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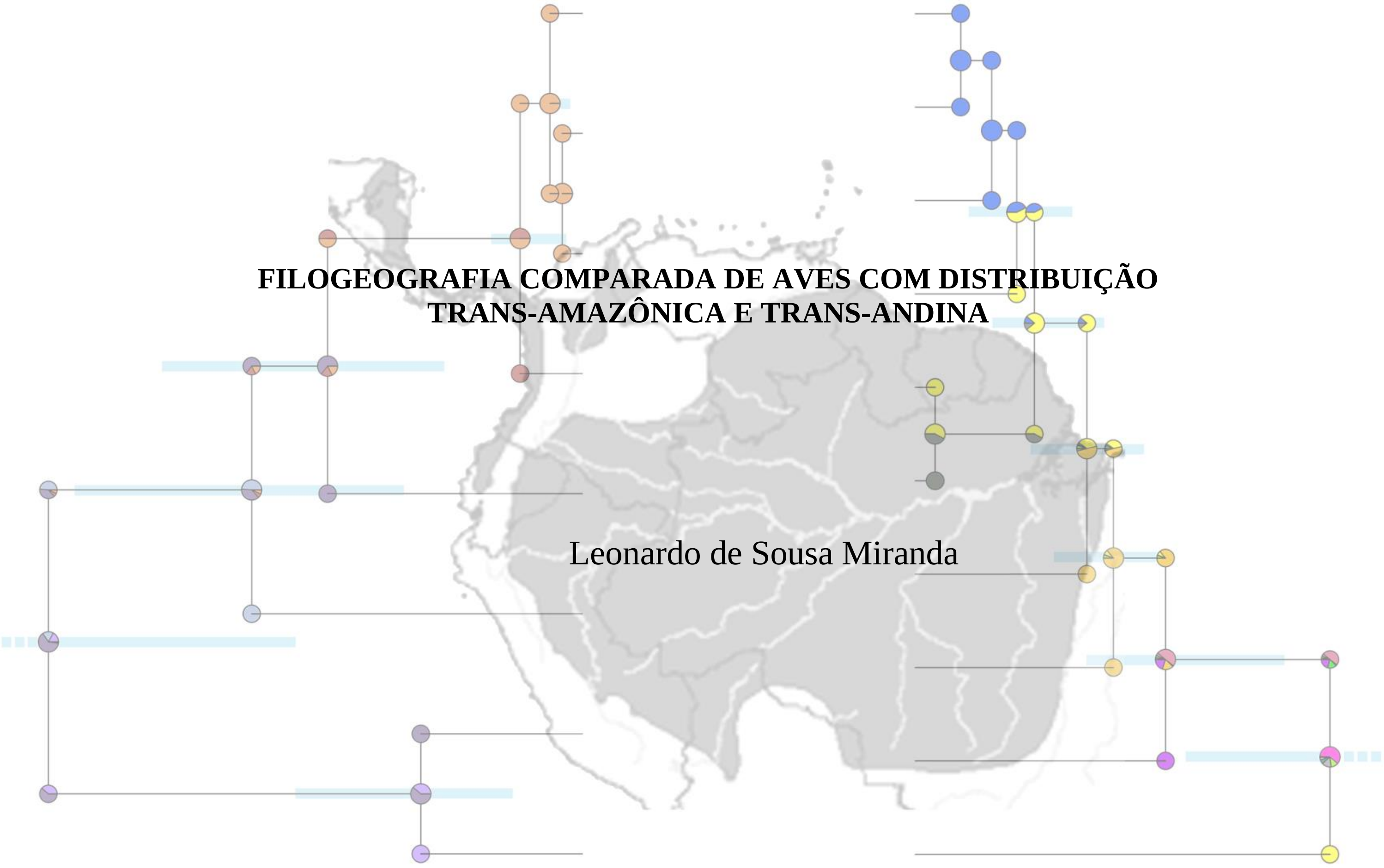
UNIVERSIDADE FEDERAL DO PARÁ

PROGRAMA DE PÓS-GRADUAÇÃO EM ZOOLOGIA



FILOGEOGRAFIA COMPARADA DE AVES COM DISTRIBUIÇÃO TRANS-AMAZÔNICA E TRANS-ANDINA

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Tese apresentada ao Programa de Pós-Graduação em Zoologia da Universidade Federal do Pará e Museu Paraense Emílio Goeldi como requisito parcial para obtenção do grau de Doutor em Zoologia.

Orientador: Dr. Alexandre Aleixo

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“Um intelecto que, num dado instante, conheça todas as forças que estejam atuando na natureza, e as posições de todas as coisas das quais o mundo é constituído – supondo-se que o dito intelecto fosse grande o suficiente para sujeitar esses dados à analise – abraçaria, na mesma fórmula, os movimentos dos maiores corpos do universo e os dos menores átomos; nada seria incerto para ele e o futuro, assim como o passado, estaria presente aos seus olhos”

Pierre S. Laplace

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RESUMO

As florestas Neotropicais possuem a avifauna mais rica do mundo, com muitos táxons endêmicos; e os padrões de distribuição e variação fenotípica dos organismos nessa região são muito complexos. Muitas hipóteses já foram propostas para tentar explicar os processos de diversificação que resultaram na atual diversidade e nos padrões de endemismo encontrados nessa região. A maior parte dessas hipóteses não havia sido testada devido à dificuldade de falseação das mesmas num contexto filogenético. Contudo, com o acumulo de informações e a disponibilidade de novos métodos analíticos, estão sendo desenvolvidos trabalhos voltados à análise das hipóteses biogeográficas a partir da descrição e dos testes de suas premissas. Estudos de paleoclimatologia e paleoecologia associados a informações da história geológica de uma área produzem cenários que, por sua vez, levam a formulação de hipóteses de diversificação. O contraste destas hipóteses com os padrões de distribuição e relações filogenéticas entre os organismos pode fornecer informações acerca dos eventos de diversificação que influenciaram a origem das espécies atuais. Dessa forma, o principal objetivo desse trabalho é usar dados moleculares de duas espécies de aves com distribuição trans-Amazônica e trans-Andina para contrastar as hipóteses de diversificação, incorporando informações sobre tempos relativos de divergência, áreas ancestrais e meios de diversificação, e realizando inferências sobre quais eventos no passado podem ter influenciado os processos de cladogênese. E finalmente foi avaliado se os padrões espaciais e temporais de divergência entre os filogrupos das espécies estudadas são congruentes para as mesmas barreiras consideradas (por exemplo, principais rios Amazônicos e a cordilheira dos Andes).

1. INTRODUÇÃO GERAL

1.1 FILOGEOGRAFIA

Estudos de paleoclimatologia, palinologia e paleoecologia associados a informações da história geológica do continente podem fornecer uma visão geral dos diferentes ambientes no passado (ex., Cowling et al. 2001, Rossetti e Netto 2006), produzindo cenários que, por sua vez, levam a formulação de hipóteses de diversificação, muitas das quais passíveis de serem testadas do ponto de vista filogenético (Carstens et al. 2005, Spellman e Klicka 2006). O contraste destas hipóteses com os padrões de distribuição e relações filogenéticas entre os organismos de diferentes regiões geográficas pode fornecer informações acerca dos eventos de diversificação que influenciaram a origem das espécies atuais (ex., Marks et al. 2002, Aleixo 2004, 2006, Cheviron et al. 2005, Ribas et al. 2005, 2012, Cabanne et al. 2008, Milá et al. 2009, Antonelli et al. 2010, D'Horta et al. 2012, Fernandes et al. 2012).

A filogeografia tornou-se um poderoso campo de pesquisa, revelando os princípios e processos que determinam a distribuição geográfica de linhagens genealógicas, dentro e entre espécies relacionadas (Avise 2000). Uma extensão das análises filogeográficas para uma única espécie é comparar padrões em múltiplas espécies co-distribuídas. O principal objetivo dessa filogeografia comparada tem sido o de encontrar concordância filogenética e no padrão de distribuição entre as linhagens de diferentes espécies, que possam indicar a influência de fatores históricos comuns. Estudos recentes de filogeogafia comparada têm encontrado padrões biogeográficos sugerindo que vicariância tem desempenhado um dos papeis mais importantes na formação da biota atual (Aleixo e Rossetti 2007, Antonelli et al. 2010).

Por outro lado, os avanços associados a estudos filogeográficos vão muito além do maior rigor estatístico, acompanhado de uma maior sofisticação computacional e avanços moleculares (Knowles 2009). Atualmente, tem-se empregado muitos métodos e ferramentas moleculares diferentes (Brumfield et al. 2008). Essas novas metodologias têm tornado as comparações entre padrões de diversificação de grupos diferentes menos descritivas e menos restritas a metodologias pré-definidas (Moritz et al. 2000, Zink 2002). Esse procedimento se justifica principalmente pela estocasticidade do processo de coalescência – que corresponde à perda aleatória de linhagens de genes por deriva genética, causada pelo sucesso reprodutivo diferencial entre os indivíduos de uma população (Avise 2000, Maddison e Knowles 2006). Em termos práticos, isso significa que da mesma forma que um histórico populacional comum pode gerar diferentes padrões genealógicos em diferentes loci, uma árvore de gene também pode ser consistente com muitas historias populacionais, levando a falsas inferências (Klicka et al. 2001, Carling e Brumfield 2008). Levando esses aspectos em consideração, estudos recentes têm revelado que muitas vezes os eventos de diversificação em grupos que ocupam áreas comuns ocorreram em tempos distintos, não podendo, portanto, serem tomados como evidências de um único evento de vicariância (Ribas e Miyaki 2007).

Até recentemente, as análises estatísticas utilizadas refletiam uma divisão entre os campos da genética de populações e inferências filogenéticas, em outras palavras, ou eram utilizadas árvores de genes como base para inferências (Moritz et al. 2000) ou estimativas de parâmetros populacionais baseado em amostras de genealogias (Kuhner 2009). No primeiro caso, comparando a estrutura filogeográfica de várias espécies co-distribuidas, pode-se inferir sobre a estabilidade histórica da fauna atual, como evidenciado pela freqüência relativa de grupos reciprocamente monofiléticos e geograficamente congruentes (Aleixo et al. 2010). É possível, portanto, decifrar a história evolutiva de cada taxon se a topologia da árvore está, ou não, estruturada. Podem-se observar quatro tipos principais de estruturação filogeográfica (Avise 2000): Tipo 1 – árvores com ramos longos que são geograficamente estruturadas; Tipo 2 – árvores com ramos longos sem estruturação geográfica; Tipo 3 – árvores com ramos curtos com estrutura geográfica; e Tipo 4 – árvores com ramos curtos sem estrutura geográfica. Cada tipo é consistente com uma história particular. Grupos geograficamente estruturados (Tipos 1 e 3) em uma área são, normalmente, resultado de isolamento devido a uma barreira ambiental, ecológica ou física; por outro lado, um padrão do tipo 4 é consistente com uma expansão populacional recente e ausência de barreiras (Zink 2002).

Em uma abordagem clássica a comparação de padrões filogeográficos entre táxons co-distribuidos mostra como estes têm respondido a eventos recentes de isolamento (Figura 1). O primeiro passo, nesse sentido, seria verificar se existem grupos reciprocamente monofiléticos dentro de uma amostra de táxons, em uma comunidade. O próximo passo seria avaliar se os grupos mutuamente monofiléticos e geograficamente concordantes são grupos-irmãos. Neste caso, pode-se determinar quanto tempo os grupos estão isolados, em um sentido relativo (Klicka e Zink 1999, Ribas e Miyaki 2007, Carling e Brumfield 2008). Por outro lado, assumindo que há congruência indicando uma barreira histórica para, pelo menos alguns táxons, pode-se examinar as razões pelas quais outros táxons da fauna atual não mostram monofilia recíproca entre as mesmas barreiras. Nesse caso, ou as espécies eram amplamente distribuídas e, simplesmente, não responderam à barreira, ou essas espécies expandiram recentemente suas áreas de distribuição, cruzando as barreiras (Figura 1). Dessa forma, podem-se distinguir essas duas alternativas através das assinaturas genéticas das populações consideradas (Moritz 1996, Crandall e Templeton 1996). Por exemplo, se as espécies eram amplamente distribuídas por um longo período de tempo, a diversidade nucleotídica deveria ser equivalente, bem como o tamanho efetivo nas populações em ambos os lados da barreira. Alternativamente, se ocorreu expansão recente da área de distribuição, a diversidade genética nos dois lados da barreira deveriam ser diferentes, com a área recentemente colonizada apresentando menos variabilidade e um tamanho efetivo menor (Figura 1).

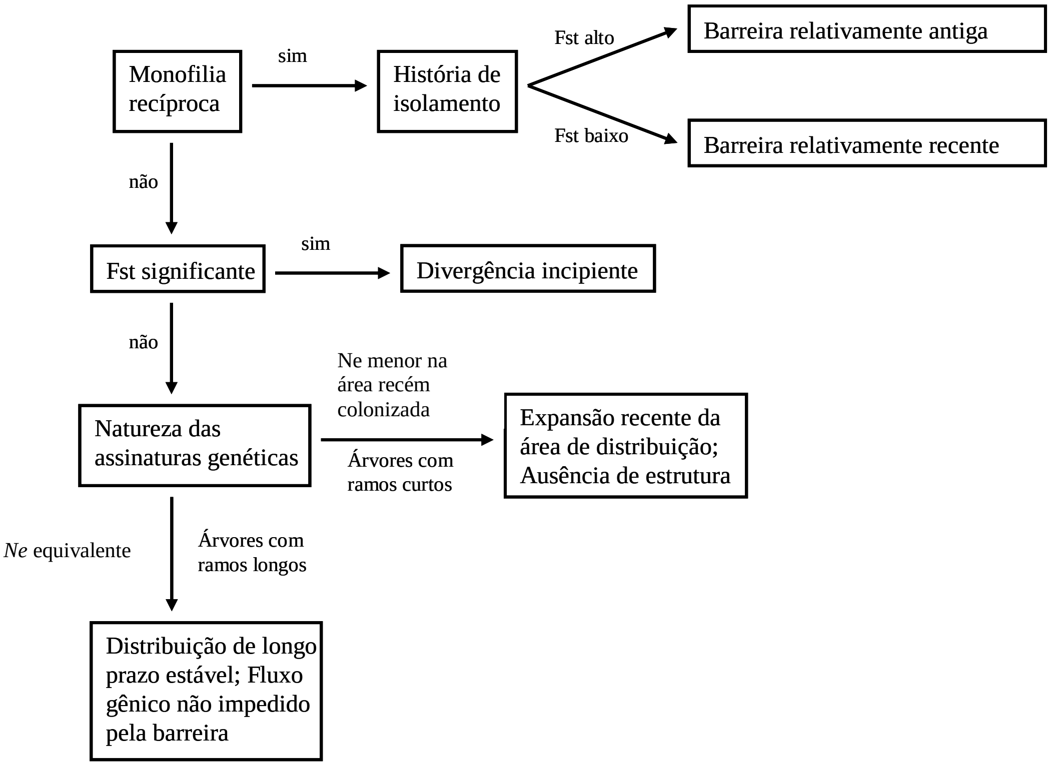

Figura 1 – Esquema demonstrando as conclusões que se pode chegar a partir dos resultados (assinaturas genéticas) observados a partir de análises de filogeografia comparada (Adaptado de Zink, 2002)

Contudo, essa divisão tradicional está sendo substituída por uma abordagem de filogeografia estatística, que considera tanto a variância mutacional quanto o processo de coalescência, possibilitando uma série de metodologias que podem ser utilizadas para estimar os parâmetros genéticos da população e testar hipóteses (para uma revisão do assunto ver Knowles 2009). Esses métodos têm se tornado cada vez mais populares devido ao seu potencial para explicar histórias complexas como, por exemplo, distinguir a divergência com fluxo gênico de retenção de polimorfismo ancestral (Pinho e Hey 2010). Além disso, essas abordagens são muito flexíveis, podendo acomodar situações complexas envolvendo muitos processos combinados (ex. divergência populacional, flutuações no tamanho da população e migração), comparação de cenários concorrentes e estimativas de parâmetros, sob diferentes tamanhos de populações e amostras (Carnaval et al. 2009, Knowles 2009).

1.2 HIPÓTESES FILOGEOGRÁFICAS

As florestas Neotropicais possuem a avifauna mais rica do mundo, com muitos táxons endêmicos. Os padrões de distribuição e variação fenotípica dos organismos nessa região são muito complexos, contudo áreas de endemismo comuns a vários grupos (Figura 2) foram reconhecidas com base nas congruências entre esses padrões (Cracraft 1985, Silva et al. 2005, Borges e Silva 2012).

Figura 2 – Áreas de endemismos para aves amazônicas segundo Cracraft (1985) eSilva et al. (2005). Os números correspondem às localidades comparadas quanto a composição da avifauna por Borges e Silva (2012).

Muitas hipóteses já foram propostas para tentar explicar os processos de diversificação que resultaram na diversidade única e nos padrões de endemismo encontrados atualmente nas florestas Neotropicais, principalmente na bacia Amazônica (revisado em Haffer 1997). A maior parte dessas hipóteses de diversificação histórica propostas para a região Neotropical não havia sido testada devido à dificuldade inerente de falsificação das mesmas num contexto sistemático / filogenético (Patton e Silva 1998). Entretanto, devido ao acumulo de informações e com a disponibilidade de novos métodos analíticos, começaram a ser desenvolvidos trabalhos voltados à análise das hipóteses biogeográficas a partir da descrição de suas premissas, seguido dos testes dessas predições (Moritz et al. 2000), relacionadas, principalmente, à distribuição geográfica, relações evolutivas, tempo de divergência e história demográfica das linhagens (Aleixo 2004, 2006, Ribas et al. 2005, 2012, D’Horta et al. 2012, Fernandes et al. 2012).

Alguns dos modelos mais difundidos que tentam explicar a distribuição espacial das áreas de endemismo na Amazônia são as hipóteses dos refúgios (Haffer 1969) e de mudanças na paisagem mediadas por eventos tectônicos no neogeno (Hoorn et al. 2010). As filogenias de grupos com distribuição mais ampla (Região Neotropical) têm mostrado que muitas das linhagens de espécies são anteriores ao Quaternário e que, portanto, eventos paleogeográficos, principalmente as alterações na bacia amazônica, causadas pela orogênese dos Andes e incursões marinhas, podem ter tido um papel maior do que tradicionalmente proposto na sua diversificação (Marks et al. 2002, Aleixo 2004, Ribas et al. 2012, Fernandes et al. 2012). Esses eventos pré-quaternários podem ter estabelecido as principais divisões regionais, com subseqüente diferenciação geográfica e redistribuição de espécies durante o Pleistoceno (Ribas e Miyaki 2007).

Portanto, de um ponto de vista mais abrangente, o soerguimento dos Andes teve um grande impacto sobre a diversificação nos táxons de terras baixas da região Neotropical. Essa influência sobre a diversidade é evidente tanto pelos atuais dados de distribuição dos organismos, para os quais a porção leste dos Andes geralmente delimita a distribuição dos organismos de terras baixas (Ridgely e Tudor 1994); como por estudos de filogeografia, os quais têm demonstrado uma divisão filogenética entre clados *trans*- (oeste dos Andes) e *cis*-Andinos (leste dos Andes) (Brumfield e Capparella 1996). Ainda assim, existe muita discussão a respeito do papel dos Andes sobre a diversificação de aves neotropicais (Brumfield e Edwards 2007), seja: (1) atuando diretamente através de vicariância dos táxons que, antes do soerguimento, estariam distribuídos continuamente desde a bacia amazônica até o oceano pacífico; ou (2) de uma forma indireta, pela criação de um estreito corredor na margem norte que permitiria dispersão entre os indivíduos associados a terras baixas de lados opostos, durante ciclos de umidade e/ou baixos níveis dos oceanos; e isolamento, durante ciclos de seca e/ou elevados níveis dos oceanos.

Entretanto, apesar dos diversos esforços para encontrar uma explicação geral para as origens dos padrões atuais de diversidade Neotropical, os resultados obtidos até hoje são controversos e apontam para uma história complexa, influenciada por muitos fatores diferentes em diversos períodos de tempo (Aleixo e Rossetti 2007, Antonelli et al. 2010). Diante dessas informações, o estudo proposto constitui mais um esforço no sentido de compreender melhor os processos de diversificação responsáveis pela origem dos padrões de diversidade encontrados hoje na região Neotropical.

1.3 OS MODELOS

Como detalhado acima, a maior parte dos modelos de diversificação partem da idéia de isolamento geográfico como o modo primário de diferenciação. Baseado no fato de que a maioria dos grupos de vertebrados Neotropicais está distribuída parapatrica ou alopatricamente, é possível sugerir que isolamento geográfico pode representar a hipótese nula de diversificação para vertebrados na região Neotropical. Entretanto, gradientes ecológicos podem produzir diferenciação genética entre populações de aves, mas demonstrações empíricas de especiação através desses gradientes são escassas (Smith 2005). Testes para essas hipóteses na região Neotropical usam diferentes abordagens (genética de populações e filogenias), mas geralmente corroboram isolamento geográfico como a força dominante em aves (Ribas et al. 2012, D'Horta et al. 2012, Fernandes et al. 2012). Contudo, estudos de táxons abrangendo um conjunto de habitats são necessários para gerar um teste completo para os mecanismos de diversificação Neotropical (Burney e Brumfield 2009, Smith et al. 2014).

Os pares de espécies biológicas *Cymbilaimus lineatus / sanctaemariae* (Aves: Thamnophilidae) e *Microcerculus bambla / marginatus* (Aves: Troglodytidae) são bons modelos para inferir a história de formação da biota Neotropical principalmente pelo fato de estarem amplamente distribuídos por esta região. Esses táxons são uniformemente abundantes e relativamente restritos ao tipo de habitat mais comum, as florestas de terra-firme. Aliado a esses fatores, há a disponibilidade de tecidos em diferentes coleções científicas para as análises moleculares.

Ainda que estudos recentes encontraram suporte para o monofiletismo da família Thamnophilidae (Irestedt et al. 2004, Brumfield et al. 2007, Moyle et al. 2009), o posicionamento filogenético do gênero *Cymbilaimus* dentro da família permanece incerto, devido a incompleta – ou mesmo ausência – amostragem de mais gêneros representantes dessa familia; ora sendo colocado como clado irmão de *Frederickaena* (Brumfield e Edwards 2007), ora como grupo-irmão de *Taraba* (Brumfield et al. 2007, Moyle et al. 2009). Além disso, caracteres moleculares, morfológicos e vocais ainda não foram avaliados a nível intra-específico (Zimmer e Isler 2003). Atualmente são reconhecidas três subespécies de *Cymbilaimus lineatus*, que se distribuem da seguinte maneira (Zimmer e Isler 2003, Figura 3):

- *C. l. lineatus* Leach, 1814 – leste da Venezuela; Guianas; e nordeste da Amazônia brasileira.
- *C. l. fasciatus* Ridgway, 1884 –sudeste de Honduras (El Paraíso); costa caribenha na Nicaragua e Costa Rica, no Panamá; norte e oeste da Colômbia; noroeste da Venezuela; e Noroeste do Equador.

- *C. l. intermedius* Hartert e Goodson, 1917 –sul da Venezuela; sul da Colômbia; Leste do Equador; leste do Peru; noroeste e sul da Amazônia brasileira (ao sul do rio Amazonas desde o rio Tocantins até o rio Negro e ao norte desde o rio Negro até o rio Branco); e nordeste e extremo leste da Bolívia.

Figura 3 – Mapa da região Neotropical com área de distribuição da espécie biológica *Cymbilaimus lineatus* (Aves: Thamnophilidae). Fonte: Natureserve 2007.

Anteriormente, a espécie *C. lineatus* era tratada como co-específica de *C. sanctaemariae*, entretanto esta última é simpátrica em parte da área de ocorrência de *C. lineatus*; além disso, caracteres de plumagem e vocais separam esses táxons (Pierpont e Fitzpatrick 1983). *Cymbilaimus sanctaemariae* ocorre a sudeste do Peru (departamentos de Cusco e Madre de Dios); noroeste da Bolívia (departamentos de Pando e norte de La Paz); e sudoeste da Amazônia brasileira (estados do Acre, Rondônia e Amazonas) (Zimmer e Isler 2003, Whittaker 2009; Figura 4).

