landscape evolution in Roraima, north Amazonia - plantation, paleosols and paleoclimates. Zeitschrift für Geomorphologie 39(1): 1-28.
- Shirley, M.H., Villanova, V.L., Vliet, K.A. and Austin, J.D. (2014a). Genetic barcoding facilitates captive and wild management of three cryptic African crocodile species complexes. Animal Conservation 18(4): 322-330.
- Shirley, M.H., Vliet, K.A., Carr, A.N. and Austin, J.D. (2014b). Rigorous approaches to species delimitation have significant implications for African crocodilian systematics and conservation. Proceedings of the Royal Society B: Biological Sciences 281: 20132483.
- Souza, B.C. (2010). Ocorrência, uso de habitats e distribuição de jacarés (Alligatoridae) na Estação Ecológica de Maracá, Roraima, Amazônia brasileira. Dissertação mestrado. Universidade Federal de Roraima. 72p.
- Fábio Muniz<sup>1,2</sup>, Pedro Senna Bittencourt<sup>1,2</sup>, Izeni Pires Farias<sup>1</sup>, Tomas Hrbek<sup>1</sup> and Zilca Campos<sup>3</sup>; <sup>1</sup>Laboratory of Evolution and Animal Genetics, Department of Genetics, Institute of Biological Sciences, Federal University of Amazonas, Manaus 69077-000, AM, Brazil; <sup>2</sup>Graduate Program in Genetics, Conservation and Evolutionary Biology, INPA - National Institute for Amazon Research, Manaus 69011-900, AM, Brazil; <sup>3</sup>Embrapa Pantanal, CP 109, Corumbá 79320-900, MS, Brazil.
- 
- ## Europe
- ### United Kingdom
- TOMISTOMA IN THE UK. There is renewed interest within UK zoos to display Tomistoma (*Tomistoma schlegelii*). For the past few years, Paignton Zoo has displayed the species within its Crocodile Swamp exhibit. These animals are from the successful breeding program established by Fuengirola Zoo in Spain. In August 2015 Chester Zoo unveiled its 'Sunda Gharial' exhibit within its expansive Islands section, with animals from La Ferme aux Crocodiles in France.
- Crocodiles of the World (COTW), the UK's only crocodile zoo, located near Oxford, also has a pair of adult Tomistoma, which were imported from Johnson Jong's Crocodile Park in Sarawak, Malaysia, and were destined for the Rare Species Conservation Centre in Kent. Until a suitable enclosure could be prepared, the Tomistoma were being held at COTW. Earlier in 2015, the pair were officially transferred to COTW, where they will remain.
- The Tomistoma arrived in the UK on 3 September 2014. The male, hatched in 1995, was 3.4 m long and weighed 132.9 kg. The female, hatched in 1999, was 2.3 m long and weighed 37.5 kg. As of now the Tomistoma are off-display at COTW, and have settled in nicely. These animals will allow COTW to begin raising awareness - and therefore funds - in order to support *in-situ* conservation projects for the species and the CSG Tomistoma Task Force. COTW will also be the focal zoo