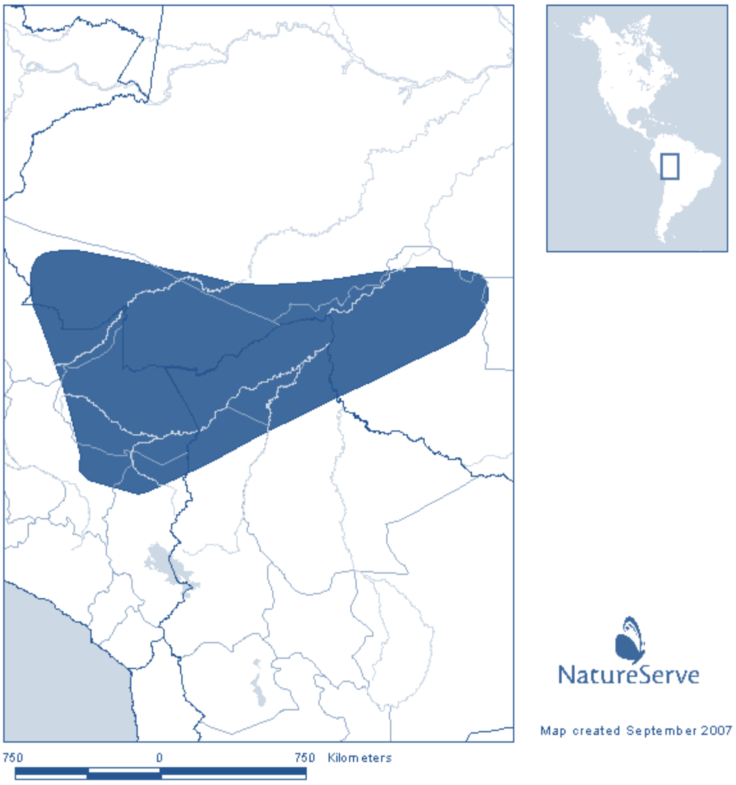

Figura 4 – Mapa da região Neotropical com área de distribuição da espécie biológica *Cymbilaimus sanctaemariae* (Aves: Thamnophilidae). Fonte: Natureserve 2007.

O complexo *Microcerculus bambla / marginatus* (Aves: Troglodytidae) corresponde a espécies que possuem distribuições complementares, onde *M. bambla* distribui-se ao norte do rio Amazonas, desde o rio Branco até o Amapá, com algumas populações ocorrendo em pontos isolados na porção *cis*-andina da América do sul (no Peru e no Equador); e *M. marginatus*, que ocorre na América Central, na região oeste da Amazônia e ao sul do rio Amazonas (Kroodsma e Brewer 2005). Atualmente são reconhecidas seis subespécies em *M. marginatus*, distribuídas da seguinte maneira (Kroodsma e Brewer 2005; Figura 6):

- *M. m.marginatus* Sclater, 1855 –oeste da Amazônia, desde o leste do Equador, leste da Colômbia e sul da Venezuela até o leste do Peru, oeste e sul da Amazônia brasileira e norte-nordeste da Bolívia.
- *M. m. luscinia* Salvin, 1866 - Costa Rica e Panamá
- *M. m. squamulatus* Sclater e Salvin, 1875 – norte da Colômbia (exceto a região de Santa Maria) e montanhas do norte e noroeste da Venezuela.
- *M. m. taeniatus* Salvin, 1881 – oeste do Equador.
- *M. m. corrasus* Bangs, 1902 – região de Santa Marta no norte da Colômbia.
- *M. m. occidentalis* Hellmayr, 1906 – oeste da Colômbia e noroeste do Equador.

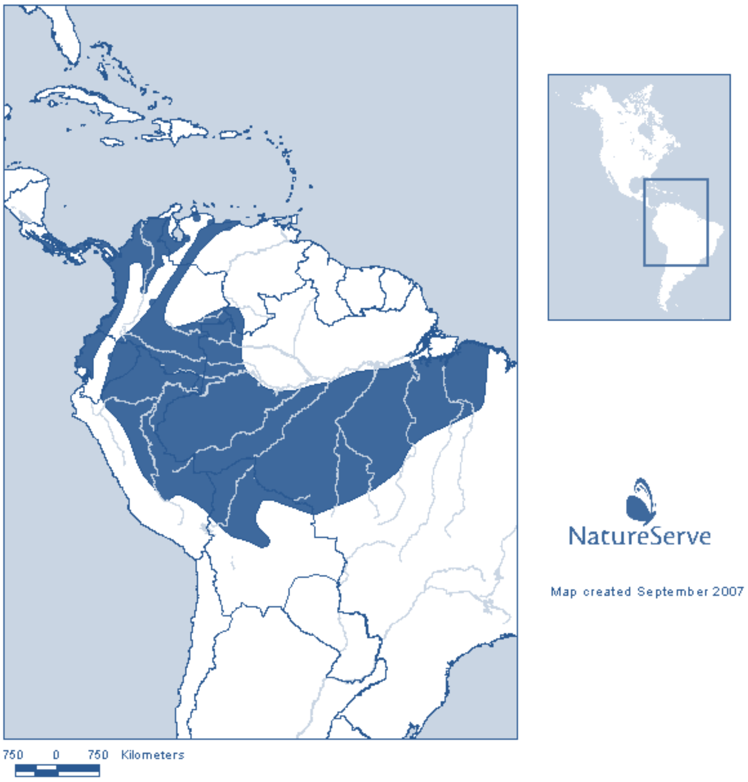

Figura 6 – Mapa da região Neotropical com área de distribuição da espécie biológica *Microcerculus marginatus* (Aves: Troglodytidae). Fonte: InfoNatura 2007.

Em *M. bambla* são reconhecidas três subespécies, as quais apresentam a seguinte distribuição (Kroodsma e Brewer 2005; Figura 7):

- *M. b. bambla* Boddaert, 1783 –extremo leste da Venezuela, Guianas e norte do Brasil (a partir do rio Branco até o Amapá).
- *M. b. albigularis* Sclater, 1858 – representada por populações isoladas a lestedo Equador, leste do Peru e noroeste do Brasil.
- *M. b. caurensis* Berlepsch e Hartert, 1902 – sul e sudeste da Venezuela e extremo leste da Colômbia (no interflúvio dos rios Solimões-Branco).

Figura 7 – Mapa da região Neotropical com área de distribuição da espécie biológica *Microcerculus bambla* (Aves: Troglodytidae). Fonte: InfoNatura 2007.

2. OBJETIVOS

O principal objetivo desse trabalho é usar dados moleculares para testar, através de uma abordagem de filogeografia estatística, as hipóteses de diversificação, incorporando informações sobre tempos relativos de divergência e realizando inferências sobre quais eventos no passado podem ter influenciado os processos de diversificação. Para tanto, dois cenários serão testados: (1) O tempo e padrões espaciais de divergência entre filogrupos co-distribuídos das espécies estudadas serão estatisticamente congruentes para as mesmas barreiras consideradas (principais rios Amazônicos e a cordilheira dos Andes); ou (2) o tempo e padrões espaciais de divergência entre filogrupos co-distribuídos das espécies estudadas não serão estatisticamente congruentes para as mesmas barreiras.

Especificamente, esse trabalho se propõe a:

1) Testar a monofilia dos táxons incluídos nas espécies biológicas *Cymbilaimus lineatus*, *Cymbilaimus sanctaemariae*, *Microcerculus bambla* e *Microcerculus marginatus*, abrangendo toda as suas respectivas áreas de distribuição;

2) Reconstruir a estrutura filogeográfica das diferentes populações naturais identificadas nas espécies estudadas, quantificando a diversidade genética, padrões e intensidade de fluxo gênico entre elas;

3) Estimar tempos de divergência entre as diferentes populações naturais identificadas nas espécies estudadas, verificando a existência ou não de congruência nos tempos e padrões espaciais de diversificação entre espécies distintas.

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Capitulo 1

Manuscrito a ser submetido para
Molecular Ecology

Diversification of a widespread lowland Neotropical avian lineage and the balance between dispersal and vicariance

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Running head: Balance between dispersal and vicariance

Abstract

We used Bayesian coalescent-based methods to infer population structure, divergence time and diversification of the *Cymbilaimus lineatus* / *sanctaemariae* species complex (Aves, Thamnophilidae) in the northern Neotropics. Our dataset consisted on sequences of two protein-coding mitochondrial genes and three autosomal nuclear loci representing all species and recognized subspecies in the complex. Our goal was to contrast the spatio-temporal diversification pattern of *Cymbilaimus* with the landscape history of South America. The monophyly of *Cymbilaimus* species was strongly supported and, within *C. lineatus*, two well supported major clades were recovered: (1) a basal lineage from the Guianan shield, and (2) a second clade grouping populations from western and southern Amazonia, and Central America. Our analyses are consistent with a recent and rapid diversification with both incomplete lineage sorting and gene flow shaping the evolutionary history of *Cymbilaimus*. The spatio-temporal pattern suggests that *Cymbilamus* originated in the northern and western portion of cis-Andean South America and then proceeded into the Brazilian Shield and Central America probably after the consolidation of the modern Amazonian drainage, the Andean range, and the Isthmus of Panama. Our results also support a scenario of evolution explained by cycles of expansion, followed by isolation during the last 0.8 Mya. We conclude that vicariance alone cannot explain the patterns we recovered, but instead that events of dispersal across important barriers such as the Andes and major Amazonian rivers might have occurred, with subsequent differentiation on opposite sides of these barriers probably enhanced by sequential founder events.

Introduction

The origin of Neotropical biodiversity is a complex subject. A modern attempt to explain it was the refuge hypothesis (originally developed as a speciation model for Palearctic birds by Stresemann 1919, but adapted for Neotropics speciation by Haffer 1969), stating that climatic and vegetational changes promoted cladogenesis between populations in lowland rain forests by cyclical glacial contractions (fragmenting) and interglacial expansions (reuniting their ranges). Support for the refuge hypothesis was based mostly on palaeoecological studies (see reviews in Burnham & Graham 1999; Haffer & Prance 2001; but see Colinvaux *et al.* 2000 for contrary evidences), whereas most phylogeographic studies have undermined its role (Ribas *et al*. 2012). In contrast, advances derived from DNA molecular phylogenetics using temporal inferences have supported the origin of Neotropical diversity as pre-Quaternary, suggesting that other hypotheses of diversification such as that the Andean uplift and associated palaeogeographic rearrangements are the most likely speciation drivers (Hoorn *et al.* 2010). Thus, currently there are two lines of thoughts: the Pleistocene-refugial hypothesis and the Neogene-orogenic hypothesis. The defenders of the latter assert that the Pleistocene should no longer be considered a time of diversification, due simply to chronological reasons, and by assuming Pleistocene speciation as refuge speciation (Rull 2015), but forgetting a variety of mechanisms that could also be assigned to this period (e.g., the riverine barrier hypothesis, the gradient hypothesis, the disturbance-vicariance hypothesis, to name some, see Antonelli *et al.* 2010).

Regardless of this debate, one would expect concordant histories for an assemblage of largely co-distributed lineages sharing a similar habitat (humid lowland forests) under both models of Pleistocene-refuge and Neogene-orogenic landscape changes. However this was not found in a recent work conducted by Smith *et al.* (2014) with more than 20 Neotropical birds lineages, which showed that idiosyncratic responses of co-distributed lineages across common barriers are the predominant pattern. Smith *et al.* (2014) also showed that even though overall diversification occurred continuously, it varied across lineages depending on the local of origin, timing of diversification, and dispersal ability. Yet, the traditional notion of areas of endemism – from which the diversification hypotheses have been built – is based on the distribution of morphologically diagnosable taxa and may not accurately reflect evolutionary relationships (Cracraft 1985, Fernandes *et al*. 2013).

Many historical biogeography studies conducted in the Neotropics have used understory birds as models because they are likely to show geographically structured populations mostly because of their poor dispersal abilities (e.g., by rivers and mountains, D'Horta *et al.* 2013; Fernandes *et al.* 2013; Batalha-Filho *et al.* 2014; Thom & Aleixo 2015). However, despite the known effect of dispersal on geographical range expansion and gene flow, empirical evidence for the relationship between dispersal ability and speciation remains scarce (Bohonak 1999; Burney & Brumfield 2009; Smith *et al.* 2014). The *Cymbilaimus lineatus* / *sanctaemariae* species complex has a wide distribution in the Neotropics ranging from Central America, west to the Andes, and throughout nearly the entire Amazon Basin. It is a relatively common insectivorous species inhabiting the mid-story canopy and forest edge (Stotz *et al.* 1996, Zimmer & Isler 2003). These characteristics make *Cymbilaimus* an uncommon model organism to investigate the influence of biogeographical barriers on population structure. Here, we present a comprehensive study on the evolutionary history, divergence time, and diversification patterns of the *C. lineatus*

/ *sanctaemariae* species complex to address the following questions: (1) what are the evolutionary relationships among the populations/subspecies of *Cymbilaimus*? (ii) what are the relationships between the time and location of cladogenetic events in *C. lineatus* / *sanctaemariae* with those related to major landscape changes in South America?

Materials and methods

Samples

We sequenced 86 *C. lineatus* / *sanctaemariae* tissue samples collected throughout the species' range, encompassing all major biogeographic areas as well as every recognized subspecies / taxa (i.e., *C. l. lineatus*, *C. l. fasciatus*, *C. l. intemedius*, and *C. sanctaemariae*; Zimmer & Isler 2003; Fig. 1). All tissues sequenced had associated voucher specimens housed at different ornithological collections (Table S1, Supporting Information). We also included representatives of the most closely related genera *Taraba*, *Hypoedaleus*, *Batara*, *Frederickena*, *Mackenziaena* and a *Thamnophilus* as outgroups (Brumfield *et al*. 2007, Moyle *et al*. 2009).

DNA extraction, amplification and sequencing

Whole genomic DNA was extracted from tissue samples following procedures described in Sambrook *et al.*(1989). Two mitochondrial genes, cytochrome b (cyt b) and NADH dehydrogenase subunit 2 (ND2); and three nuclear genes, b-fibrinogen intron 5 (bf5), glyceraldehyde 3-phosphate dehydrogenase intron 11 (g3pdh) and myoglobin intron 2 (myo) were amplified via polymerase chain reaction (PCR).

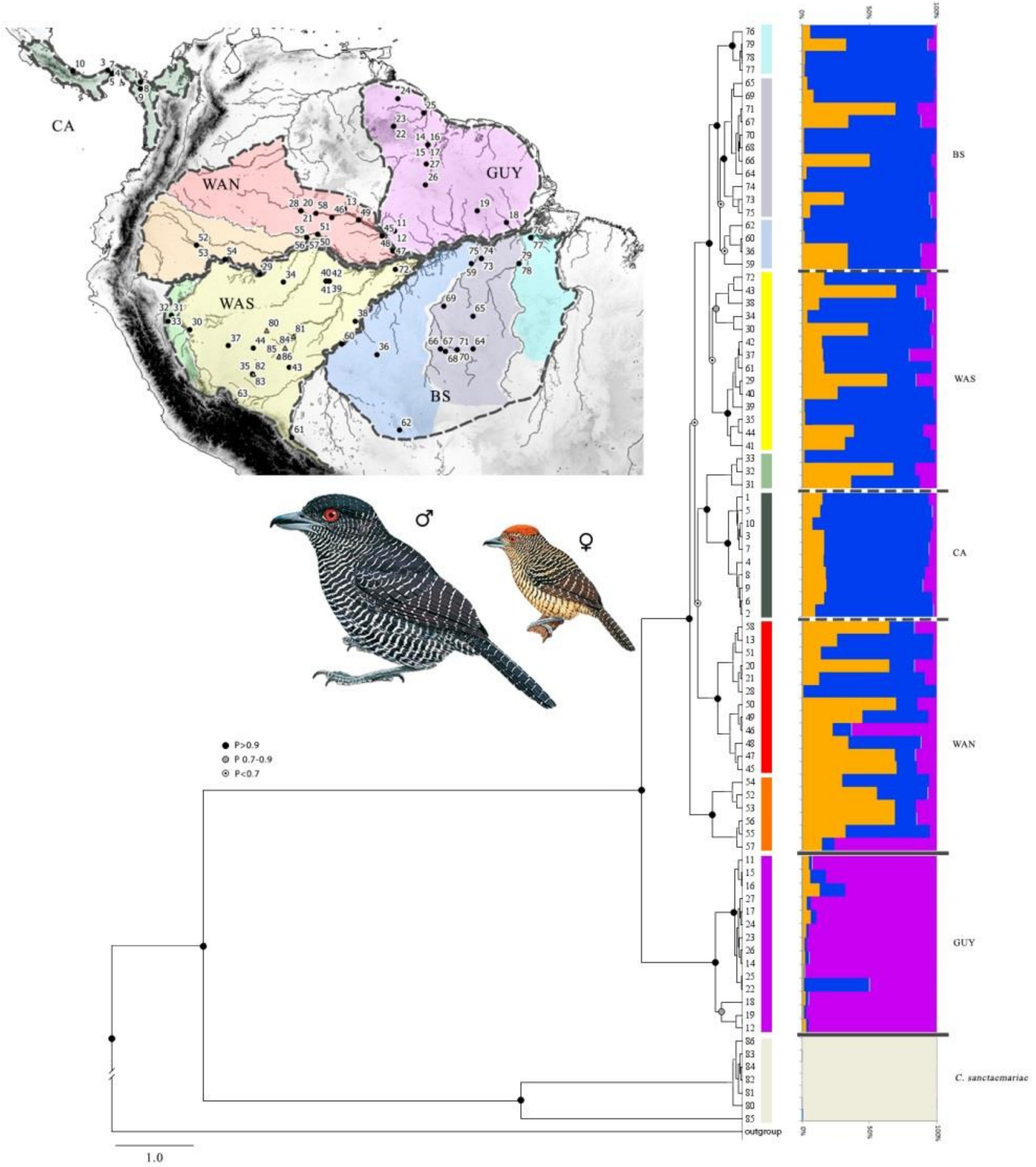
CA
WAN
WAS
GUY
BS
♂
♀
● P>0.9
● P 0.7-0.9
⊙ P<0.7
1.0
outgroup
0%
50%
100%
BS
WAS
CA
WAN
GUY
C. sanctaemariae

Fig.1 – Map showing the distribution of tissue sampling localities and their numbers corresponding to the sequences in the tree – black circles: *Cymbilaimus lineatus* and gray triangles: *C. sanctaemariae*. The gradient of gray color in the map represents the elevation gradient (the darker the higher is the altitude). Bayesian inferred maximum clade credibility mtDNA tree with posterior probabilities support on nodes (see legend). The colors on the tree match those in the map. Bar charts to the right denote STRUCTURE results obtained based on nDNA only: the most likely number of genetic groups is four (denoted as grey, purple, orange, and blue). The proportion of different colors in bar charts associated with each individual on the tree depicts the probability of membership to a particular nuclear genetic group as defined by STRUCTURE. Full and dashed lines with respective capital letters refer to major phylogroups recovered by the STRUCTURE and mtDNA tree, respectively, that show geographic correspondence to biogeography and geologic provinces: Guianan shield (GUY), Western sedimentary Amazon basin north of the Amazon River (WAN), Western sedimentary Amazon basin south of the Amazon River (WAS), the Brazilian Shield (BS) and Central America (CA). High admixture levels between two distinct nuclear groups recovered by STRUCTURE prompted the treatment of phylogroups WAN, WAS, BS and CA as a single unit based on nDNA. *Cymbilaimus* illustrations are courtesy of Lynx Edicions (Handbook of the birds of the world, Vol. 8, 2003).

Amplifications were performed in 12.5µl volumes, containing 1.25µl 10x reaction buffer, 1.5mM $MgCl_2$, 0.4mM each dNTP, 0.2µM each primer (Table S2, Supporting Information), 1 unit of taq DNA polymerase (Invitrogen) and 10 – 25 ng of genomic DNA. Thermocycling conditions for mitochondrial DNA (mtDNA) started with an initial denaturation at 95 ºC for 5 min, followed by 35 cycles consisting of: 30 s denaturation at 95 ºC, 30 s annealing at 50 ºC (cytb) or 55 ºC (ND2), and 1 min extension at 72 ºC; a final extension of 5 min at 72 ºC. Touchdown cycling parameters, differing only in annealing temperatures, was used for nuclear DNA (nDNA) with 50 °C for four cycles, 49 °C for four cycles, 48 °C for 35 cycles (for primer details, see Table S2, Supporting Information). PCR products were purified using 20% polyethylene glycol 8000 (PEG) before sequencing. For each molecular marker, sequences for both forward and reverse strands were performed in the ABI Prism BigDye Terminator Cycle sequencing protocol in an ABI PRISM 3130 XL Genetic Analyser (Applied Biosystems®). All DNA sequences generated are available on GenBank (Table S1, Supporting Information, numbers pending).

Sequence analysis

Sequences were visually inspected and aligned using ClustalW (Thompson *et al.* 1994) as implemented in BioEdit 7.0.5.3 (Hall 1999). Protein-coding mtDNA were translated into amino acids to verify the absence of stop codons or other anomalous residues. nDNA heterozygous nucleotide positions were identified by double peaks in the electropherograms and heterozygous indels positions were identified by a transition from neat to a series of double peaks in the electropherogram. Allelic phases were determined using PHASE 2.1.1 (Stephens & Scheet 2005; Stephens *et al*. 2001). Input files were produced with the online software SeqPHASE (Flot 2010). We kept the complete dataset including some low-probability calls, since PHASE has been shown to generate a very low number of false positives (Garrick *et al*. 2010), to avoid biasing estimates of population genetic parameters. Also, the three nuclear loci was check for recombination ($p > 0.05$) using the Phi test implemented in SPLITSTREE 4 (Bruen *et al*. 2006; Huson & Bryant 2006). Arlequin 3.5 (Excoffier & Lischer 2010) was used to calculate the diversity statistics and the neutrality test indexes Tajima’s D (Tajima 1989) and Fu’s Fs (Fu 1997).

Bayesian phylogenetic analyses and molecular dating

All phylogenetic analyses were conducted using BEAST (Drummond & Rambaut 2007). The mitochondrial genes (cyt b and ND2) were concatenated in a single data matrix to produce a mtDNA tree, while all nuclear genes were analyzed independently. The evolutionary models were selected with jModelTest 2.1.3 (Darriba *et al*. 2012) using the Bayesian information criterion (BIC, Posada 2008). The best fitting models incorporated as prior information in the

Bayesian analyses were summarized in Table S3, Supporting Information. We generated a mtDNA gene tree using uncorrelated lognormal relaxed clock, a UPGMA topology as a starting tree and a coalescent constant-size for the tree prior. Default priors were used except when uniform prior distribution was involved; in this case we used a lognormal prior distribution. The cyt b mutational rate of 2.1% sequence divergence per lineage per million years was applied (Weir & Schluter 2008). We ran two independent MCMCs for $2x10^8$ generations (sampling every 10^4 generations and discarding the first 10% as burn-in); and to check the analysis performance (ESS values > 200) and convergence of parameters between runs we used TRACER 1.5 (http://beast.bio.ed.ac.uk/Tracer). The maximum clade credibility trees were computed with TreeAnnotator (part of the BEAST package). The consensus tree was visualized in FigTree 1.2.3 (http://tree.bio.ed.ac.uk/software/figtree/). For the species trees reconstruction, we used both nuclear and mtDNA and the *BEAST algorithm (Heled & Drummond 2010). We unlinked substitution model parameters for each gene. We used the phylogroups that were genetically differentiated in the mtDNA tree (Fig. 1) to designate the units in *BEAST. This analysis uses the same parameter settings, except that implemented a Yule process on the species tree prior and was ran via Cipres Portal (Miller *et al.* 2010). We generated a cloudogram of the species trees with DensiTree 2.0.1 (Bouckaert 2010).

Biogeographical reconstruction

We used the R package BioGeoBEARS (Matzke 2013; http://cran. r-project.org/web/packages/BioGeoBEARS/index.html), that is designed to infer biogeographic history and model how biogeography may evolve on a phylogeny. In this inference, ancestral areas are optimized onto the internal nodes by calculating maximum likelihood of the ancestral states (range inheritance scenarios) at speciation events as a function of time. It allows model testing and model choice of many different possible models (dispersal, vicariance, founder-event speciation, among others). We defined five geographical areas for the BioGeoBEARS analysis according to major geological discontinuities and biogeographical provinces recognized within *C. lineatus* / *sanctaemariae*' s range (see Fig. 1; Aleixo & Rossetti 2007; Roddaz *et al*. 2010): Guianan shield (Guy), Western sedimentary Amazon basin north of the Amazon River (WAN), Western sedimentary Amazon basin south of the Amazon River (WAS), Central America (CA), and the Brazilian Shield (BS).

Population structure,gene flow and demographic analyses

We performed analyses using phased nDNA only, to assign individuals to populations using the Bayesian clustering program STRUCTURE 2.3.3 (Pritchard *et al*. 2000). Based on observed mtDNA phylogeographic structure, we performed two runs for values of K ranging from 1 to 14 (totalizing 7 iterations for each K). We used the admixture model, correlated allele frequencies, a 5 iterations run with burn-in period of $5x10^4$ steps and MCMC length of $4x10^5$ steps, and a 2 iterations run with a burn-in of 10^5 steps and MCMC length of $9x10^5$ steps. Different iterations of the optimal K value were combined in CLUMPP 1.1.2 (Jakobsson & Rosenberg 2007), and plotted results with individuals in the order of their appearance on the mtDNA gene tree (Fig. 1). To examine the geographic distribution relationship of the haplotypes we also constructed median-joining networks (Bandelt *et al*. 1999) using NETWORK 4.5.1.0 (www.fluxus-engineering.com).

As reconstruction methods used above lean on the assumption that no gene-flow occurred between species, we used the isolation–migration model (Hey & Nielsen 2004; Nielsen & Wakeley 2001) implemented in IMa2 (Hey 2010) to pairs of phylogroups. Units of analysis (Fig. 1) were selected according to specific phylogenetic clades with geographic correspondence (Fig.1, see the geographical areas for the BioGeoBEARS analysis). For all analyses we used the complete sequences of mtDNA and nDNA together; the HKY model (Hasegawa *et al*. 1985) was applied for all markers; an inheritance scale of 0.25 for the mtDNA and 1 for nDNA; substitution rates of the cyt b gene of 2.1% sequence divergence per million years per generation; and a generation time of one year. Several runs were performed to establish the best priors for effective population sizes, times of divergence and migration parameters. Three final runs were performed using 10^5 generations of burn-in, 10^5 trees sampled during 10^6 generations, and use of 20 chains. To test whether a model of isolation without gene flow fitted the data better than a model with gene flow we used the Nielsen & Wakeley (2001) approach and also the likelihood-ratio tests of different models implemented in IMa2's L mode.

Finally, we used the Extended Bayesian Skyline Plot method (EBSP, Heled & Drummond 2008) implemented in BEAST to analyze population size dynamics through time for both loci combined. The EBSP analysis was performed for each unit used in the IMa2 analysis. The best fit substitution model for each marker, the substitution rates, priors and the MCMC run strategy were the same as described above for others Bayesian phylogenetic analysis.

Results

Data characteristics

Fragments of 1042 and 1051 base pairs were obtained for cyt b and ND2, respectively. No indels in unexpected positions, neither stop or nonsense codons were detected in both alignments. Sequence chromatographs of the mitochondrial ND2 marker from *C. sanctaemariae* only contained double peaks indicating the presence of multiple different sequence products and possibly heteroplasmy. As heteroplasmy is yet little reported in birds and may complicate interpretations, we regarded these positions as missing data in all analyses. For nuclear markers, we obtained fragments of 598 bp for bf5, including 2 indels in a heterozygous state (ranging between 1 and 3 bp); 409 bp for g3pdh, including 6 indels in a heterozygous state (ranging between 1 and 20 bp); and 531 bp for myo, including 2 indels in a heterozygous state (ranging between 1 and 2 bp). We found no evidence of recombination (data not shown). The diversity statistics and the neutrality test indexes Tajima's D (Tajima 1989) and Fu's Fs (Fu 1997) are shown in Table 1.

Table 1 – Descriptive statistics and neutrality tests for each marker separated by lineage. (N) number of phased alleles; (s) number of segregating sites; (k) average number of nucleotide differences; (π) nucleotide diversity; (h), number of haplotypes; (Hd) haplotype diversity; (θ) diversity parameter per sequence [95% confidence interval]; (Fs) Fu's Fs (Fu, 1997); (D) Tajima's D test (Tajima,1989); (*) Significance levels for Tajima's D and Fu's Fs $P < 0.05$

Lineage / Locus	**position**	**bp**	**N**	**s**	**k**	**π**	**#haps**	**Hd**	**θ [95% CI]**	**Fs**	**D**
C. sanctaemariae											
cytb	mtDNA	1042	7	104	30,095	0.029	4	0.714	42.449 [8.975 - 66.938]	6.840	**-1.700***
nd2	mtDNA	1051	5	-	-	-	1	-	-	-	-
bf5	ch 4	598	10	25	7,400	0.001	10	1,000	8.837 [2.121 - 16.260]	**-4.431***	-0.774
g3pdh	ch 1	409	14	6	1,835	0.006	5	0.780	1.886 [0.314 - 4.402]	-0.137	-0.098
myo	ch 1	531	16	5	2,041	0.004	6	0.817	1.506 [0.301 - 4.520]	-0.634	1,169

Table 1 (cont.)

Lineage / Locus	**position**	**bp**	**N**	**s**	**k**	**π**	**#haps**	**Hd**	**θ [95% CI]**	**Fs**	**D**
Guyana											
cytb	mtDNA	1042	12	13	3,121	0.004	4	0.561	4.304 [0.662 - 6.953]	2,096	-1.164
nd2	mtDNA	1051	12	8	1,848	0.003	6	0.758	2.649 [0.331 - 4.635]	-1.540	-1.206
bf5	ch 4	598	20	7	1,358	0.002	8	0.795	1.973 [0.281 - 3.100]]	-3.680	-1.025
g3pdh	ch 1	409	16	9	1,650	0.005	9	0.767	3.013 [0.301 - 3.917]	**-5.075***	**-1.687***
myo	ch 1	531	26	7	1,929	0.003	7	0.735	2.096 [0.524 - 4.192]	-0.808	-0.251
WAN											
cytb	mtDNA	1042	20	35	9,889	0.009	13	0.953	9.865 [3.946 - 18.885]	-0.962	0.009
nd2	mtDNA	1051	19	26	6,479	0.007	16	0.982	7.438 [2.288 - 12.875]	**-7.260***	-0.506
bf5	ch 4	598	30	17	1,492	0.002	13	0.749	4.291 [0.252 - 3.281]	**-9.144***	**-2.231***
g3pdh	ch 1	409	38	1	0.053	0.0001	2	0.053	0.238 [2.142 - 9.758]	-1.407	-1.128
myo	ch 1	531	36	3	0.219	0.0004	4	0.211	0.723 [0.0 - 0.723]	**-3.010***	**-1.561***
CA											
cytb	mtDNA	1042	9	9	3,666	0.003	6	0.889	3.311 [0.735 - 8.462]	-0.697	0.493
nd2	mtDNA	1051	9	1	0.222	0.0002	2	0.222	0.367 [0.0 - 1.103]	-0.263	-1.088
bf5	ch 4	598	16	5	0.733	0.001	4	0.442	1.506 [0.0 - 2.109]	-0.943	**-1.691***
g3pdh	ch 1	409	18	1	0.294	0.0007	2	0.294	0.291 [0.0 - 1.162]	0.463	0.022
myo	ch 1	531	20	3	1,068	0.002	5	0.758	0.845 [0.0 - 2.536]	-0.945	0.703
WAS											
cytb	mtDNA	1042	10	21	5,933	0.005	10	1,000	7.423 [1.767 - 13.079]	**-5.231***	-0.946
nd2	mtDNA	1051	11	18	4,545	0.004	8	0.927	6.145 [1.365 - 9.901]	-1.724	-1.171
bf5	ch 4	598	14	1	0.439	0.0007	2	0.440	0.314 [0.0 - 1.572]	0.944	0.842
g3pdh	ch 1	409	24	4	0.409	0.001	4	0.308	1.071 [0.0 - 1.339]	**-1.854***	**-1.689***
myo	ch 1	531	24	5	0.492	0.001	5	0.377	1.338 [0.0 - 1.339]	**-2.799***	**-1.831***
BS											
cytb	mtDNA	1042	20	27	4,931	0.005	13	0.916	7.610 [1.691 - 10.147]	**-3.844***	-1.370
nd2	mtDNA	1051	17	11	2,367	0.005	11	0.904	3.253 [0.591 - 5.028]	**-6.111***	-1.010
bf5	ch 4	598	32	5	0.764	0.001	5	0.601	1.241 [0.0 - 1.986]	-1.270	-1.032
g3pdh	ch 1	409	34	2	0.117	0.0004	3	0.116	0.489 [0.0 - 0.489]	**-2.516***	**-1.499***
myo	ch 1	531	36	2	0.163	0.0003	3	0.110	0.482 [0.0 - 0.723]	**-1.839***	**-1.284***

Phylogenetic analyses based on mtDNA and the species tree

The Bayesian inferred phylogenetic analyses of the mitochondrial dataset strongly support the monophyly of the genus *Cymbilaimus*. Two clades were observed in the phylogeny: *C. sanctaemariae* and *C. lineatus*. Within *C. lineatus*, two well supported major clades were recognized. A basal lineage consisted of the *C. lineatus* population from the Guiana shield, while a second lineage included *C. lineatus* populations from western and southern Amazonia, and Central America (Fig. 1). Some relationships within lineages of this latter clade, which coincide with known Amazonian areas of endemism, were poorly resolved; nevertheless some nodes showed strong support, as follows: (1) populations coinciding with the Napo and Imeri lineages consisted well supported monophyletic lineages, which are treated together (see STRUCTURE results) as the Western Amazonian phylogroup (WAN); (2) a clade of southern Amazon phylogroups including individuals from Xingu, Tapajos and Rondonia areas of endemism (called the Brazilian Shield-BS phylogroup), which is sister to a paraphyletic Inambari area group (called the southern Western sedimentary Amazon basin clade - WAS); and (3) the Central American phylogroup (CA). Except for the WAS phylogroup, whose individuals from the western part of Inambari area of endemism (hereafter called the Andes foothill lineage) group as sister to CA; and the WAN phylogroup, whose internal relationships are poorly supported, the remaining phylogroups (GUY and CA) were found to be monophyletic in the mitochondrial tree (Fig. 1). In fact, mitochondrial phylogroups are bounded by known physiographic barriers, such as the Andes, and the Madeira, Negro, and Amazon/Solimões rivers.

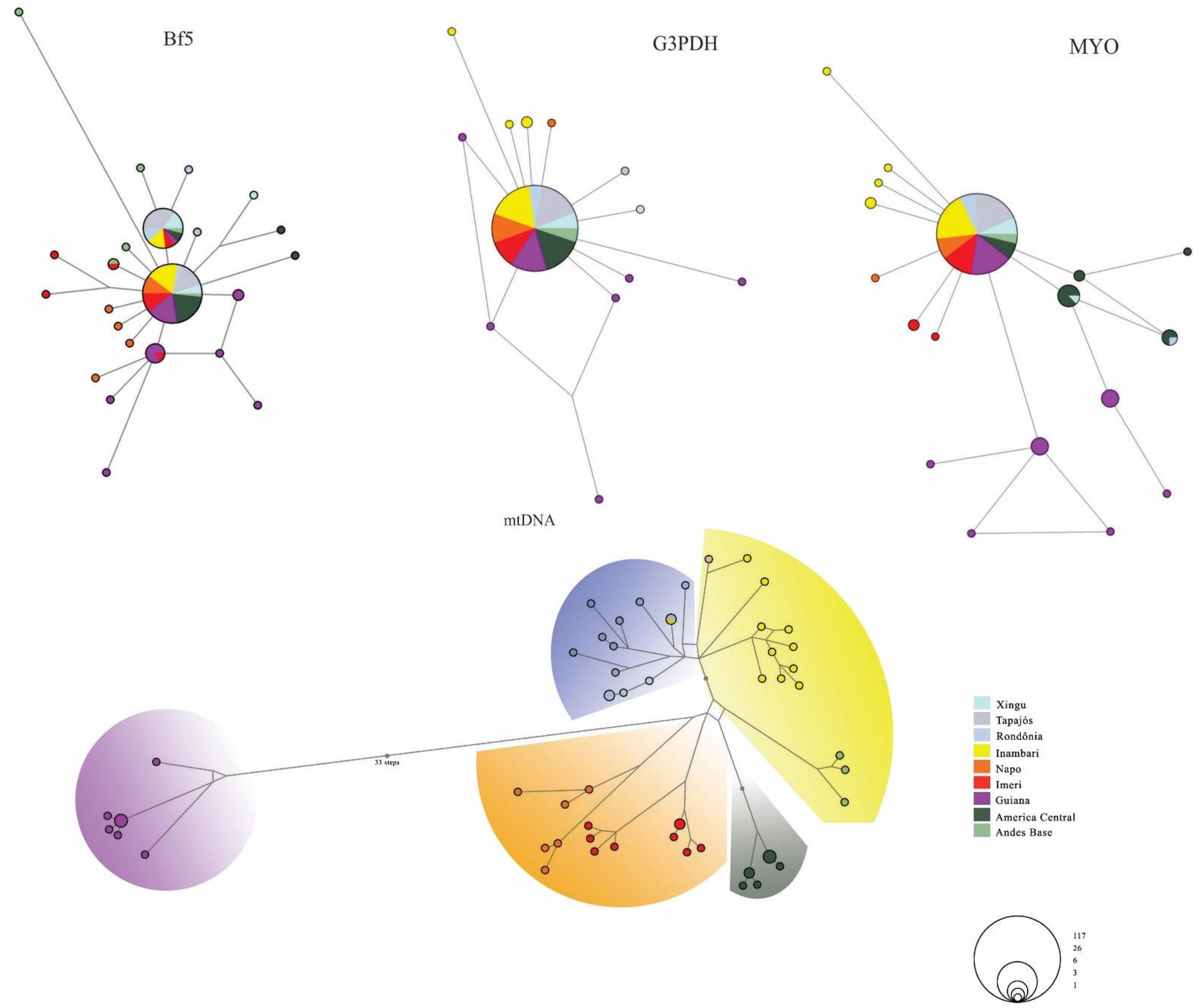

Fig. 2 – *Cymbilaimus lineatus* median-join haplotype networks for the different genes sequenced. For each marker, *C. sanctaemariae* haplotypes (not shown) were separated by more mutational steps from those of *C. lineatus* than any found within the latter species. Colors in the network correspond to each of the well-supported lineages / phylogroups according to their biogeographical provinces (see legend). The size of each circle represents the number of individuals sharing that haplotype (see legend at bottom right).

Population structure

STRUCTURE analyses based on nDNA recovered four clusters that match allocation of individuals to *C. sanctaemariae, C. lineatus* from the Guianan shield and the remaining *C. lineatus* populations, irrespective of the number of K used (data not shown). Even though results from STRUCTURE under the admixture ancestry model with correlated allele frequencies identified 4 genetic groups in the data set (Fig. 1, LnPr(X|K) supported a value of K = 4), the software identified *C. lineatus* populations from western and southern Amazonia, and those in Central America as two clusters with high admixture levels. For *C. sanctaemariae* and *C. lineatus* from the Guiana shield most individuals showed high membership probability to a cluster (Fig. 1).

The network of *C. lineatus* mtDNA resulted in lineages congruent with the phylogenetic analysis in Fig. 1. On the other hand, haplotype networks based on each of the three nuclear markers showed overall absence of geographic structure. The commonest and internal haplotypes were found in several geographically distant locations (Fig. 2).

Divergence times and ancestral distribution

The divergence times in *Cymbilaimus* revealed an initial divergence of *C. sanctaemariae* and *C. lineatus* (~0.77 Mya, Fig. 3) and the radiation within *C. lineatus*, with the splitting of the Guiana shield population from the remaining phylogroups of *C. lineatus* (~0.48 Mya, Fig. 3) occurring during the late Pleistocene. The split between the WAN phylogroup and those south of the Amazon (WAS and BS) was ~0.39 Mya, whereas that between these two latters groups was only ~0.18 Mya (Fig. 3).

The most likely ancestral area reconstruction with respective node pie charts likelihoods are given in Fig. 3. The best statistical fit to the data was DIVALIKE+J (ln L = -12.3). Almost all the recent evolutionary history of *Cymbilaimus* can be explained through dispersal, but vicariant events are more likely for the first splits (see legend Fig.3). The most likely center of origin for *Cymbilaimus* recovered in this biogeographic reconstruction was a widely distributed ancestral population inhabiting western and northern of Amazonia. Our analyses favored a vicariant event for the split between *C. sanctaemariae* and the ancestor of *C. lineatus*, as well as for the split between Guianan shield and all remaining populations of *C. lineatus* during the late Pleistocene. The ancestor of the remaining *C. lineatus* phylogroups inhabited northwestern Amazonia (WAN area), from which it colonized Amazonia south of the Amazon River (WAS and BS). Another putative splitting event involved the dispersal (or range expansion) of *C. lineatus* from WAS toward the CA. Nevertheless, this ancestral area reconstruction should be interpreted with caution, as some associated nodes were poorly resolved in our phylogenetic reconstructions (Fig. 1, Fig. 3, Fig. S1, Supporting Information).

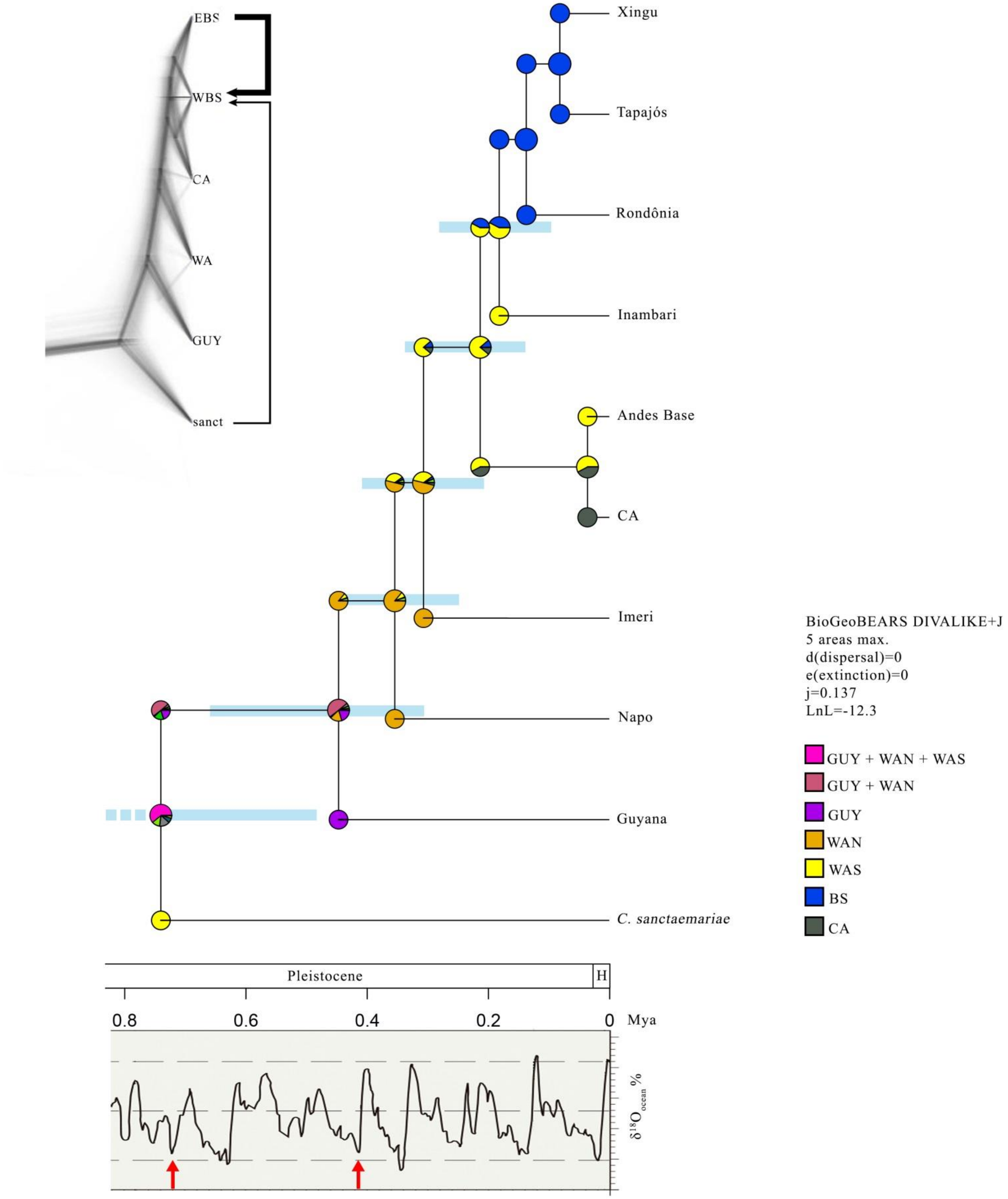
EBS
WBS
CA
WA
GUY
sanct
Xingu
Tapajós
Rondônia
Inambari
Andes Base
CA
Imeri
Napo
Guyana
C. sanctaemariae
BioGeoBEARS DIVALIKE+J
5 areas max.
d(dispersal)=0
e(extinction)=0
j=0.137
LnL=-12.3
GUY + WAN + WAS
GUY + WAN
GUY
WAN
WAS
BS
CA
Pleistocene
H
0.8
0.6
0.4
0.2
0
Mya
δ18Oocean %

Fig. 3 – Chronogram with divergence times of the *Cymbilaimus lineatus* / *sanctaemariae* species complex. Tree topologies were estimated with *BEAST species tree analysis based on all markers. Bars on the nodes represent 95% of high posterior density of divergence times. Pie charts at node represent probabilities of the ancestral distributions from BioGEoBEARS, with areas coded by colors in the pie chart (legend on the right). Quaternary climate curve derived from δ ^{18}O (modified from Head & Gibbard 2005) and scaled against the divergence time analysis. Red arrows correspond to major glacial cycles possibly related to vicariant events.

Gene flow and demography

In all IMa2 runs, the posterior distributions of parameters had a clear peak and the right tails converged on zero (Fig. S2, Supporting Information). IMa2 analyses indicated significant unidirectional and paraphyletic gene flow from *C. sanctamariae* into the WAS phylogroup, with posterior distributions peaking at 0.1 migrants per genereation, with the 95% highest posterior density (HPD) interval including zero (95% HPD: 0.0–0.4, Table 2, Fig. S2A, Supporting Information). Furthermore, the WAS phylogroup receives migrants from BS on an average of 5 individuals per generation (95% HPD: 0.296 - 20.07, Table 2, Fig. S2B, Supporting Information) – the highest migration rate detected and the only one not including a lower bond likelihood of zero. Both rates are significantly different from zero according to the likelihood ratio test of Nielsen & Wakeley (2001). All others species pairwise comparisons revealed no significant gene flow (Table 2, Fig. S2C and D, Supporting Information). IMa2 showed similar effective population sizes (Ne) for all phylogroups of *C. lineatus*, except for the CA, which shows the smaller one; and for *C. sanctaemariae*, with larger values (Table 2, Fig. S2E, Supporting Information). The posterior distributions of ancestral populations showed small sizes for all phylogroups (Fig. S2F, Supporting Information).

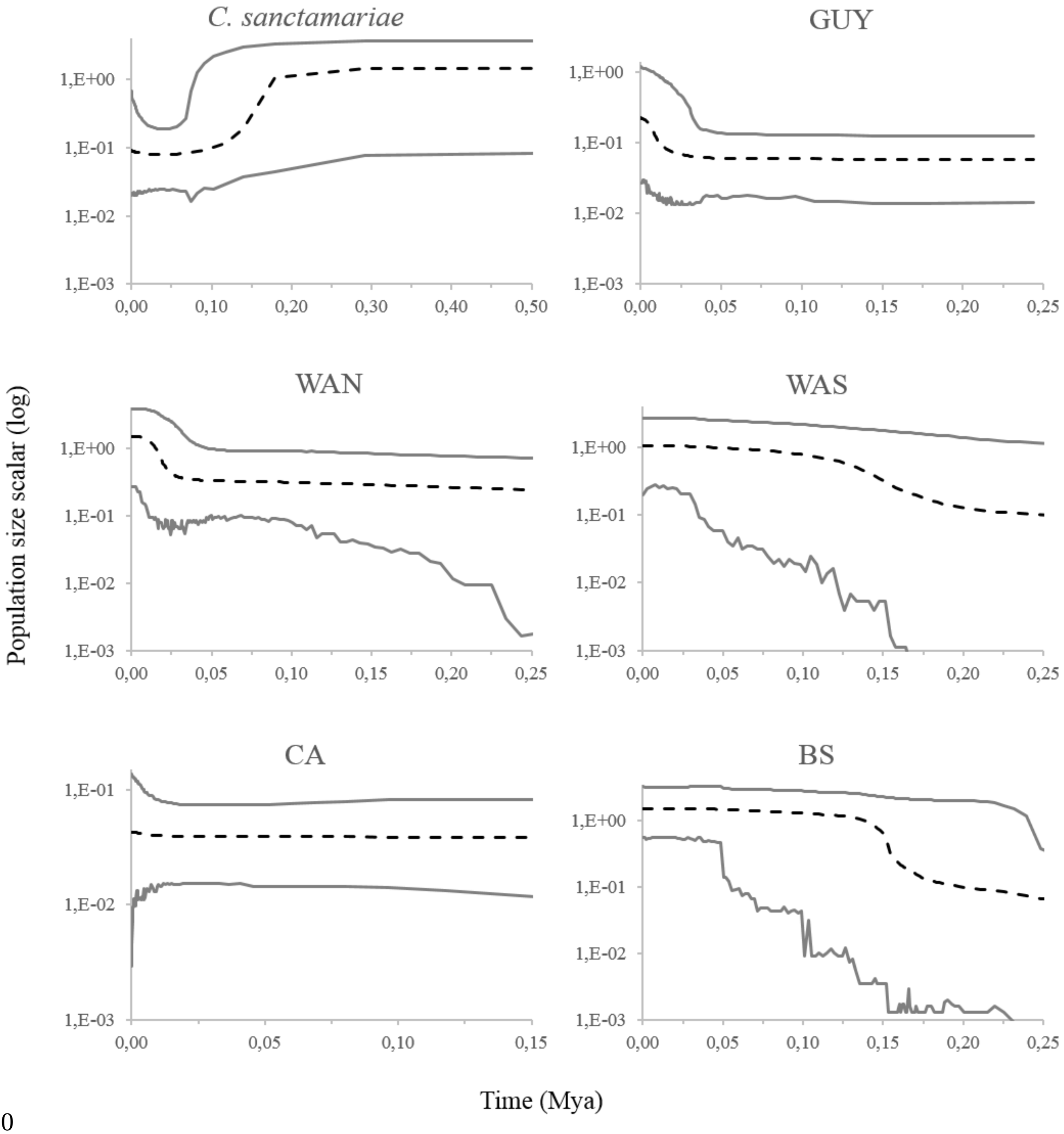

Fig. 4 – Demographic histories of major phylogroups of the *Cymbilaimus lineatus* / *sanctaemariae* species complex inferred through Extended Bayesian Skyline Plots based on all markers. Black solid lines represent median values, while the gray area corresponds to 95% confidence intervals. The X axis corresponds to time in million years before present while the Y axis represents log scaled *Ne*.

The Extended Bayesian Skyline Plots (EBSPs) estimated for the *C. sanctaemariae* and *C. lineatus* phylogroups agree with the neutrality tests in inferring histories of demographic fluctuations during the Late Pleistocene. Tajima's D and Fu's Fs were negative for most loci but were significant only for nDNA markers (Table 1), indicating signs of demographic expansion. *C. sanctaemariae* is inferred to have maintained a relatively high and stable population size during the Pleistocene, followed by a sudden decline during the last 0.10 Mya, despite large confidence intervals (Fig. 4). Other two phylogroups, GUY and WAN, also appeared to have maintained stable population sizes during the Pleistocene, but with small sizes and a slight expansion during the last 0.10 Mya (Fig. 4). A simultaneous, significant and sudden population expansion was inferred for those phylogropus south of the Amazon around 0.15 Mya (Fig. 4). The CA phylogroup is inferred to have maintained a very small and stable population size during the last 0.15 Mya (Fig. 4).

Discussion

Incomplete lineage sorting, gene flow and divergence date estimates

Incomplete lineage sorting (ILS), wherein ancestral polymorphisms persist through species divergence and gene flow across species limits can generate impacts on phylogenetic inferences (Leaché *et al*. 2014). First of all, it is important to distinguish shared ancestral variation from gene flow once both scenarios produce similar patterns. On the one side, ILS can be accommodated in coalescent approaches such as that implemented in BEAST (Heled & Drummond 2010), but this approach does not model gene flow. In fact, the impacts of gene flow on phylogenetic inference remain little studied (Pinho & Hey 2010), even less methods that can jointly consider ILS and gene flow. Since here we explicitly test for gene flow and it was verified an asymmetric gene flow involving just two out seven pairs of populations – i.e., from *C. sanctaemariae* into *C. lineatus* WAS phylogroup (less than 1 migrant / generation) and from *C. lineatus* phylogroups BS to WAS (more than 2 migrants / generation, Table 2); our study shows that *C. lineatus* phylogroups WAN, WAS, BS and CA probably have not completed the lineage sorting process in their autosomes. Additional lines of evidence in this direction also include the rapid radiation, short internal branches in the phylogenetic analyses, and the high degree of shared polymorphic sites and haplotypes among these groups (Figs. 1 and 2). Based on predictions from coalescence theory, it is necessary about four to seven *Ne* generations to complete the lineage sorting of autosomal genes, which means that species with a large population size could be even more affected by ILS (Nichols 2001). Furthermore, as evidenced by the EBSP analysis, the retention of ancestral polymorphism is seemingly associated with recent and rapid demographic expansion. Thus, considering intraspecific variation, here we demonstrate that both ILS and limited gene flow have shaped the evolutionary history of *Cymbilaimus* phylogroups.

It has been explicitly demonstrated the impact of gene flow on species tree inferences (e.g. by decreasing posterior clade probabilities and underestimating divergence time estimates; Leaché *et al*. 2014). Our divergence date estimates among phylogroups according to the *BEAST species tree were not consistent with those inferred with the IM model, with those estimated by the latter being around 3 times older than those obtained by the former (Fig. S2G, see also Table S4 for comparisons, Supporting Information). We note that our *BEAST estimates may underestimate actual divergence times, since we observed a paraphyletic gene flow from *C. sanctaemariae* into WAS phylogroup and from BS into WAS. Notwithstanding, we kept the *BEAST divergence time inferences for scenario reconstructions, since they are apparently more trustworthy inasmuch as the effects of this pattern of gene flow are predictable and do not alter drastically the tempo and mode of diversification. Furthermore, it was recently reported by simulations data (Strasburg & Rieseberg 2010) and in terms of the probability of genealogies (Sousa *et al*. 2011) that the divergence time estimated under a model with migration (IM) is statistically unreliable as the different migration timings could show misleading genealogies with the same probability. In fact, it is noteworthy to remind that more complex evolutionary scenarios are more poorly described in a tree-like manner; so all these inferences should be interpreted with caution for various reasons. On the other hand, regardless of the exact timing and even considering the confidence intervals, splitting times converge on the Pleistocene, an epoch characterized by changes in habitat characteristics – such as habitat fragmentation – promoted by climatic fluctuations, and resulting in population differentiation.

A scenario for the evolution of Cymbilaimus *phylogroups*

Having highlighted the mechanisms that played a role in the diversification process, we can now trace the scenario. Our results provide support for diversification for *Cymbilaimus* occurring between 0.8 and 0.1 Mya (Fig. 3), and for patterns of speciation being strongly associated with environmental changes, probably due Pleistocene climatic fluctuations – as the main splits apparently coincided with glacial maxima following the Quaternary climate curve derived from $\delta\ ^{18}O$ (modified from Head & Gibbard 2005 and used as a phylogenetic test of the refuge hypothesis, Ribas *et al*. 2012). And even though some relationships within mtDNA lineages were poorly resolved, the majority of the lineages was separated by major rivers, highlighting the role of rivers as barriers - even if secondary, depending on the dispersal abilities of the group as will be discussed later (Burney & Brumfield 2009).

From this perspective, our analyses suggested a first vicariant event for the split between *C. sanctaemariae* and the ancestral of *C. lineatus* occurring around 0.77 Mya. The estimated age indicates that this first split into two lineages north and south of the present-day Amazon River is highly associated with a major glacial cycle (Fig. 3). Yet, it is important to mention *C. sanctaemariae* is a strict bamboo specialist (Pierpont & Fitzpatrick 1983) and – as evidenced by a recent thorny bamboo similar to *Guadua* macrofossil found in Peru (Olivier *et al*. 2009) – it has been suggested the presence of bamboo in Amazonia since at least the Pleistocene.

Another vicariant event was the split of *C. lineatus* populations from Guiana and the ancestor of all remaining phylogroups – which probably inhabited the WAN area – occurring about approximately 0.48 Mya, and that also overlapped in time with a major glacial cycle (Fig. 3). The interfluve delimited by the upper Negro and Orinoco rivers and the Branco River is occupied by the largest patches of savannas and white-sand forests (campinas and campinaranas) known in Amazonia, which may have expanded during the cooler period, hence isolating areas to the east and west and functioning as a barrier (Naka *et al*. 2012). Secondly, the modern Branco River originated from a recent Pleistocene (ca. 1 Ma) capture of a previous ancient proto-basin that flowed north into the Essequibo River into the Caribbean (Schaefer & Vale 1997). Furthermore, there is strong evidence that the Negro River’s mouth migrated from west to east (~150 km of its current position, Almeida-Filho & Miranda 2007) as a result of tectonic activity, with an estimated age of about 0.4 Mya for the initial sedimentation of the lower Negro course (Soares *et al*. 2010). All these evidences predated the estimated age for the split and are therefore consistent with the hypothesis of having influenced it. This also could explain the stronger "barrier effect" of the lower part of the Negro river in contrast to its middle and upper parts, as indicated by the fact that populations from the Negro-Branco interfluvium (locality 13) belong to the WAN rather than the Guianan shield phylogroup. This patterns contrasts with those recovered by many studies whereby the Negro-Branco interfluvium as part of the Guiana area of endemism (e.g., Ribas *et al*., 2012), whereas the role of the Branco River as barrier has been less investigated (but see Naka *et al*. 2012, Boubli *et al*. 2014).

Our data also support a pattern of sequential dispersal episodes for the occupancy of the south bank of the Amazon River and the Brazilian Shield. The distribution of the ancestor of the remaining *C. lineatus* phylogroups (WAS and BS) was inferred to be in northwestern Amazonia (WAN), following a dispersal movement south and eastward between 0.39 and 0.18 Mya (Fig. 3), and which promoted the secondary contact between different non-sister lineages that diverged in allopatry, the WAS *C. lineatus* phylogroup and *C. sanctaemariae*. Despite the detected phylogeographic structure recovered for both WAS and BS phylogroups, the high degree of gene flow between them suggests that distinct geological and/or climatic events could have led to multiple dispersal and vicariance events between these phylogroups. One possible explanation for the mixing of sister populations separated by rivers, such as the WAS and BS phylogroups, is tectonically mediated drainage capture as well as megafan formation (Wilkinson *et al*. 2010) – as documented for the Madeira (Hayakawa & Rossetti 2015), and Tocantins (Rossetti & Valeriano 2007) river basins. At the same time, the borders of Amazonia may have experienced repeated contractions and expansions during glacial and interglacial periods, respectively, as suggested by the river refuge hypothesis (Haffer 1993). A population expansion was indicated by the Extended Bayesian Skyline plots for both the WAS and BS phylogroups occurring around 0.15 Mya (Fig. 4), suggesting the influence of a glacial cycle on population dynamics (Head & Gibbard 2005). When they expanded their ranges, the phylogroups became secondarily in contact but, even with gene flow, may have kept on diverging due to the barrier effect of the Madeira River. Interestingly, our estimates of gene flow are significant only westward (from BS to WAS; Table 2), which is consistent with the migration of the lower channel of the Madeira River eastward (Hayakawa & Rossetti 2015), therefore apparently transferring in a relative short period of time entire populations of the BS phylogroup from the eastern to the western bank of the barrier, where they entered in direct contact with members of the WAS phylogroup. The presence of phylogroups west of the lower Madeira River that are nevertheless more closely related to those occurring across the river rather than any co-distributed phylogroups on the west bank, has been reported for at least two other avian lineages associated with the same habitat as *Cymbilaimus* (upland terra-firme forest; Aleixo 2004, Patané *et al*. 2009) and may reflect a community wide response to the shifting of the lower Madeira River eastward. .

Finally, our estimates involved the dispersal / range expansion of the *C. lineatus* from WAS towards CA dating ca. 0.15 Mya (Fig. 3). Studies of some avian lineages have identified a widely range for the estimated time of speciation across the Andes (Smith *et al*. 2014). Divergence times estimated for the separation between cis- and trans-Andean lineages of lowland lineages ranged from the late Miocene and early Pliocene (*Pyrilia* [6.84–7.06 Mya, Ribas *et al*. 2005] and *Sclerurus* [3.69–6.68 Mya, d'Horta *et al*. 2013]), or late Pliocene (*Brotogeris* [ca. 3.0

Mya, Ribas *et al*. 2009], *Ramphastos* [2.60–4.15 Mya, Patané *et al*. 2009] and *Pteroglossus torquatus* species complex [2.05–3.28 Mya, Patel *et al*. 2011]), to the late Pleistocene (*Pionus* [0.34–1.41 Ma; Ribas *et al*. 2007]). In fact, the Andes apparently does not serve as a strong barrier even to the dispersal of small passerines inhabiting the forest understory, such as *Mionectes* (Miller *et al*. 2008), and therefore would be an even more permeable barrier to canopy birds such as *Cymbilaimus* (Burney & Brumfield 2009). In contrast to all previous studies whereby trans-Andean lineages are sister to those north of the Amazon River, in *C. lineatus* the trans-Andean clade shows a sister relationship to another clade occurring south of that river – a pattern just recently found in the *Hylophylax naevius* / *naevioides* species complex (Fernandes *et al*. 2014). This could be explained by a dispersal event across the central Andes, perhaps across the Marañon valley in northern Peru, which has historically consisted of a barrier to the dispersal of humid forest species during glacial maxima but acting like a corridor connecting both sides of the Andean cordillera for these species during interglacials (see Winger & Bates 2015).

Ecology and dispersal ability in the diversification process

Effective population sizes and, consequently, the patterns of gene flow among such populations are influenced by the ecology of a species (e.g., Bohonak 1999). For example, species with stringent habitat requirements may have a low ability for moving individuals between populations (hence reducing gene flow), but such difficulty can be surpassed with the increase of the dispersal ability (Phillipsen *et al.* 2015). Despite limitations in gathering data at population level for a large numbers of species (see Burney & Brumfield 2009), one link between the evolution of a species and ecology is the relationship among levels of population structure and dispersal ability – which are usually inferred through species attributes associated with habitat, diet, and relative abundance. Herein, we demonstrate a rapid radiation for *Cymbilaimus* during the last 0.8 My, which proceeded with some degree of gene flow and incomplete lineage sorting. These birds inhabit the mid-story canopy and forest edges, attributes associated with a better ability to cross habitat gaps, hence functioning as an indirect measure of dispersal ability (Stotz *et al*. 1996, Zimmer & Isler 2003). Given this timing of diversification and in accordance with Smith *et al.* (2014), landscape change (a basic assumption of most diversification hypotheses) is not a requirement for cladogenesis and speciation among geographically separated populations. Following those authors, the "strongest predictors of speciation are the amount of time a lineage has persisted in the landscape and the ability of birds to move through the landscape matrix" (Smith *et al.* 2014).

In *Cymbilaimus*, landscape change and the appearance and shifting of physical barriers account for two splits, whereas cladogenesis resulting from dispersal followed by founder events were favored for other five nodes (Fig. 3), indicating that a balance between vicariance and genetic differentiation resulting from dispersal and founder events across pre-existing barriers together explain the mode of diversification in this lineage. Furthermore, landscape change cannot only be associated with vicariance, but also with promotion of admixture, as inferred for the asymmetric gene flow documented between the phylogroups BS and WAS in *C. lineatus* (Table 2), which probably resulted from the shifting of the lower Madeira River during the 0.18 Mya (Hayakawa & Rossetti 2015).

Phylogeographic structure and taxonomy

Two species of *Cymbilaimus* and three subspecies in *C. lineatus* are currently recognized on the basis of bioacoustical, morphological and morphometric data (Zimmer & Isler 2003), although the phenotypic diagnosis of *C. lineatus* subspecies is not straightforward and it is mostly based on the barring throughout the body. Genetically, our multi-locus analysis supported the existence of three major groups in *Cymbilaimus*: *C. sanctamariae*, *C. lineatus lineatus* (nominate *lineatus* is the taxon name applied to the Guianan shield phylogroup; Peters 1951, Zimmer & Isler 2003) and a third *C. lineatus* unity which groups all remaining phylogroups (WAN, WAS, BS and CA), including populations to which the taxa names *fasciatus* and *intermedius* apply (Fig. 1). Despite the existence of eight reciprocally monophyletic lineages in the latter group according the the mtDNA genes, analyses based only on the nuclear genes indicated lack of structuring among these groups (Fig. 1), which could be explained either by lack of lineage sorting or asymmetrical gene flow between mtDNA and nDNA genes or both factors (Tajima 1983, Madson & Knowles 2006, Pinho & Hey 2010). When pairwise rates of gene flow are estimated among the WAN, WAS, BS and CA phylogroups in *C. lineatus*, only one (involving the parapatric WAS and BS phylogroups) recovered significant values (and a high migration rate as well; Table 2). Hence, evidence indicates that ancestral polymorphism rather than gene flow accounts for most of the comparatively lower differentiation in the nDNA than the mtDNA in *Cymbilaimus*, which is consistent with the notion that at least the CA, WAN, and WAS+BS phylogroups are diverging from each other with little gene flow between them. However, our sampling is too sparse to document more localized and potentially high rates of gene flow among the *Cymbilaimus* phylogroups uncovered by this study. Therefore, under the biological species concept, at least three species could be recognized in *Cymbilaimus*: C. *sanctaemariae*, *C. lineatus*, and C. *fasciatus*, following the International Code for Zoological Nomenclature (ICZN 1999), which states the valid name of a taxon is the oldest available name applied to it. Interestingly, gene flow still takes place in western Amazonia between C. *sanctaemariae* and *C. fasciatus* (WAS clade), even though at a very low rate (Table 2), which is nevertheless surprising considering the high degree of vocal, ecological, and genetic differentiation already acquired by these lineages (Zimmer & Isler 2003). This finding supports the notion that complete reproductive isolation takes a very long time to evolve and that it represents more of an ancestral character that is lost only after two groups have evolved independently for a long time (Zink 2004). Thus, within *C. fasciatus*, more limited evidence support the evolutionary independence of phylogroups CA, WAN, and WAS+BS, and hence their status as phylogenetic or evolutionary species (de Queiroz 2007). From a taxonomic standpoint, the name *intermedius* applies to the WAS+BS phylogroups, which are connected by a high gene flow rate (Table 2), but no existing names relate specifically to the evolutionary independent WAN phylogroup, which makes difficult the immediate implementation of an alternative taxonomic treatment based on the evolutionary species concept. Regardless of alternative taxonomic treatments, evidence exist for splitting the former polytypic *C. lineatus* into four major evolutionary units (Guiana, CA, WAN, and WAS+BS phylogroups), which should be considered basal units in both biogeographic studies and conservation assessments.

Conclusions

Several studies have attributed an important role to the formation of the Amazon drainage and uplift of the Andes in accounting for patterns of diversification in several humid forest avian lineages (e.g., Aleixo & Rossetti 2007, Ribas *et al*. 2012, d' Horta *et al*. 2013), and in *Cymbilaimus* the Andes and Amazonian rivers also coincide with major phylogeographic breaks. However, the timing of the *Cymbilaimus* radiation in the northern Neotropics (0.8–0.1 Mya) is at odds with most time estimates for the origin of the modern Amazon drainage (i.e., at ca. 2.5 Mya or older; Figueiredo *et al*. 2009; Campbell *et al*. 2006, Latrubesse *et al*. 2010, but see Rossetti *et al*. 2005) and establishment of the central and northern Andes (Gregory-Wodzicki 2000). If these time estimates are all correct, the relatively recent splits of *Cymbilaimus* phylogroups across these relatively old barriers are explained by dispersal following vicariance and were probably mediated by climate change, which may have created dispersal corridors at different time frames and between different populations. In this respect, our results contribute to a growing number of studies favoring dispersal events as the initiators of geographical isolation and speciation.

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Supporting Information

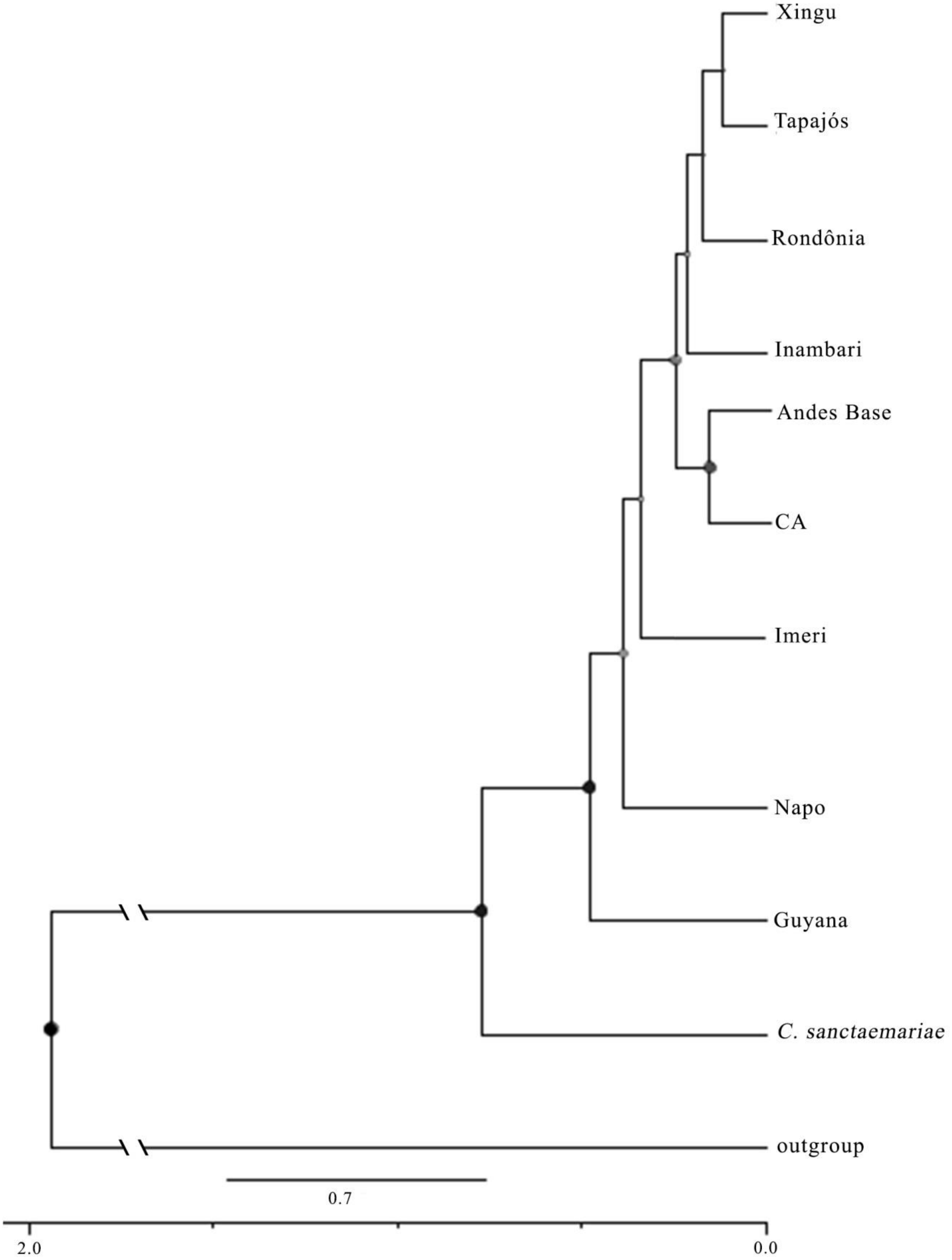
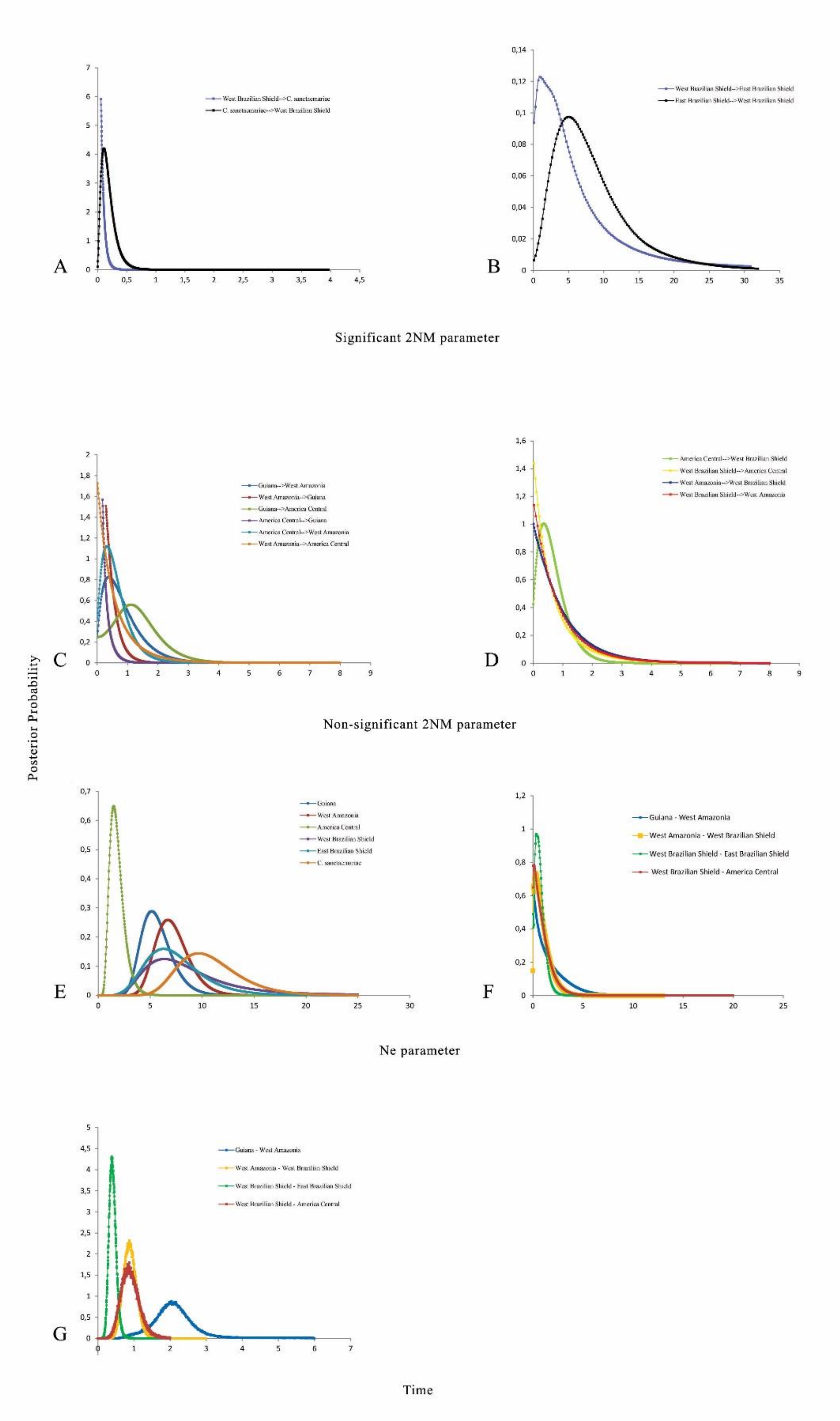


Fig. S1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4
3,5
3
2,5
2
1,5
1
0,5
0
0
1
2
3
4
5
6
7
Guiana - West Amazonia
West Amazonia - West Brazilian Shield
West Brazilian Shield - East Brazilian Shield
West Brazilian Shield - America Central
Time

Fig. S2

Table S1

							Map	coordenates		genbank accession number				
Field_Numb	Museum	Tombo	Species		mtDNA lineages	Major_Area	Loc	Lat	Long	cytb	ND2	BF5	G3PDH	Myo
B2205	LSUMZ	2205	*Cymbilaimus*	*lineatus*	Central America	Central America	1	8.4724	-77.7206					
B2252	LSUMZ	2252	*Cymbilaimus*	*lineatus*	Central America	Central America	2	8.4724	-77.7206					
B28582	LSUMZ	28582	*Cymbilaimus*	*lineatus*	Central America	Central America	3	9.2601	-79.9430					
B28690	LSUMZ	28690	*Cymbilaimus*	*lineatus*	Central America	Central America	4	9.0500	-79.6500					
B28749	LSUMZ	28749	*Cymbilaimus*	*lineatus*	Central America	Central America	5	9.0500	-79.6500					
B28750	LSUMZ	28750	*Cymbilaimus*	*lineatus*	Central America	Central America	6	9.0500	-79.6500					
B28751	LSUMZ	28751	*Cymbilaimus*	*lineatus*	Central America	Central America	7	9.0500	-79.6500					
B46621	LSUMZ	46621	*Cymbilaimus*	*lineatus*	Central America	Central America	8	8.0167	-77.7167					
B46622	LSUMZ	46622	*Cymbilaimus*	*lineatus*	Central America	Central America	9	8.0167	-77.7167					
USNM612365	USNM	612365	*Cymbilaimus*	*lineatus*	Central America	Central America	10	9.1828	-82.2849					
A1547	INPA	1547	*Cymbilaimus*	*lineatus*	Guiana	Guiana	11	-1.4667	-60.7333					
A1551	INPA	1551	*Cymbilaimus*	*lineatus*	Guiana	Guiana	12	-1.4667	-60.7333					
A1631	INPA	1631	*Cymbilaimus*	*lineatus*	Guiana	Guiana	13	-0.0500	-64.0833					
ANSP21254	ANSP	21254	*Cymbilaimus*	*lineatus*	Guiana	Guiana	14	4.2833	-58.5167					
ANSP21585	ANSP	21585	*Cymbilaimus*	*lineatus*	Guiana	Guiana	15	4.2833	-58.5167					
ANSP21705	ANSP	21705	*Cymbilaimus*	*lineatus*	Guiana	Guiana	16	4.2833	-58.5167					
ANSP21794	ANSP	21794	*Cymbilaimus*	*lineatus*	Guiana	Guiana	17	4.2833	-58.5167					
CN1174	MPEG	66485	*Cymbilaimus*	*lineatus*	Guiana	Guiana	18	-0.9333	-53.2333					
CN394	MPEG	65410	*Cymbilaimus*	*lineatus*	Guiana	Guiana	19	-0.1500	-55.1833					
USNM609151	USNM	609151	*Cymbilaimus*	*lineatus*	Guiana	Guiana	22	5.5000	-60.7833					
USNM609152	USNM	609152	*Cymbilaimus*	*lineatus*	Guiana	Guiana	23	5.5000	-60.7833					
USNM621430	USNM	621430	*Cymbilaimus*	*lineatus*	Guiana	Guiana	24	7.3667	-60.4833					
USNM622939	USNM	622939	*Cymbilaimus*	*lineatus*	Guiana	Guiana	25	6.4000	-58.7667					
USNM625465	USNM	625465	*Cymbilaimus*	*lineatus*	Guiana	Guiana	26	1.5833	-58.6333					
USNM637094	USNM	637094	*Cymbilaimus*	*lineatus*	Guiana	Guiana	27	2.9667	-58.5833					
A1111	INPA	1111	*Cymbilaimus*	*lineatus*	Imeri	West Amazonia	28	-0.1667	-66.9833					

								coordenates		genbank accession number				
Field_Numb	Museum	Tombo	Species		mtDNA lineages	Major_Area	Map Loc	Lat	Long	cytb	ND2	BF5	G3PDH	Myo
A1953	INPA	1953	*Cymbilaimus*	*lineatus*	Imeri	West Amazonia	45	-1.8333	-61.5833					
A6614	INPA	6614	*Cymbilaimus*	*lineatus*	Imeri	West Amazonia	46	-0.5833	-64.9167					
AMZ108	MPEG	59507	*Cymbilaimus*	*lineatus*	Imeri	West Amazonia	47	-2.8500	-60.8500					
AMZ170	MPEG	59508	*Cymbilaimus*	*lineatus*	Imeri	West Amazonia	48	-2.8500	-60.8500					
AMZ265	MPEG	59509	*Cymbilaimus*	*lineatus*	Imeri	West Amazonia	49	-0.7833	-63.1500					
JAP631	MPEG	62743	*Cymbilaimus*	*lineatus*	Imeri	West Amazonia	50	-1.7167	-65.8667					
JAP812	MPEG	62744	*Cymbilaimus*	*lineatus*	Imeri	West Amazonia	51	-1.7167	-65.8667					
SGC074	MPEG	77134	*Cymbilaimus*	*lineatus*	Imeri	West Amazonia	20	-0.1333	-67.0167					
SGC173	MPEG	77234	*Cymbilaimus*	*lineatus*	Imeri	West Amazonia	21	-0.1333	-67.0167					
SGC759	MPEG	77818	*Cymbilaimus*	*lineatus*	Imeri	West Amazonia	58	-0.3000	-66.0167					
B4157	LSUMZ	4157	*Cymbilaimus*	*lineatus*	Napo	West Amazonia	52	-2.4492	-74.0149					
B4308	LSUMZ	4308	*Cymbilaimus*	*lineatus*	Napo	West Amazonia	53	-2.4492	-74.0149					
B6890	LSUMZ	6890	*Cymbilaimus*	*lineatus*	Napo	West Amazonia	54	-3.3695	-72.1095					
JAP443	MPEG	62747	*Cymbilaimus*	*lineatus*	Napo	West Amazonia	55	-1.9333	-66.6000					
JAP457	MPEG	62746	*Cymbilaimus*	*lineatus*	Napo	West Amazonia	56	-1.9333	-66.6000					
JAP458	MPEG	62745	*Cymbilaimus*	*lineatus*	Napo	West Amazonia	57	-1.9333	-66.6000					
B27679	LSUMZ	27679	*Cymbilaimus*	*lineatus*	Andes Base	West Brazilian Shield	31	-7.1333	-75.6833					
B27958	LSUMZ	27958	*Cymbilaimus*	*lineatus*	Andes Base	West Brazilian Shield	32	-7.1333	-75.6833					
B40200	LSUMZ	40200	*Cymbilaimus*	*lineatus*	Andes Base	West Brazilian Shield	33	-7.5500	-75.9000					
AMA146	MPEG	72703	*Cymbilaimus*	*lineatus*	Inambari	West Brazilian Shield	29	-4.4000	-69.7333					
B11156	LSUMZ	11156	*Cymbilaimus*	*lineatus*	Inambari	West Brazilian Shield	30	-8.0908	-74.4447					
B1129	LSUMZ	1129	*Cymbilaimus*	*lineatus*	Inambari	West Brazilian Shield	61	-15.2900	-67.5900					
CUJ176	MPEG	60168	*Cymbilaimus*	*lineatus*	Inambari	West Brazilian Shield	34	-4.9333	-68.1667					
ESEC212	MPEG	59835	*Cymbilaimus*	*lineatus*	Inambari	West Brazilian Shield	35	-11.0500	-70.2667					
FMNH389890	FMNH	389850	*Cymbilaimus*	*lineatus*	Inambari	West Brazilian Shield	36	-9.7500	-61.9167					
JRD019	MPEG	71384	*Cymbilaimus*	*lineatus*	Inambari	West Brazilian Shield	37	-9.1833	-71.8500					
OM009	MPEG	71090	*Cymbilaimus*	*lineatus*	Inambari	West Brazilian Shield	38	-7.5167	-63.3333					

							Map	coordenates		genbank accession number				
Field_Numb	Museum	Tombo	Species		mtDNA lineages	Major_Area	Loc	Lat	Long	cytb	ND2	BF5	G3PDH	Myo
PUC096	MPEG	57075	*Cymbilaimus*	*lineatus*	Inambari	West Brazilian Shield	39	-4.8500	-65.0833					
PUC114	MPEG	57077	*Cymbilaimus*	*lineatus*	Inambari	West Brazilian Shield	40	-4.8667	-65.3167					
PUC115	MPEG	57076	*Cymbilaimus*	*lineatus*	Inambari	West Brazilian Shield	41	-4.8667	-65.3167					
PUC209	MPEG	57074	*Cymbilaimus*	*lineatus*	Inambari	West Brazilian Shield	42	-4.8500	-65.0667					
UFAC1752	MPEG	64455	*Cymbilaimus*	*lineatus*	Inambari	West Brazilian Shield	43	-10.6333	-67.8000					
UFAC559	MPEG	60626	*Cymbilaimus*	*lineatus*	Inambari	West Brazilian Shield	44	-9.3500	-70.2000					
A5275	INPA	5275	*Cymbilaimus*	*lineatus*	Rondonia	East Brazilian Sield	59	-3.7000	-55.5833					
A6241	INPA	6241	*Cymbilaimus*	*lineatus*	Rondonia	East Brazilian Sield	60	-9.0333	-64.2333					
B18168	LSUMZ	18168	*Cymbilaimus*	*lineatus*	Rondonia	East Brazilian Sield	62	-14.8333	-60.4167					
BR163_116	MPEG	59140	*Cymbilaimus*	*lineatus*	Tapajos	East Brazilian Sield	64	-9.4000	-55.4833					
BR163_161	MPEG	59139	*Cymbilaimus*	*lineatus*	Tapajos	East Brazilian Sield	65	-7.1833	-55.4833					
LGEMA12333	LGEMA	12333	*Cymbilaimus*	*lineatus*	Tapajos	East Brazilian Sield	66	-9.3833	-57.6333					
LGEMA12334	LGEMA	12334	*Cymbilaimus*	*lineatus*	Tapajos	East Brazilian Sield	67	-9.3833	-57.6333					
LGEMA12621	LGEMA	12621	*Cymbilaimus*	*lineatus*	Tapajos	East Brazilian Sield	68	-9.5333	-57.3000					
MPDS1233	MPEG	65209	*Cymbilaimus*	*lineatus*	Tapajos	East Brazilian Sield	69	-6.5167	-57.4333					
TLPA061	MPEG	67380	*Cymbilaimus*	*lineatus*	Tapajos	East Brazilian Sield	70	-9.4167	-56.5500					
TLPA062	MPEG	67381	*Cymbilaimus*	*lineatus*	Tapajos	East Brazilian Sield	71	-9.4167	-56.5500					
TUP030	MPEG	68882	*Cymbilaimus*	*lineatus*	Tapajos	East Brazilian Sield	72	-4.0833	-60.6500					
WM324	MPEG	56058	*Cymbilaimus*	*lineatus*	Tapajos	East Brazilian Sield	73	-3.3500	-54.9333					
WM325	MPEG	56059	*Cymbilaimus*	*lineatus*	Tapajos	East Brazilian Sield	74	-3.3500	-54.9333					
WM382	MPEG	56060	*Cymbilaimus*	*lineatus*	Tapajos	East Brazilian Sield	75	-3.3500	-54.9333					
PPBio018	MPEG	61831	*Cymbilaimus*	*lineatus*	Xingu	East Brazilian Sield	76	-1.9500	-51.6000					
PPBio108	MPEG	61832	*Cymbilaimus*	*lineatus*	Xingu	East Brazilian Sield	77	-1.9500	-51.6000					
USNM541625	USNM	541625	*Cymbilaimus*	*lineatus*	Xingu	East Brazilian Sield	78	-3.6500	-52.3667					
USNMB06907	USNM	B06907	*Cymbilaimus*	*lineatus*	Xingu	East Brazilian Sield	79	-3.6500	-52.3667					
FMNH321763	FMNH	321763	*Cymbilaimus*	*sanctaemariae*	Inambari	West Brazilian Shield	63	-12.8773	-71.3865					
A1425	INPA	1425	*Cymbilaimus*	*sanctaemariae*	Inambari	West Brazilian Shield	80	-8.1667	-69.3000					

								coordenates		genbank accession number				
Field_Numb	Museum	Tombo	Species		mtDNA lineages	Major_Area	Map Loc	Lat	Long	cytb	ND2	BF5	G3PDH	Myo
A4787	INPA	4787	*Cymbilaimus*	*sanctaemariae*	Inambari	West Brazilian Shield	81	-8.5500	-67.5167					
ESEC015	MPEG	58888	*Cymbilaimus*	*sanctaemariae*	Inambari	West Brazilian Shield	82	-11.0500	-70.2000					
ESEC036	MPEG	58889	*Cymbilaimus*	*sanctaemariae*	Inambari	West Brazilian Shield	83	-11.0500	-70.2000					
UFAC832	MPEG	61293	*Cymbilaimus*	*sanctaemariae*	Inambari	West Brazilian Shield	84	-9.3500	-68.0833					
UFAC833	MPEG	61295	*Cymbilaimus*	*sanctaemariae*	Inambari	West Brazilian Shield	85	-9.9000	-68.4667					
UFAC900	MPEG	61294	*Cymbilaimus*	*sanctaemariae*	Inambari	West Brazilian Shield	86	-9.9000	-68.4667					
Tmajor	NRM	956694	*Taraba*	*major*	outgroup									
Bcinerea	LSUMZ	B18607	*Batara*	*cinerea*	outgroup									
Hguttatus	LSUMZ	B25895	*Hypoedaleus*	*guttatus*	outgroup									
Funduligera	ZMCU	S1776	*Frederickena*	*unduligera*	outgroup									
Fviridis	LSUMZ	B9259	*Frederickena*	*viridis*	outgroup									
Mleachii	LSUMZ	B5985	*Mackenziaena*	*leachii*	outgroup									
Msevera	NRM	956630	*Mackenziaena*	*severa*	outgroup									
Tdoliatus	NRM	956691	*Thamnophilus*	*doliatus*	outgroup									
USNM354	USNM	354	*Thamnophilus*	*doliatus*	outgroup									
USNM356	USNM	356	*Thamnophilus*	*doliatus*	outgroup									

Table S2

Primers	Sequências (5' – 3')	Referências
Cytochrome *b* (cyt b)		
L14841	AAC TGC AGT CAT CTC CGG TTT ACA AGA C	Kocher et al. (1989)
H16065	AAC TGC AGT CAT CTC CGG TTT ACA AGA C	Sorenson et al. (1999)
NADH Deidrogenase 2 (ND2)		
L5215	TAT CGG GCC CAT ACC CCG AAA T	Hackett (1996)
H6313	CTC TTA TTT AAG GCT TTG AAG GC	Sorenson et al. (1999)
β-fibrinogen intron 5 (bf5)		
S713	CGC CAT ACA GAG TAT ACT GTG ACA T	Weissbach et al. (1991)
AS767	GCC ATC CTG GCG ATC TGA A	Weissbach et al. (1991)
Glyceraldehyde-3-phospho-dehydrogenase intron 11 (G3PDH)		
G3P-13b	TCC ACC TTT GAT GCG GGT GCT GGC AT	Fjeldså et al. (2003)
G3P-14b	AAG TCC ACA ACA CGG TTG CTG TA	Fjeldså et al. (2003)
Myoglobin intron 2 (Myo)		
Myo2	GCC ACC AAG CAC AAG ATC CC	Slade et al. (1993)
Myo3F	TTC AGC AAG GAC CTT GAT AAT GAC TT	Heslewood et al. (1998)

Table S3

Primers	Best fit model (BIC)	nst	gamma	pinvar	-ln(L)
Cytochrome *b* (cyt b)	TIM2+I+G	6	3.0980	0.6030	4860.6096
NADH dehydrogenase subunit 2 (ND2)	HKY+I+G	2	2.7050	0.5270	4688.9497
b-Fibrinogen intron 5 (bf5)	HKY+I	2	-	0.3810	1894.3712
Glyceraldehyde-3-phospho-dehydrogenase intron 11 (G3PDH)	K80+G	2	0.6870	-	1335.0720
Myoglobin intron 2 (Myo)	K80+G	2	0.4040	-	1246.2377

Table S4

	*BEAST		IMa2	
Nodes	**TMRCA**	**95%HPD**	**TMRCA**	**95%HPD**
C. sanctaemariae - C. lineatus	0.684	0.44 - 1.01	-	-
Guiana - West Amazonia	0.42	0.29 - 0.57	2,036	0.7 - 3.67
West Amazonia - Brazilian Shield	0.3	0.2 - 0.4	0.862	0.53 - 1.24
West Brazilian Shield - East Brazilian Shield	0.18	0.11 - 0.26	0.381	0.22 - 0.61

Capitulo 2

Manuscrito a ser submetido para
Journal of Biogeography

Founder event speciation and divergence with gene flow in the *Microcerculus bambla* / *marginatus* (Aves: Troglodytidae) species complex: a different framework for thinking about diversification processes in the Amazon

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ABSTRACT

Aim A robust biogeographic analysis to elucidate centers of origin and dispersal, as well as the effects of physiographic barriers and climatic events in shaping the evolutionary history of an understory avian oscine lineage.

Location Neotropical lowlands

Methods Mitochondrial and nuclear genes of individuals collected throughout the species' ranges were sequenced and then analyzed using Bayesian approaches. Species tree analysis, divergence time inference, and historical demography tests were used to reconstruct the evolutionary history of the *Microcerculus bambla* / *marginatus* species complex. The isolation-with-migration coalescent model was employed to determine whether genetic divergence among *Microcerculus bambla* / *marginatus* lineages occurred in the presence of gene flow.

Results Our analyses support the monophyly of the *M. bambla / marginatus* species complex, with only five out of 10 reciprocally monophyletic lineages recovered being entirely consistent with currently recognized subspecies limits in this group. The evolutionary history of these lineages is best explained by a sequential series of cladogenetic events resulting from dispersal and founder events originating from a Western Amazonia ancestor during the last 2.4 Myr BP. Demographic expansions and low levels of asymmetrical gene flow have also been detected.

Main conclusions Our results highlight a different diversification scenario from those proposed so far for Amazonia, whereby sequential colonization (founder events) of adjacent interfluves during the consolidation of the modern Amazonian drainage account for the observed patterns of cladogensis and gene flow.

Keywords: phylogeography, population structure, gene flow, founder event speciation

INTRODUCTION

Basic understanding of biodiversity patterns are necessary to investigate the processes driving the origin of Neotropical diversity, ecosystem functioning and thus to underpin conservation efforts (Sites & Marshall, 2003, 2004). However, even birds, which are among the most diverse and best studied groups of Neotropical vertebrates, are still incompletely understood, with recent studies frequently showing incongruences between evolutionary history and inter-specific species limits, as well as several gaps in the taxonomy, resulting in the description of new species level taxa (Batista *et al*., 2013; Miranda *et al*., 2013; Rodrigues *et al*., 2013); consequently, such a cryptic diversity can affect our perception of biodiversity structure.

Amazonian landscape evolution has probably been influenced by a combination of tectonic events – i.e., uplift of the Andes and drainage formation in the Amazon Basin –and climatic changes (Hoorn *et al*., 2010; Rull, 2011). Historically, they have been proposed as important environmental drivers of biotic diversification mainly under the river barrier and refuge hypothesis, although none is widely accepted and it is recognized that different explanations may not be mutually exclusive (Rull, 2011, 2013). Rull (2013) has listed some problems that could be hampering the progress in the understanding of the origin of Neotropical diversity: the basic sampling units’ issue (species versus Evolutionary Significant Units dating inferences), the tendency to shift from one paradigm to another (river barrier *versus* refuge hypothesis), and the over-generalizations from one or a few case studies (i.e., Ribas *et al*., 2012; Garzón-Orduña *et al*., 2014). On the other hand, a more recently proposed model posits that rather than being directly linked to landscape change, the strongest predictors of speciation are the local of origin, timing of diversification and the ability to move through the landscape matrix (Smith *et al*., 2014; Miranda *et al*., *in prep*; Chapter 1).

Ultimately, what is needed are densely sampled and fully resolved time-calibrated phylogenies to improve our understanding of how and when lineages accumulated in this hyper diverse region and an approach that seeks to explore any possible explanation for the observed phenomena.

The *M. bambla* / *marginatus* species complex is a typical sedentary understory insectivorous avian lineage that is found mainly in lowland rainforest, including forest with wooded ravines and other dense tangles, rich in rotting logs (Kroodsma & Brewer, 2005). They show a geographic pattern of replacement where *M. bambla* occurrs mostly north of the Amazon and east of the Japurá rivers (but with an isolated population in eastern Ecuador and southeastern Peru; *M. b. albigularis*), while *M. marginatus* has a wide distribution ranging from the west bank of the Japurá River, across most of Amazonia south of the Amazon River (*M. m. marginatus*), as well as west of the Andes throughout Central America (six subspecies with restricted ranges, including one recently split as separate species: *M. m. philomela*; Kroodsma & Brewer, 2005).

Here we present the first comprehensive phylogeographic reconstructions for the *M. bambla* / *marginatus* species complex. We used multi-locus coalescent approaches to molecular dating, ancestral area reconstructions and population genetics to answer the following questions: a) how many basal taxa / evolutionary independent units can be recognized in the complex?; b) what are the ages of emergence of these extant basal taxa?; c) what have been the roles of tectonically driven phenomena such as the Andean uplift and drainage evolution in accounting for the diversification in this groups?; and d) could climatic change be also related to the same diversification scenario?

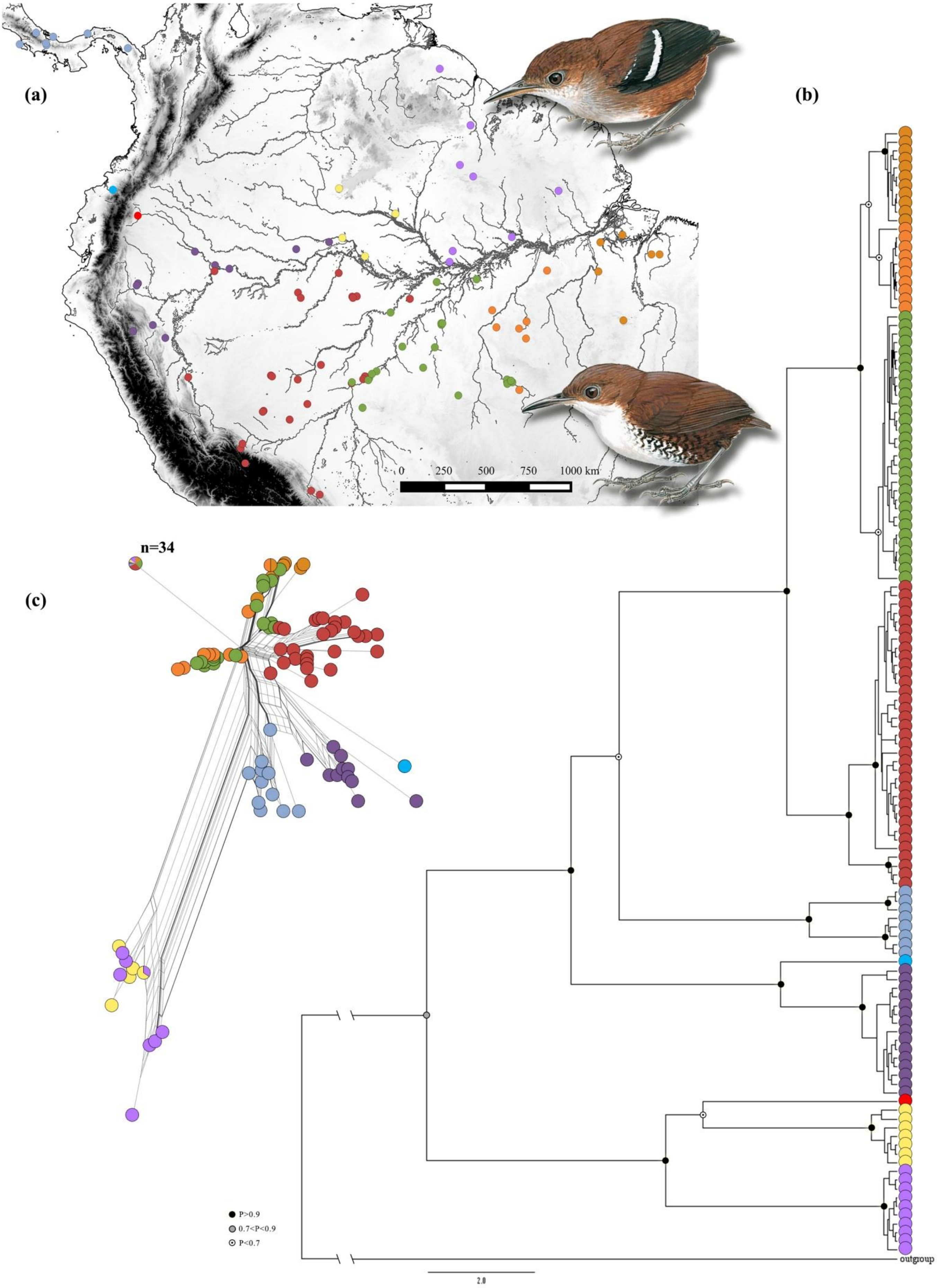
(a)
(b)
0
250
500
750
1000 km
n=34
(c)
● P>0.9
● 0.7<P<0.9
⊙ P<0.7
outgroup
2.0

Fig.1 – (a) Map showing the distribution of sampling localities in the *Microcerculus bambla / marginatus* range, with colors corresponding to lineages in the tree. The gradient of gray color in the map represents topographic elevation (the darker the higher the altitude). (b) Bayesian inferred maximum clade credibility mtDNA tree with posterior probabilities on nodes (see legend). (c) multilocus network constructed from the three nuclear loci.

MATERIALS AND METHODS

Sampling and laboratory methods

Altogether, 143 tissue samples of the *M. bambla* / *marginatus* species complex were obtained from throughout its range. This sampling scheme covered opposite sides of the Andes, Central America, and all major Amazonian river basins, including 7 out 10 recognized subspecies (Kroodsma & Brewer, 2005; Fig 1a). Three recognized *Microcerculus marginatus* subspecies, distributed in northwestern Venezuela, northern Colombia and northwestern Ecuador (*M. m. squamulatus*, *M. m. corrasus*, *M. m. occidentalis*) were not sampled due to the lack of available tissues. Detailed information on specimens, voucher information, and GenBank accession numbers are provided in Appendix S1, Supporting Information.

Total genomic DNA was extracted using procedures described in Sambrook *et al.* (1989). For most samples, we sequenced fragments of five loci: two protein-coding mitochondrial DNA (mtDNA) genes – cytochrome b (cyt b) and NADH dehydrogenase subunit 2 (ND2) – and three autosomal nuclear loci – β-fibrinogen intron 5(bf5), glyceraldehyde 3-phosphate dehydrogenase intron 11 (g3pdh) and myoglobin intron 2 (myo). PCR amplifications were performed in 12.5µl volumes, containing 1.25µl 10x reaction buffer, 1.5mM $MgCl_2$, 0.4mM each dNTP, 0.2µM each primer (Appendix S2, Supporting Information), 1 unit of taq DNA polymerase (Invitrogen) and 10–25 ng of genomic DNA. Thermocycling conditions started with 5min denaturation at 95 ºC, and a final extension of 5 min at 72 ºC. Annealing temperature and number of cycles varied by primer pair (for primer details, see Appendix S2, Supporting Information). PCR products were purified using 20% polyethylene glycol 8000 (PEG) before sequencing. Sequencing was carried out using an ABI PRISM 3130 XL Genetic Analyzer (Applied Biosystems®) with the ABI Prism BigDye Terminator Cycle sequencing protocol. For each molecular marker, sequences for both forward and reverse strands were performed and all DNA sequences generated are available on GenBank (Appendix S1, Supporting Information, numbers pending).

Sequences were visually inspected and aligned using ClustalW (Thompson *et al.*, 1994) as implemented in BioEdit7.0.5.3 (Hall, 1999). To confirm the absence of stop codons and other anomalous residues, protein-coding mtDNA were translated into amino acids. We resolved the gametic phase of nuclear alleles using PHASE 2.1.1 (Stephens *et al.*, 2001; Stephens & Scheet, 2005) with the input files produced in the online software SeqPHASE (Flot, 2010). To avoid biasing estimates of population genetic parameters, each dataset was analyzed using default values and including some low-probability calls, since PHASE has been shown to generate a very low number of false positives (Garrick *et al.*, 2010). Also, the three nuclear loci were checked for recombination ($p > 0.05$) using the Phi test implemented in SPLITSTREE 4.12.8 (Bruen *et al.*, 2006; Huson & Bryant, 2006). DnaSP 5 (Librado & Rozas, 2009) was used to calculate the diversity statistics.

Phylogeographic estimation

To delineate geographical lineages, we generated a mtDNA phylogeny using the BEAST package (Drummond & Rambaut, 2007). Lineages were defined as genetically distinct geographical clusters and some single divergent samples from unique geographical areas were also referred to as lineages for convenience. jModelTest 2.1.3 (Darriba *et al.*, 2012) was used to select the best-fit evolutionary models with the Bayesian information criterion (BIC; Posada, 2008). The best fitting models incorporated as prior information in the Bayesian analyses were summarized in Appendix S2, Supporting Information. We used a coalescent constant-size for the tree prior and uncorrelated lognormal relaxed clock. To calibrate the mtDNA gene tree we used the cyt b mutational rate of 2.1% sequence divergence per lineage per million years (Weir & Schluter, 2008). Default priors were used, except when uniform prior distributions were involved; in this case we used a lognormal prior distribution. Two independent runs were performed for 100 million generations (sampling every 10,000 generations and discarding the first 20% as burn-in). To check the likelihood stationarity, adequate effective sample size (> 200) and convergence of parameters between runs, we used TRACER 1.5 (http://beast.bio.ed.ac.uk/Tracer). Trees were combined and summarized using LogCombiner v1.5.2 and TreeAnnotator v1.5.2, respectively (both softwares are included along with the BEAST package). The consensus tree was visualized in FigTree 1.2.3 (http://tree.bio.ed.ac.uk/software/figtree/).

A multilocus network of the nuclear data was used to examine the geographic distribution and relationships among haplotypes. To produce the network, first we used POFAD 1.03 (Joly & Bruneau, 2006) to combine individual uncorrected *p*-distances matrices for each nuclear locus (calculated in PAUP 4.0b10; Swofford, 2003) into a single distance matrix of specimens. The genetic network among specimens was constructed using the NeighborNet algorithm (Bryant & Moulton, 2004) in SPLITSTREE 4.12.8 (Huson& Bryant, 2006).

We also investigated population structure with a Bayesian probabilistic genetic clustering implemented by STRUCTURE version 2.3 (Pritchard *et al.*, 2000) using nuclear markers only. First, all samples from both species were examined, then each species was analyzed separately. We adopted the admixture and correlated allele frequencies model. Analyzes were performed with two runs (short and long runs) for values of K ranging from 1 to 10. Short runs consisted of 5 iterations with a burn-in period of 50,000 steps and MCMC length of 450,000 steps, whereas long runs included 2 iterations with burn-in of 100,000 steps and MCMC length of 900,000 steps (totalizing 7 iterations). Different iterations of the optimal K value were averaged in CLUMPP 1.1.2 (Jakobsson & Rosenberg, 2007).

Divergence times and ancestral area reconstruction

We used the *BEAST algorithm (Heled & Drummond, 2010) to reconstruct a time-calibrated multilocus species tree. We used the lineages that were genetically differentiated in the mtDNA tree (Fig 1b) to designate the basal units in *BEAST. This analysis used the same parameter settings as in the estimated mtDNA gene tree, but with a Yule process on the species tree prior. Runs were performed for 200 million generations with substitution model parameters unlinked for each gene. Analyzes were run via the Cipres Portal (Miller *et al.*, 2010). We generated a cloudogram of the species trees with DensiTree 2.0.1 (Bouckaert, 2010).

We used the likelihood based methods implemented in the R statistical package BioGeoBearS (Matzke, 2013) to determine the biogeographic history of the *M. bambla* / *marginatus* complex. In this inference, model testing and choice of many different possibilities of ancestral areas reconstruction are evaluated by calculating the maximum likelihood of ancestral states at speciation events as a function of time. The models evaluated included the Dispersal-Extinction Cladogenesis (DEC), Dispersal-Vicariance Analysis (DIVALIKE), and Bayesian inference of historical biogeography for discrete areas (BAYAREALIKE) – each one including the founder parameter +J. Each model allows for a different subset of biogeographic possibilities, such as dispersal, vicariance and extinction, but differ in their treatment of cladogenetic events in which ancestral and daughter ranges overlap (see Matzke, 2013). Our estimates were based on the ultrametric species tree and included the following parameters: a) the maximum number of areas that a species level lineage could occupy was limited to two; and b) five possible geographical areas that show landscape features previously recognized as barriers were recognized (Miranda *et al*., *in prep*.; Chapter 1; Guianan shield (Guy), Western sedimentary Amazon basin north of the Amazon River (WAN), Western sedimentary Amazon basin south of the Amazon River (WAS), Central America (CA), and the Brazilian Shield (BS).

Demographic history and gene flow

For population genetic analyses, groups were defined according to the lineages recovered in the phylogeographic estimates. Historical population size dynamics were reconstructed using the Extended Bayesian Skyline Plot method (EBSP, Heled & Drummond, 2008) implemented in BEAST. The best fit substitution model for each marker, the substitution rate, priors and the MCMC run strategy were the same as described above for other Bayesian phylogenetic analyses.

We used the isolation–migration model (Hey & Nielsen, 2004; Nielsen & Wakeley, 2001) implemented in IMa2 (Hey, 2010) to estimate whether gene flow has occurred among those parapatric lineages of *Microcerculus bambla / marginatus* potentially in contact (Fig 1). In this way, one could test whether the observed absence of phylogenetic signal in the nuclear markers is better explained by incomplete lineage sorting as a result of recent population splitting (m= 0) or by the presence of gene flow (m> 0). For all analyses the HKY model (Hasegawa *et al*., 1985) was applied for all markers; other parameters selected were: a) an inheritance scale of 0.25 for the mtDNA and 1 for nDNA; b) substitution rates based on that of the cyt b gene (2.1% sequence divergence per million years per generation; Weir & Schluter, 2008); and c) a generation time of one year. We performed preliminary runs to establish the best priors for effective population sizes, times of divergences, and migration parameters. We then performed three final runs discarding 100,000 generations as burn-in, sampling 100,000 trees during 1,000,000 generations, and using 20 chains. Finally, to test whether a model of isolation without gene flow fitted the data better than a model with gene flow, we used the Nielsen & Wakeley (2001) approach and also likelihood-ratio tests associated with different models implemented in IMa2's L mode.

RESULTS

Fragments of 1010 and 1041 base pairs were obtained for cytb and ND2, respectively. No indels in unexpected positions, neither stop or nonsense codons were detected in both alignments. For nuclear markers, we obtained fragments of 589bp for bf5, including 2 indels of 1bp in a heterozygous state; 370bp for g3pdh, including 1bp indel in a heterozygous state (shared only by *M. bambla* specimens); and 521 bp for myo, including 1bp indel in a heterozygous state. We found no evidence of recombination (data not shown). The summary statistics for each sequenced gene is presented in Table 1.

Table 1 – Descriptive statistics and neutrality tests for each marker separated by *Microcerculus bambla / marginatus* lineage. (N) number of phased alleles; (s) number of segregating sites; (k) average number of nucleotide differences; (π) nucleotide diversity; (h), number of haplotypes; (Hd) haplotype diversity; (θ) diversity parameter per sequence [95% confidence interval]; (Fs) Fu's Fs (Fu, 1997); (D) Tajima's D test (Tajima,1989); (*) Significance levels for Tajima's D and Fu's Fs P < 0.05.

Locus / Lineage	**N**	**s**	**k**	**π**	**h**	**Hd**	**θ [95% CI]**	**Fs**	**D**
cytb									
Guyana	8	7	2.571	0.004	4	0.821	2.699 [0.385 - 6.556]	0.633	0.429
Imeri	4	6	3.000	0.004	4	1.000	3.272 [0.0 - 8.727]	0.085	0.148
Central America	6	17	9.400	0.013	5	0.933	7.445 [2.189 - 22.335]	0.564	0.973
Rondônia	24	10	1.054	0.002	10	0.620	2.677 [0.0 - 2.410]	**0.000***	**0.002***
Tapajós	7	3	1.048	0.001	3	0.667	1.224 [0.0 - 2.857]	0.485	0.303
Napo	14	26	6.393	0.009	9	0.786	9.641 [1.542 - 14.655]	0.271	0.056
Inambari	23	13	2.526	0.004	10	0.895	3.719 [0.572 - 5.436]	**0.045***	0.156
Xingu	9	4	1.222	0.001	5	0.806	1.471 [0.0 -3.311]	**0.025***	0.275
nd2									
Guyana	9	2	0.722	0.001	3	0.639	0.735 [0.0 - 2.207]	0.414	0.384
Imeri	7	9	3.810	0.004	6	0.952	3.673 [0.816 - 9.387]	0.239	0.588
Central America	7	29	18.800	0.024	5	1.000	15.840 [3.840 - 46.080]	0.515	0.673
Rondônia	26	23	2.960	0.003	13	0.870	5.826 [0.794 - 6.091]	**0.002***	**0.018***
Tapajós	9	9	2.167	0.002	4	0.583	3.311 [0.367 - 5.151]	0.624	**0.045***
Napo	16	57	10.867	0.014	13	0.967	17.780 [4.219 - 21.095]	0.136	**0.037***
Inambari	27	48	6.926	0.012	22	0.907	10.022 [2.826 - 13.105]	**0.004***	0.187
Xingu	7	8	2.476	0.003	6	0.952	3.265 [0.408 - 6.530]	**0.004***	0.215
bf5									
Guyana	18	9	2.562	0.004	12	0.948	2.616 [0.581 - 5.523]	**0.000***	0.507
Imeri	14	2	1.022	0.002	3	0.692	0.628 [0.0 - 2.830]	0.729	0.960
Central America	18	9	3.190	0.005	9	0.876	2.907 [0.872 - 6.686]	0.150	0.671
Rondônia	42	5	1.123	0.002	7	0.757	1.162 [0.232 - 2.556]	0.142	0.509
Tapajós	10	4	1.667	0.003	4	0.778	1.413 [0.0 - 4.241]	0.534	0.758
Napo	32	14	1.722	0.003	10	0.679	3.724 [0.496 - 3.724]	**0.020***	**0.014***
Inambari	76	13	1.560	0.003	16	0.826	2.856 [0.408 - 3.060]	**0.000***	0.078
Xingu	12	3	1.121	0.002	4	0.758	0.993 [0.0 - 2.980]	0.355	0.679
g3pdh									
Guyana	24	8	2.656	0.007	8	0.826	2.142 [0.803 - 5.623]	0.354	0.805
Imeri	16	4	1.154	0.003	5	0.824	1.257 [0.0 - 2.830]	0.150	0.434
Central America	22	12	1.558	0.004	7	0.636	3.291 [0.274 - 3.566]	0.119	**0.015***
Rondônia	60	2	0.554	0.002	3	0.527	0.428 [0.0 - 1.501]	0.662	0.742
Tapajós	20	1	0.521	0.001	2	0.521	0.281 [0.0 - 1.409]	0.861	0.951
Napo	32	5	0.786	0.002	6	0.556	1.241 [0.0 - 1.986]	0.057	0.192
Inambari	78	14	1.158	0.003	15	0.632	2.848 [0.203 - 2.441]	**0.000***	**0.011***
Xingu	24	4	1.036	0.003	5	0.678	1.071 [0.0 - 2.410]	0.294	0.492

Table 1 – Cont.

Locus / Lineage	N	s	k	π	h	Hd	θ [95% CI]	Fs	D
myo									
Guyana	18	24	3.642	0.007	10	0.767	7.232 [0.904 - 7.835]	0.078	**0.005***
Imeri	12	14	3.288	0.006	7	0.773	4.637 [0.662 - 7.285]	0.243	0.112
Central America	22	10	1.026	0.002	7	0.621	1.973 [0.0 - 2.536]	0.059	**0.024***
Rondonia	50	4	0.160	0.000	5	0.155	0.893 [0.0 - 0.669]	**0.000***	**0.000***
Tapajós	18	8	1.444	0.003	7	0.739	2.325 [0.290 - 3.488]	**0.033***	0.093
Napo	26	15	1.837	0.004	11	0.748	4.192 [0.524 - 3.931]	**0.001***	**0.004***
Inambari	60	15	0.926	0.002	12	0.545	3.216 [0.214 - 2.144]	**0.000***	**0.000***
Xingu	16	5	1.042	0.002	6	0.733	1.506 [0.0 - 2.712]	**0.013***	0.200

Phylogeographic estimates

BI analyses based on the mtDNA showed that *Microcerculus bambla* and *M. marginatus* are reciprocally monophyletic sister taxa. This analysis revealed ten lineages (denoted as different colors in Fig. 1b), with strong support for the monophyly of eight of them. As all these lineages coincide with known Amazonian areas of endemism, we decided to use their names (*sensu* Silva *et al*., 2005) for mitochondrial lineage designations. Two lineages were recovered within *M. bambla* formed by Guyana and Imeri specimens (Fig. 1b). Also, a unique sampled specimen of *M. bambla* from the Andean foothill disjunct population in eastern Ecuador appeared as sister to Imeri lineage. *Microcerculus marginatus* can be divided into six lineages in following branching order (Fig. 1b): (1) Napo, here including a unique sampled specimen from the west side of the Andes in Ecuador that is more closely related to this group than to the Central America lineage; (2) Central America, which exhibits well-supported sub-clades congruent with both sides of Panama isthmus; (3) Inambari; (4) Rondônia; (5) Tapajós; and (6) Xingu, including two specimens from the Belém area of endemism. Despite the formation of these mostly well-supported mitochondrial clades, lineages separated by the Madeira, Tapajós, and Tocantins rivers were not recovered as reciprocally monophyletic, indicating a mismatch between clade limits and modern riverine barriers (Fig. 1b). Relationships among the six mitochondrial lineages of *M. marginatus* were well-resolved and supported overall, with the exception of the sister relationship of the Central American clade and those basal ones involving the eastern most clades Rondônia, Tapajós, Xingu, and Belém (Fig 1b).

The multilocus network generated using the three nuclear loci supported five phylogeographic groups whose limits coincided to some extent with those well supported lineages in mtDNA gene tree (Fig 1c). These phylogroups include both *M. bambla* lineages, plus *M. marginatus* Napo, Central America, Inambari and east of Madeira river lineages (hereafter Brazilian Shield – BS, and which groups Rondônia, Tapajós, Xingu and Belém mtDNA lineages). Furthermore, 34 specimens from all aforementioned lineages grouped together.

STRUCTURE analyses supported a value of K = 2, representing the most basal hierarchical structure in the data (*M. bambla* and *M. marginatus*; Fig 2a). Although there is no sign of admixture, one *M. marginatus* specimen located at the contact regions between clusters (MPEG 63199; Fig 2a, b & c; map locality 60; Appendix S1, Supporting information) was assigned to a *M. bambla* cluster. When we ran analyses within each species separately, STRUCTURE recovered a value of K = 3 and K=7 for *M. bambla* and *M. marginatus*, respectively (Fig 2b). There is overall concordance between identified nuclear clusters and mtDNA lineages for *M. marginatus*, but the same is not true for *M. bambla*. For the latter, the three recovered clusters are not structured geographically and exhibit high levels of admixture (Fig 2b & c). For *M. marginatus* clusters, specimens from Napo, Central America and Inambari showed a high membership probability to their own areas, although Inambari appeared with two clusters in admixture in some extent.

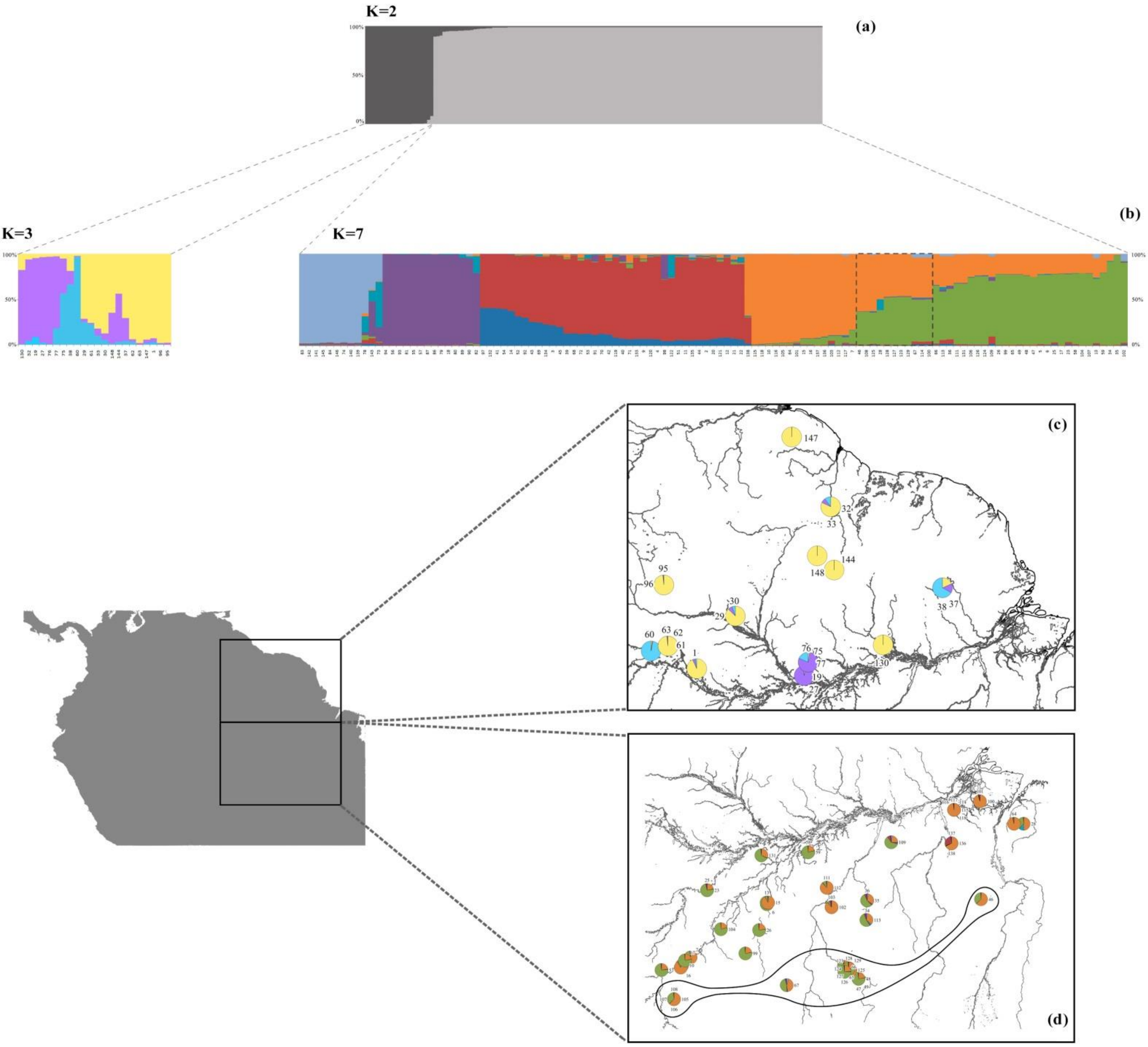

Fig. 2 – (a) STRUCTURE plot depicting population structure according to the best K between samples of the entire *Microcerculus bambla* / *marginatus* complex. (b) Population clusters for *M. bambla* (left; K=3) and *M. marginatus* (right; K=7) each represented by a different color, with vertical bars representing individuals and the posterior probability that a given individual is assigned to a particular cluster. Geographic distribution of Bayesian clustering results for *M. bambla* (c) and *M. marginatus* lineages occurring on the Brazilian Shield (d).

The remaining Brazilian Shield lineages grouped in two clusters with high admixture levels (Fig 2b). More specifically in the case of BS lineages, specimens with around 50% admixture came from headwater regions across the Madeira, Tapajós, and Xingu rivers (Fig 2b & d).

Divergence times and ancestral area reconstruction

The *BEAST derived species tree (Fig 3a & b) topology was largely consistent with the mtDNA gene tree analyses, yet some differences included: (1) the position of Central America lineage as a first split in the *M. marginatus* diversification; and (2) strong support for the Rondônia lineage as sister to Tapajós lineage (instead of a Tapajós – Xingu sister relationship; see Figs 1a & 3a for comparisons). Estimated median divergence times recovered that *M. bambla* and *M. marginatus* diverged at *ca*. 2.4 Ma, whereas the two lineages of *M. bambla* diverged at *ca*. 0.7 Ma, with the initial divergence of the main lineages within *M. marginatus* dated between 1.5 and 0.08 Ma (Fig 3a), all during the Pleistocene.

The statistical model chosen to ancestral area reconstructions in the *M. bambla* / *marginatus* complex was BAYAREALIKE+J (Fig 3a; ln L = -11.3). This analysis indicated that the relatively recent evolutionary history of the *M. bambla* / *marginatus* complex could be explained through a series of founder events leading to cladogenesis. Under this model, the *M. bambla* / *marginatus* ancestor is more likely to have originated in northwestern Amazonia (equivalent to the Napo and Imeri areas of endemism see Fig 1 Miranda *et al.*, *in prep.*; Chapter 1) before dispersing to inhabit both the Guiana and

Brazilian shields, as well as Central America. Nevertheless, this ancestral area reconstruction should be interpreted with caution, as some nodes were poorly resolved in our phylogenetic reconstructions (Fig 3b).

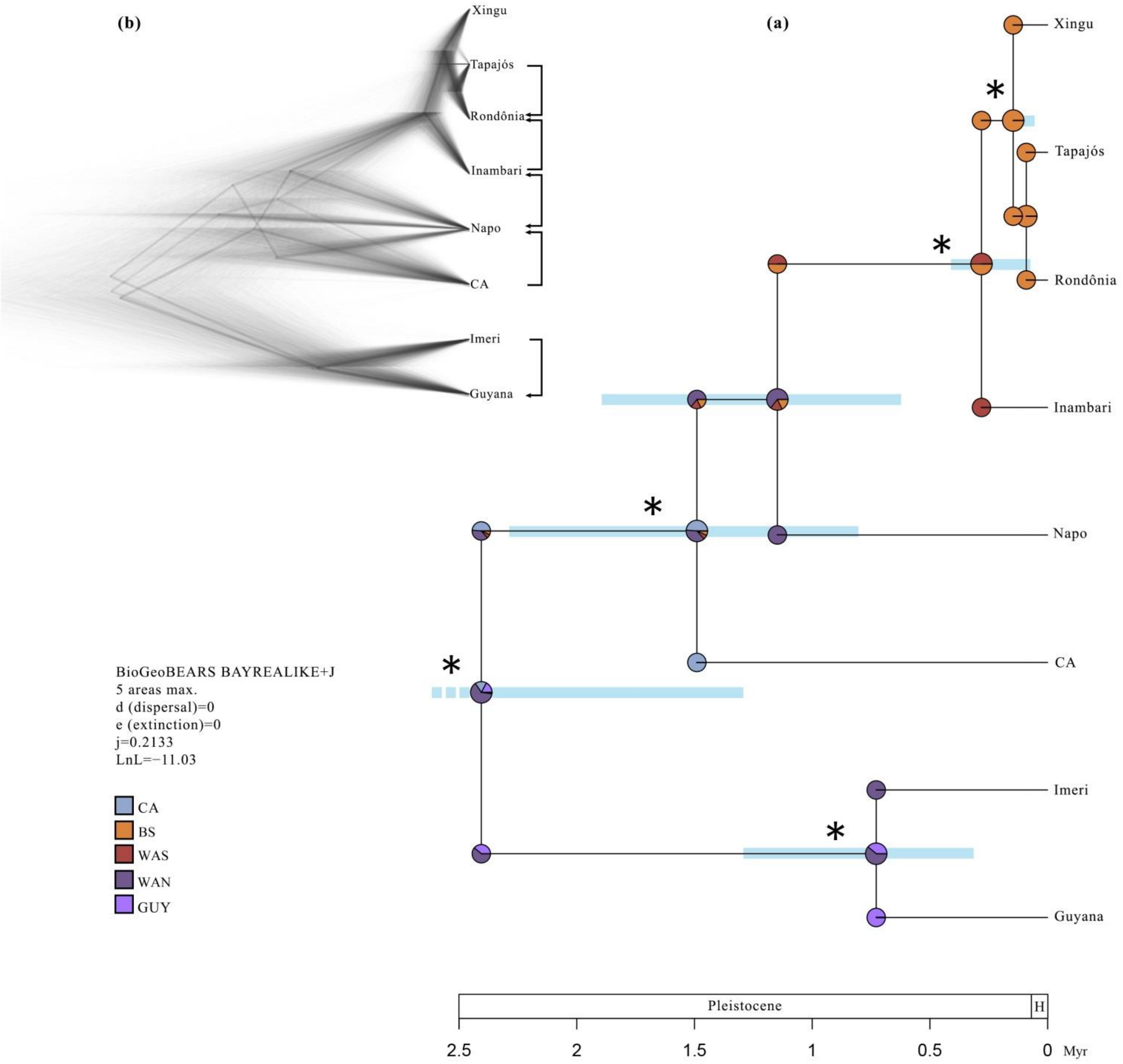

Fig. 3 – (a) Chronogram with divergence times for the *Microcerculus bambla* / *marginatus* complex. Tree topologies were obtained from a *BEAST species tree analysis base on all sequenced genes with time scale. Asterisks represent values of posterior probability ≥95%. Bars on nodes represent 95% high posterior density of divergence times. Pie charts at nodes represent probabilities of geographic ancestral distributions estimated by BioGEoBEARS, with each region coded by a different color (see legend at bottom left). (b) Cloudogram of the of all gene

trees generated by *BEAST analysis with the most frequently occurring topology highlighted in dark gray lines.

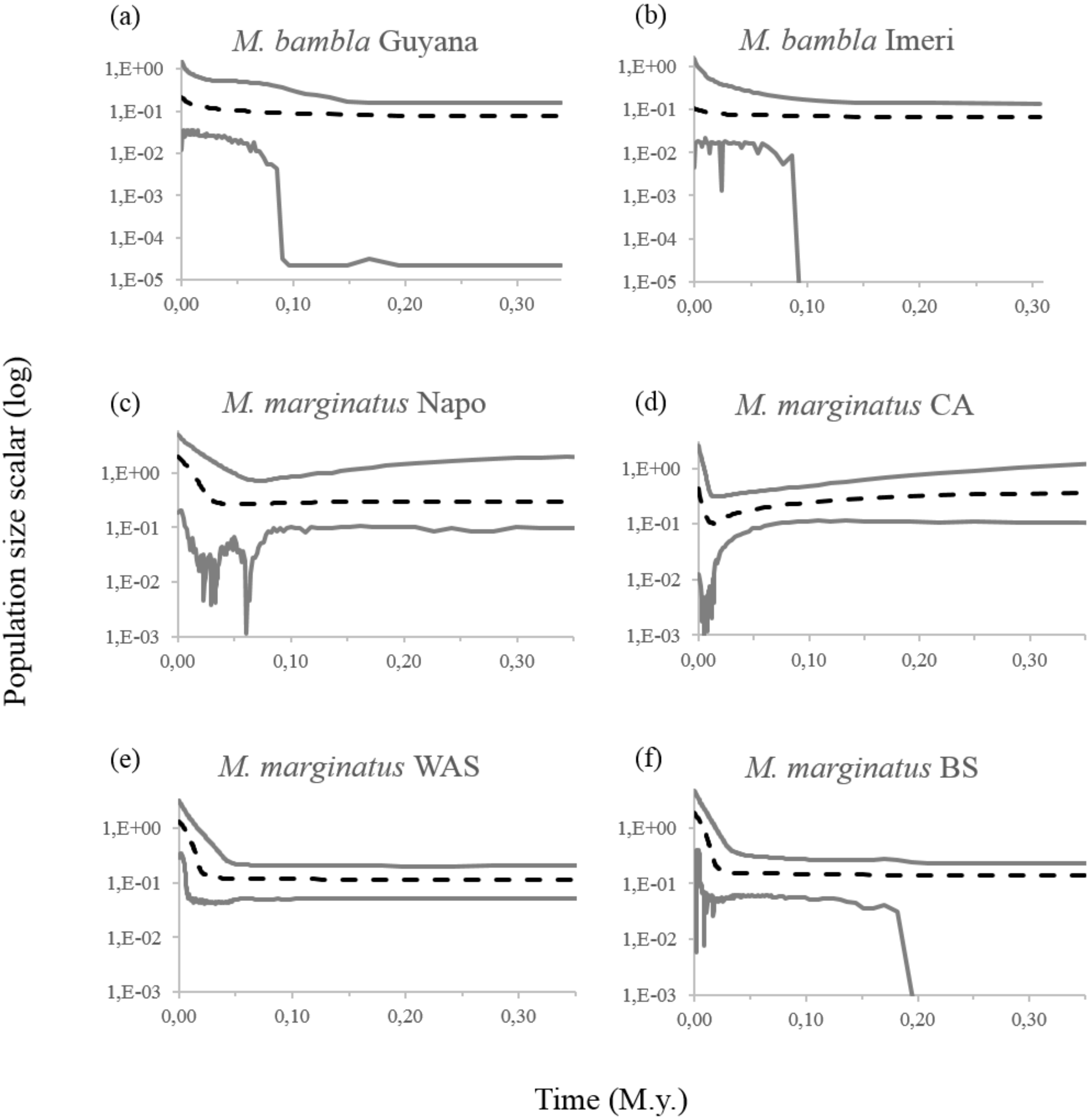

Fig. 4 – Demographic histories of the major phylogroups of the *Microcerculus bambla* / *marginatus* complex inferred through Extended Bayesian Skyline plots based on all markers. Black traced lines represent median values, while the gray area corresponds to 95% confidence intervals. The X axis corresponds to time in million years before present, while the Y axis represents log scaled effective populations sizes (*Ne*).

Demographic history and gene flow

Analyses conducted to detect demographic-change histories for the *M. bambla* lineages indicated that they have maintained relatively stable sizes during the late Pleistocene (Fig 4a & b). In contrast, *M. marginatus* lineages show a strong signal of population expansion in the last 0.02 Mya (Fig 4c-f).

In all IMa2 runs, the posterior distributions of parameters had a clear peak and the right tails converge on zero (data not shown). Among almost all lineages of *M. bambla* / *marginatus* potentially in contact, IMa2 analyses favored scenarios of very low levels of asymmetrical gene flow (Table 2; Fig. 3b). In contrast, gene flow between *M. bambla* and *M. marginatus* and between the Tapajós and Xingu *M. marginatus* lineages was rejected by LRT tests. The highest migration rate detected (not including a lower bond likelihood of zero) was from the Rondônia into the Tapajós lineage of *M. marginatus* (Table 2).

Table 2 – Demographic Parameters (modal values; 95% HPD interval in parentheses)

Pairwise Analyses	**Ne_1**	**Ne_2**	**2N1M1>2**	**2N2M2>1**
M. bambla Imeri x *M. bambla* Guyana	4.77 (2.77 - 8.25)	7.95 (4.85 - 13.27)	0.002 (0.0 - 0.367)	0.772 (0.241 - 1.736)*
M. bambla Imeri x *M. marginatus* Napo	5.21 (3.01 - 8.93)	12.63 (9.25 - 17.31)	0.001 (0.0 - 0.196)	0.001 (0.0 - 0.175)
M. marginatus Napo x *M. marginatus* CA	10.75 (6.71 - 15.71)	8.51 (4.97 - 15.37)	0.162 (0.0 - 0.953)	0.378 (0.0 - 1.019)*
M. marginatus Napo x *M. marginatus* Inambari	12.88 (9.16 - 18.11)	9.92 (7.34 - 13.28)	0.121 (0.0 - 0.644)*	0.089 (0.003 - 0.378)*
M. marginatus Inambari x *M. marginatus* Rondonia	10.85 (7.84 - 14.87)	3.10 (1.96 - 4.78)	0.272 (0.036 - 0.986)*	0.001 (0.0 - 0.206)
M. marginatus Rondonia x *M. marginatus* Tapajos	4.57 (2.77 - 7.29)	1.48 (0.60 - 3.44)	0.002 (0.0 - 0.143)	1.298 (0.279 - 3.861)*
M. marginatus Tapajos x *M. marginatus* Xingu	1.48 (0.60 - 3.44)	2.44 (1.16 - 4.92)	0.004 (0.0 - 0.902)	0.004 (0.0 - 1.179)

Ne_1 and Ne_2, effective population sizes for populations 1 and 2, respectively;

2N1M1>2, population migration rate into populations 1 from populations 2 per generation; 2N2M2>1,population migration rate into populations 2 from populations 1 per generation.

*Migration rates that are significantly different from zero at the $P<0.01$ level in LLR tests (Nielsen and Wakeley 2001; Hey 2010).

Posterior probability distributions for parameters are shown in Fig S3, Supporting Information

DISCUSSION

We assembled a relatively large dataset consisting on several specimens of most taxa and populations of the *M. bambla* / *marginatus* species complex to generate the first comprehensive molecular phylogenetic reconstruction for the group. Although our analyses support the monophyly of the *M. bambla* / *marginatus* species complex, of the 10 reciprocally monophyletic lineages recovered in the mtDNA gene tree (including single divergent samples from unique geographical areas), only five are entirely consistent with inter-specifics and subspecific limits currently recognized in this group, as follows: (1) nominate *M. bambla bambla* (Guyana lineage; light purple circles in Fig 1); (2) *M. bambla caurensis* (Imeri lineage; yellow circles in Fig 1); (3) *M. bambla albigularis* (the disjunct population from the Andean foothill only amplified for mtDNA; light red circle in Fig 1); (4) *M. marginatus luscinia* (Central America lineage; gray circles in Fig 1); and (5) *M. marginatus taeniatus* (*trans*-Andean specimen from western Ecuador which grouped as sister to the Napo lineage; light blue circle in Fig 1). Owing to the lack of samples of *M. marginatus marginatus* from the type locality (Bogotá, Colômbia; Mayr & Greenway, 1960), it is not possible to know with certainty to which phylogroup this taxon name would correspond; in any event, at least five lineages currently attributed to this taxon can be recognized as a distinct evolutionary units (Napo [dark purple circles], Inambari [dark red circles], Rondônia [green circles], Tapajós [light orange circles], and Xingu [dark orange circles]; Fig 1). When the multilocus species tree is considered, strong support was provided for the monophyly of those lineages sampled more densely. The only lineages of *M. marginatus* for which low speciation probabilities were recovered include those associated with the Napo and the Brazilian Shield (Rondônia, Tapajós and Xingu lineages) phylogroups. Although the position of Napo lineage varied (Fig 3b), it was nevertheless involved in one of the earliest splits among *M. marginatus* lineages. Faster sorting times of mtDNA relative to nuclear genes (Figs 1b & 2) may explain these discrepancies between the mtDNA gene tree and the species tree, especially considering the existence of gene flow between most parapatric lineages of the *M. bambla* / *marginatus* complex (Leaché *et al*., 2014; Table 2; Fig 3b).

Regarding the overall, asymmetrical and very low rates of gene flow estimated among almost all lineage pairs potentially in contact, they occurred mainly between non-sister lineages according to the mtDNA tree and hence are probably better explained by secondary contact rather than primary intergradation. Following a neutral model, values of 2NM (the effective number of migrants received by a population per generation; Table 2) greater than one can prevent populations from diverging (Wright, 1931; Pinho & Hey, 2012). The scenario of a migration rate > 1 was supported only for the Rondônia x Tapajós pairwise comparison (Table 2), with all others lineages pairs fitting better a scenario of divergence with very low levels or even absence of gene-flow (Pinho & Hey, 2010). Similarly, despite no signal of gene flow (Table 2; Fig 3b) or even admixture (Fig 2a), we detected an interspecific hybrid between *M. bambla* and *M. marginatus* where they come into contact along the Japurá-Solimões interfluve (MPEG 63199). This particular specimen has an mtDNA and plumage pattern of *M. marginatus*, but was assigned to a *M. bambla* cluster according to the nDNA STRUCTURE analyses (Fig 2a, b & c; map locality 60; Appendix S1, Supporting information), suggesting that eventual and apparently asymmetric events of hybridization take place between these two otherwise phenotypically divergent lineages (both according to vocal and morphological characters), and which have always been treated as separate species (Kroodsma & Brewer, 2005). As demonstrated for another Neotropical avian lineage (the antshrike genus *Cymbilaimus*), where two phenotypically and ecologically divergent species still hybridize where they meet in southwestern Amazonia (Miranda *et al*., *in prep.*; Chapter 1), our results confirm that reproductive isolation takes a very long time (in the case of *Microcerculus*, certainly more than 2.4 million years) to evolve and that it is best interpreted as an ancestral character that is lost only after two groups have evolved independently for a long time (Zink 2004).

Quaternary origin and diversification in the *M. bambla* / *M. marginatus* species complex

At a first glance, our findings are consistent with a major biogeographic pattern detected for several other avian lineages, whereby different modern interfluves in Amazonia harbor distinct evolutionary lineages (Aleixo & Rossetti, 2007; Ribas *et al*., 2012; d'Horta *et al*., 2013; Thom & Aleixo, 2015); however, the timing and mode of diversification in *Microcerculus* differ in several ways from those reported in the aforementioned studies. Our results provide statistical support for diversification within the *M. bambla /marginatus* complex occurring over the last 2.4 Myr, with ancestors giving rise to daughter lineages through founder events or peripatric speciation (instead of the commonly evoked *in-situ* vicariance), without going extinct (Fig 3a).

The first split in the *M. bambla / M. marginatus* complex separated those two species and is estimated as being of Early Pleistocene age (2.4 Myr). Our ancestral area reconstructions favored a distribution for the ancestor of the *M. bambla / M. marginatus* complex in northwestern Amazonia (=Napo and Imeri areas of endemism). According to this scenario, the first split separated the ancestor of *M. bambla* lineages (which was also most likely distributed in northwestern Amazonia) from that of *M. marginatus* lineages, which had similar probabilities for occurring in northwestern Amazonia and (more likely) in Central America, in the latter case after an inferred early dispersal episode through the Andes (Fig 3a). These results are concordant with geological data indicating that the southwestern and central Amazonian lowlands were covered with extensive lacustrine conditions until the Late Pliocene, prior to their draining by a newly formed transcontinental Amazon River which became established in Plio-Pleistocene times (Campbell *et al*., 2006; Latrubesse *et al*., 2010). Only about 1 million years later (between 1.4 – 1.1 Ma) did the next splits gave rise to the Central America and Napo lineages as well as the ancestor of the Brazilian Shield lineages from another ancestor originating in northwestern Amazonia (Fig 3a).

The Central American lineage originated around 1.4 Ma and thus greatly postdates the completion of the uplift of the central and northern Andes at 3.5 Ma (Gregory-Wodzicki, 2000) and the building up of the Panama Isthmus (Bacon *et al*., 2015). It has been demonstrated that multiple splitting events between *cis*- and *trans*-Andean lineages in several lowland restricted avian taxa spanned from the late Miocene to the late Pleistocene (Smith *et al*., 2014). Furthermore, given that *M. marginatus* occurs mainly below 1800m, but it has already recorded at 3100 m in Panama (Kroodsma & Brewer, 2005), the Andes probably did not function as a strong barrier to dispersal even nowadays, as also indicated by the ongoing gene flow estimated between the Central American and Napo lineages (Table 2; Fig 3b) as well as the presence of the same so called Inambari phylogroup on both sides of the Andes (Fig. 1). Moreover, the unsampled but supposedly closely related *trans*-Andean *M. marginatus* subspecies distributed from northwestern Venezuela, northern Colombia to north-western Ecuador could have originated from independent colonization events from *cis*-Andean populations, as demonstrated for *Dendrocincla* woodcreeper species (Weir *et al*., 2011), but sampling of these range-restricted taxa are necessary for confirmation.

Regarding the differentiation of lineages south of the Amazon River from those in northwestern Amazonia (Napo), the continuous subduction of the Nazca Ridge under the South American Plate caused the drainage in western Amazonia to evolve from a depositional system into an eastward fluvial sedimentary basin, allowing for the establishment of the modern upland *terra-firme* forest in south-western Amazonia, and organisms associated with it probably began to colonize this area (Aleixo & Rossetti, 2007). The timing of subsequent splits in *M. marginatus* (Inambari, Rondônia, Tapajós and Xingu lineages; *ca*. 0.2 Ma), given that the modern Amazon drainage was probably already in place by this time (Campbell *et al*., 2006; Latrubesse *et al*., 2010), agrees with a scenario of a major drainage reorganization during the early Pleistocene probably related to drainage capture events mediated by tectonics (Latrubesse, 2002; Rossetti & Valeriano, 2007; Wilkinson *et al*., 2010). These events could account for the occurrence of specimens of the Rondônia lineage on both sides of the Madeira River, as well as those belonging to the Tapajós lineage on both sides of the middle Tapajós and Teles Pires rivers (Fig. 1a). Interestingly, asymmetric gene flow was detected from the Inambari to the Rondônia phylogroup, which is consistent with the migration of the Madeira River channel eastward (Hayakawa & Rossetti 2015), therefore apparently transferring in a relative short period of time entire populations of the Rondônia phylogroup from the eastern to the western bank of the Madeira, where they entered in direct contact with members of the Inambari phylogroup. The presence of phylogroups west of the lower Madeira River that are nevertheless more closely related to those occurring across the river rather than any co-distributed phylogroups on the west bank, has been reported for at least three other avian lineages associated with the same habitat as *Microcerculus* (upland terra-firme forest; Aleixo, 2004; Patané *et al*., 2009; Miranda *et al*., *in prep*.; Chapter 1) and may reflect a community wide response to the shifting of the lower Madeira River eastward. A similar pattern exists for the Rondônia x Tapajós comparison, where significant asymmetric gene flow was detected from the Tapajós to the Rondônia phylogroup (Table 2). This pattern is also consistent with a change in the course of the Tapajós River and its upper tributaries such as the Teles Pires, whereby populations of the Tapajós phylogroup could have been "transferred" from the eastern to the western bank of the barrier, where they entered in direct contact with members of the Rondônia phylogroup (Fig. 1).

Alternatively, these instances of gene flow could simply result from dispersal across these rivers (which does not seem likely to occur in an understory insectivore lineage such as *Microcerculus*; see Moore *et al.*, 2008) or around the headwaters of these major Amazonian tributaries. The latter scenario also finds support in the contrasting overall low levels of gene flow detected among almost all lineages (Table 2; Fig 3b) and the presence of specimens with around 50% admixture from headwater regions (Fig 2d).

On the north bank of the Amazon, diversification within *M. bambla* into the Imeri and Guyana lineages separated by Negro and Branco rivers took place around 0.7 Mya. Geological evidence suggests the lower Negro River was already established as a barrier at this time, but its course suffered frequent shifts throughout the Quaternary in response to neotectonic events (Almeida-Filho & Miranda, 2007). Furthermore, the Negro-Branco interfluve is occupied by large patches of savannas and white-sand forests, which should also function as barriers for species associated with upland *terra-firme* forest (Borges, 2004; Naka, 2011). Even so, as verified among most phylogroups of *M. marginatus*, statistical support for gene flow at low rates from the Guyana to the Imeri was found (Table 2; Fig 3b), which indicates an advanced degree of evolutionary independence between them.

Founder event speciation and the role of river-barriers and contact zones

Herein we found strong evidence that all *Microcerculus* lineages diverged through a series of founder events, in which a small number of individuals disperse into previously unoccupied areas and founding an instantly genetically isolated population from the ancestral (Mayr, 1954). Rapid and large-scale present-day expansions are rarely observed in natural populations, which are mostly in equilibrium with their environment. Much of model systems for studying how founding events affect evolutionary potentials came from nonindigenous populations introduced to novel environments (Austerlitz *et al.*, 1997). On the one hand, it has been commonly argued that range expansions by founding events will result actually in a reduction of genetic diversity in populations at the expanding range front, due to the founder effect itself and differential selective forces (Austerlitz *et al.*, 1997); and thus, it may limit the evolutionary potential by increasing local-extinction risk. Conversely, it has been argued that such a period of relaxed selection could occur when a founder population is in an open ecological niche, allowing rapid population growth after the founder event (Slatkin, 1996). This hypothesis also fits the evolutionary scenario favored for *Microcerculus* in which the establishment of modern upland forest in southwestern Amazonia after the lacustrine system dried out, allowed the occupancy of a new uninhabited, ecologically open area. In further support of a founder event, the multilocus network (Fig 1b) shows alleles shared between lineages in ancestral positions, whereas more derived alleles tended to be species specific, although mtDNA exhibit fixed differences. This pattern is suggestive of genetic isolation following a period of low effective population sizes– consistent with founder event speciation (Mayr, 1954).

Both the mtDNA gene tree and the multilocus species tree showed phylogroups of the *M. bambla* / *marginatus* complex roughly separated by the modern courses of main Amazonian tributaries (Figs 1a & 3a), clear signals of demographic expansion in all lineages (Fig 4), as well as weak and asymmetrical gene flow among lineages in contact (Table 2; Fig 3b). Altogether, our results indicate that rivers play an important role as diversification barriers, although contact zones away from these main river courses reject simple *in situ* vicariance scenarios. The accumulation of phylogeographic data available now for Amazonian lineages support the notion that the courses of the Negro, Madeira, and Tapajós rivers have been stable for long periods of time until shifting at least some parts of them, and then remaining stable for an additional time (Ribas *et al.* 2012; Fernandes *et al.* 2013; Thom & Aleixo 2015). Hence, a dynamic drainage landscape, over long periods of time, can act like a speciation pump rather than prevent diversification, as previously thought (Haffer 1993; Colwell *et al.* 2000; Gascon *et al.* 2000). Understory avian lineages such as *Microcerclus* are particularly prone to diverge, even in the presence of gene flow, during periods in which a river barrier remains stable, therefore explaining patterns such as the ones recovered herein where different interfluvia are mostly occupied by one phylogroup, but which nevertheless exchanges migrants at very low rates with other neighbouring phylogroups where they come in contact directly or along more permeable barriers (Fig. 1; Table 2). Estimates of population expansion recovered for those phylogroups south of the Amazon (Inambari, Rondônia, Tapajós, and Xingu) post-date the timing inferred for their mutual divergences, even though 95% confidence intervals of these divergences do not falsify a possible expansion eastward following more humid conditions (Figs. 3 & 4). If this is correct, lineages dispersed through Amazonian rivers and diverged in different interfluvia more quickly due to successive founder events.

CONCLUSIONS

Only half of the 10 lineages of *M. bambla* / *marginatus* species complex revealed in this study are formally recognized taxonomically, indicating a very high degree of cryptic diversity. Our data and interpretations provide a framework for a model that integrates the phylogenetic and biogeographic history of *M. bambla* / *marginatus* lineages with current knowledge about the palaeogeographic history of Amazonia over the past 2.5 Myr. The ages of diversification inferred from a multilocus species tree for the *M. bambla* / *marginatus* complex including their confidence intervals completely overlap with the Pleistocene, thus corroborating the hypothesis that this period was very important for the diversification of many Neotropical lineages. Founder-event cladogensis, where a dispersal event across a barrier originates a new genetically isolated lineage, has long been considered crucial by many historical biogeographers, but it contrasts with classic *in situ* vicariance scenarios originally favored in the Neotropics mainly due to the concordance between distribution patterns and barriers such as Amazonian rivers and the Andes. As more phylogeographic studies pertaining to Neotropical lineages accumulate, it becomes clear that co-distributed and even endemic lineages do not necessarily differentiate in response to a common history; therefore, a different framework for thinking about diversification processes in the Neotropics needs to be considered. Under the *Microcerculus* diversification model presented herein, the likely dispersal of a small number of individuals from northwestern Amazonia across the Andes and southern Amazonian rivers, after the consolidation of the modern trans-continental drainage (ca. 2.5 Myr), led to multiple cladogenetic events and consequent divergence in allopatry, even in the presence of gene flow. Therefore, a balance between vicariance and dispersal accounts for the observed patterns of diversification documented herein. Given the stochastic and lineage-specific nature of dispersal, the increasingly important role attributed to this process in Neotropical biogeography could potentially explain the high degree of heterogeneity in inter-lineage responses to climate change and physical barriers, supporting independent rather than concerted events of diversification across the landscape.

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Supporting Information

Appendix S1 Collection locality, voucher number, institution of origin, and GenBank accession numbers.

Appendix S2 Primers and information on models.

Supporting Information

Appendix S1

Field_Numb	Museum	Tombo	Species		Lineages	Major_Area	coordenates		Map Loc	genbank accession number				
							Lat	Long		cytb	ND2	BF5	G3PDH	Myo
ANSP20859	ANSP	20859	*Microcerculus*	*bambla*	Guyana	Guyana	4.333	-58.783	32					
ANSP21375	ANSP	21375	*Microcerculus*	*bambla*	Guyana	Guyana	4.333	-58.783	33					
A 7842	INPA	A 7842	*Microcerculus*	*bambla*	Guyana	Guyana	-3.004	-59.941	19					
A 9021	INPA	A 9021	*Microcerculus*	*bambla*	Guyana	Guyana	-3.004	-59.941	27					
CN 916	MPEG	66372	*Microcerculus*	*bambla*	Guyana	Guyana	0.817	-53.917	37					
CN 947	MPEG	66373	*Microcerculus*	*bambla*	Guyana	Guyana	0.817	-53.917	38					
LSUMZ_B20203	LSUMZ	20203	*Microcerculus*	*bambla*	Guyana	Guyana	-2.417	-59.767	75					
LSUMZ_B20272	LSUMZ	20272	*Microcerculus*	*bambla*	Guyana	Guyana	-2.417	-59.767	76					
LSUMZ_B20280	LSUMZ	20280	*Microcerculus*	*bambla*	Guyana	Guyana	-2.417	-59.767	77					
TRO 006	MPEG	65996	*Microcerculus*	*bambla*	Guyana	Guyana	-1.667	-56.500	130					
USNM622171	USNM	622171	*Microcerculus*	*bambla*	Guyana	Guyana	1.583	-58.633	144					
USNM89062	USNM	89062	*Microcerculus*	*bambla*	Guyana	Guyana	7.367	-60.483	147					
USNM90868	USNM	90868	*Microcerculus*	*bambla*	Guyana	Guyana	2.200	-59.367	148					
A 072	INPA	A 072	*Microcerculus*	*bambla*	Imeri	West Amazon	-2.700	-64.617	1					
AMZ 431	MPEG	59728	*Microcerculus*	*bambla*	Imeri	West Amazon	-0.417	-62.933	29					
AMZ 453	MPEG	59729	*Microcerculus*	*bambla*	Imeri	West Amazon	-0.417	-62.933	30					
ANSP18750	ANSP	18750	*Microcerculus*	*bambla*	Imeri	West Amazon	-0.518	-77.170	150					
JAP 651	MPEG	63198	*Microcerculus*	*bambla*	Imeri	West Amazon	-1.717	-65.867	61					
JAP 660	MPEG	63197	*Microcerculus*	*bambla*	Imeri	West Amazon	-1.717	-65.867	62					
JAP 704	MPEG	63196	*Microcerculus*	*bambla*	Imeri	West Amazon	-1.717	-65.867	63					
LSUMZ_B7544	LSUMZ	7544	*Microcerculus*	*bambla*	Imeri	West Amazon	0.939	-66.047	95					
LSUMZ_B7562	LSUMZ	7562	*Microcerculus*	*bambla*	Imeri	West Amazon	0.939	-66.047	96					
A 667	INPA	A 667	*Microcerculus*	*marginatus*	Napo	West Amazon	-2.317	-68.417	18					
FMNH474396	FMNH	474396	*Microcerculus*	*marginatus*	Napo	West Amazon	-6.717	-77.417	55					
FMNH474397	FMNH	474397	*Microcerculus*	*marginatus*	Napo	West Amazon	-6.717	-77.417	56					
FMNH474398	FMNH	474398	*Microcerculus*	*marginatus*	Napo	West Amazon	-6.717	-77.417	57					

Field_Numb	Museum	Tombo	Species		Lineages	Major_Area	coordenates		Map	genbank accession number				
							Lat	Long	Loc	cytb	ND2	BF5	G3PDH	Myo
JAP 403	MPEG	63199	*Microcerculus*	*marginatus*	Napo	West Amazon	-1.933	-66.600	60					
LSUMZ_B11839	LSUMZ	11839	*Microcerculus*	*marginatus*	Napo	West Amazon	0.867	-78.550	73					
LSUMZ_B2513	LSUMZ	2513	*Microcerculus*	*marginatus*	Napo	West Amazon	-3.179	-72.904	79					
LSUMZ_B2640	LSUMZ	2640	*Microcerculus*	*marginatus*	Napo	West Amazon	-3.179	-72.904	80					
LSUMZ_B2662	LSUMZ	2662	*Microcerculus*	*marginatus*	Napo	West Amazon	-3.179	-72.904	81					
LSUMZ_B27847	LSUMZ	27847	*Microcerculus*	*marginatus*	Napo	West Amazon	-7.083	-75.650	82					
LSUMZ_B40824	LSUMZ	40824	*Microcerculus*	*marginatus*	Napo	West Amazon	-4.159	-77.160	86					
LSUMZ_B40856	LSUMZ	40856	*Microcerculus*	*marginatus*	Napo	West Amazon	-4.159	-77.160	87					
LSUMZ_B42842	LSUMZ	42842	*Microcerculus*	*marginatus*	Napo	West Amazon	-4.267	-77.233	89					
LSUMZ_B4459	LSUMZ	4459	*Microcerculus*	*marginatus*	Napo	West Amazon	-2.449	-74.015	90					
LSUMZ_B5393	LSUMZ	5393	*Microcerculus*	*marginatus*	Napo	West Amazon	-6.394	-76.340	93					
LSUMZ_B7077	LSUMZ	7077	*Microcerculus*	*marginatus*	Napo	West Amazon	-3.370	-72.110	94					
LSUMZ_B16131	LSUMZ	16131	*Microcerculus*	*marginatus*	Central America	Central America	8.684	-83.698	74					
LSUMZ_B2290	LSUMZ	2290	*Microcerculus*	*marginatus*	Central America	Central America	8.472	-77.721	78					
LSUMZ_B28551	LSUMZ	28551	*Microcerculus*	*marginatus*	Central America	Central America	9.260	-79.943	83					
LSUMZ_B28555	LSUMZ	28555	*Microcerculus*	*marginatus*	Central America	Central America	9.260	-79.943	84					
USNM607612	USNM	607612	*Microcerculus*	*marginatus*	Central America	Central America	8.639	-82.213	139					
USNM607613	USNM	607613	*Microcerculus*	*marginatus*	Central America	Central America	8.639	-82.213	140					
USNM607882	USNM	607882	*Microcerculus*	*marginatus*	Central America	Central America	9.083	-81.850	141					
USNM607883	USNM	607883	*Microcerculus*	*marginatus*	Central America	Central America	9.083	-81.850	142					
USNM614088	USNM	614088	*Microcerculus*	*marginatus*	Central America	Central America	9.296	-82.790	143					
USNM87018	USNM	87018	*Microcerculus*	*marginatus*	Central America	Central America	8.733	-82.250	145					
USNM87019	USNM	87019	*Microcerculus*	*marginatus*	Central America	Central America	8.733	-82.250	146					
A 177	INPA	A 177	*Microcerculus*	*marginatus*	Inambari	West Brazilian Shield	-9.517	-65.350	2					
A 190	INPA	A 190	*Microcerculus*	*marginatus*	Inambari	West Brazilian Shield	-9.517	-65.350	3					
A 200	INPA	A 200	*Microcerculus*	*marginatus*	Inambari	West Brazilian Shield	-9.517	-65.350	4					
A 3182	INPA	A 3182	*Microcerculus*	*marginatus*	Inambari	West Brazilian Shield	-9.156	-64.584	9					
A 3520	INPA	A 3520	*Microcerculus*	*marginatus*	Inambari	West Brazilian Shield	-9.150	-64.617	11					
A 3543	INPA	A 3543	*Microcerculus*	*marginatus*	Inambari	West Brazilian Shield	-9.300	-64.717	12					
A 4751	INPA	A 4751	*Microcerculus*	*marginatus*	Inambari	West Brazilian Shield	-8.533	-67.050	14					
A 790	INPA	A 790	*Microcerculus*	*marginatus*	Inambari	West Brazilian Shield	-3.600	-66.067	20					

Field_Numb	Museum	Tombo	Species		Lineages	Major_Area	coordenates		Map	genbank accession number				
							Lat	Long	Loc	cytb	ND2	BF5	G3PDH	Myo
A 7932	INPA	A 7932	*Microcerculus*	*marginatus*	Inambari	West Brazilian Shield	-4.983	-62.133	21					
A 8006	INPA	A 8006	*Microcerculus*	*marginatus*	Inambari	West Brazilian Shield	-4.983	-62.133	22					
CUJ 127	MPEG	60267	*Microcerculus*	*marginatus*	Inambari	West Brazilian Shield	-4.650	-68.317	39					
CUJ 183	MPEG	60268	*Microcerculus*	*marginatus*	Inambari	West Brazilian Shield	-4.933	-68.167	40					
ESEC 082	MPEG	58942	*Microcerculus*	*marginatus*	Inambari	West Brazilian Shield	-11.000	-70.217	41					
ESEC 200	MPEG	59888	*Microcerculus*	*marginatus*	Inambari	West Brazilian Shield	-11.050	-70.267	42					
ESEC 201	MPEG	59887	*Microcerculus*	*marginatus*	Inambari	West Brazilian Shield	-11.050	-70.267	43					
ESEC 232	MPEG	59889	*Microcerculus*	*marginatus*	Inambari	West Brazilian Shield	-11.050	-70.267	44					
FMNH323447	FMNH	323447	*Microcerculus*	*marginatus*	Inambari	West Brazilian Shield	-13.814	-71.234	45					
FMNH433723	FMNH	433723	*Microcerculus*	*marginatus*	Inambari	West Brazilian Shield	-13.017	-71.483	51					
FMNH433724	FMNH	433724	*Microcerculus*	*marginatus*	Inambari	West Brazilian Shield	-12.767	-71.383	52					
FMNH433725	FMNH	433725	*Microcerculus*	*marginatus*	Inambari	West Brazilian Shield	-12.767	-71.383	53					
FMNH433726	FMNH	433726	*Microcerculus*	*marginatus*	Inambari	West Brazilian Shield	-12.767	-71.383	54					
LSUMZ_B10638	LSUMZ	10638	*Microcerculus*	*marginatus*	Inambari	West Brazilian Shield	-9.193	-74.383	68					
LSUMZ_B106784	LSUMZ	106784	*Microcerculus*	*marginatus*	Inambari	West Brazilian Shield	-15.500	-67.117	69					
LSUMZ_B10697	LSUMZ	10697	*Microcerculus*	*marginatus*	Inambari	West Brazilian Shield	-9.193	-74.383	70					
LSUMZ_B1092	LSUMZ	1092	*Microcerculus*	*marginatus*	Inambari	West Brazilian Shield	-15.290	-67.590	71					
LSUMZ_B1139	LSUMZ	1139	*Microcerculus*	*marginatus*	Inambari	West Brazilian Shield	-15.290	-67.590	72					
LSUMZ_B4675	LSUMZ	4675	*Microcerculus*	*marginatus*	Inambari	West Brazilian Shield	-3.497	-72.936	91					
LSUMZ_B4734	LSUMZ	4734	*Microcerculus*	*marginatus*	Inambari	West Brazilian Shield	-3.497	-72.936	92					
LSUMZ_B8883	LSUMZ	8883	*Microcerculus*	*marginatus*	Inambari	West Brazilian Shield	-11.470	-68.779	97					
LSUMZ_B9127	LSUMZ	9127	*Microcerculus*	*marginatus*	Inambari	West Brazilian Shield	-11.470	-68.779	98					
PUC 182	MPEG	57199	*Microcerculus*	*marginatus*	Inambari	West Brazilian Shield	-4.850	-65.067	119					
PUC 256	MPEG	58312	*Microcerculus*	*marginatus*	Inambari	West Brazilian Shield	-4.867	-65.317	120					
RUR 047	MPEG	62364	*Microcerculus*	*marginatus*	Inambari	West Brazilian Shield	-4.850	-65.067	121					
RUR 089	MPEG	62366	*Microcerculus*	*marginatus*	Inambari	West Brazilian Shield	-4.933	-65.283	122					
UFAC 1442	MPEG	63416	*Microcerculus*	*marginatus*	Inambari	West Brazilian Shield	-9.100	-69.817	132					
UFAC 1451	MPEG	63417	*Microcerculus*	*marginatus*	Inambari	West Brazilian Shield	-9.150	-69.750	133					
UFAC 1730	MPEG	64477	*Microcerculus*	*marginatus*	Inambari	West Brazilian Shield	-10.633	-67.800	134					
UFAC 584	MPEG	60872	*Microcerculus*	*marginatus*	Inambari	West Brazilian Shield	-9.317	-68.333	135					
A 251	INPA	A 251	*Microcerculus*	*marginatus*	Rondonia	East Brazilian Shield	-9.465	-65.336	5					

Field_Numb	Museum	Tombo	Species		Lineages	Major_Area	coordenates		Map Loc	genbank accession number				
							Lat	Long		cytb	ND2	BF5	G3PDH	Myo
A 281	INPA	A 281	*Microcerculus*	*marginatus*	Rondonia	East Brazilian Shield	-6.377	-60.389	6					
A 303	INPA	A 303	*Microcerculus*	*marginatus*	Rondonia	East Brazilian Shield	-8.876	-63.973	7					
A 3262	INPA	A 3262	*Microcerculus*	*marginatus*	Rondonia	East Brazilian Shield	-9.246	-64.379	10					
A 457	INPA	A 457	*Microcerculus*	*marginatus*	Rondonia	East Brazilian Shield	-6.300	-60.400	13					
A 480	INPA	A 480	*Microcerculus*	*marginatus*	Rondonia	East Brazilian Shield	-6.300	-60.333	15					
A 6212	INPA	A 6212	*Microcerculus*	*marginatus*	Rondonia	East Brazilian Shield	-9.350	-64.433	16					
A 6249	INPA	A 6249	*Microcerculus*	*marginatus*	Rondonia	East Brazilian Shield	-9.017	-64.233	17					
A 8129	INPA	A 8129	*Microcerculus*	*marginatus*	Rondonia	East Brazilian Shield	-5.717	-63.200	23					
A 8240	INPA	A 8240	*Microcerculus*	*marginatus*	Rondonia	East Brazilian Shield	-5.717	-63.200	25					
A 901	INPA	A 901	*Microcerculus*	*marginatus*	Rondonia	East Brazilian Shield	-7.590	-60.753	26					
FMNH392166	FMNH	392166	*Microcerculus*	*marginatus*	Rondonia	East Brazilian Shield	-9.876	-56.086	47					
FMNH392168	FMNH	392168	*Microcerculus*	*marginatus*	Rondonia	East Brazilian Shield	-9.876	-56.086	48					
FPR 036	MPEG	67102	*Microcerculus*	*marginatus*	Rondonia	East Brazilian Shield	-3.933	-58.450	58					
FPR 042	MPEG	67101	*Microcerculus*	*marginatus*	Rondonia	East Brazilian Shield	-3.933	-58.450	59					
LGEMA13809	LGEMA	13809	*Microcerculus*	*marginatus*	Rondonia	East Brazilian Shield	-9.433	-56.767	65					
LGEMA14010	LGEMA	14010	*Microcerculus*	*marginatus*	Rondonia	East Brazilian Shield	-9.317	-56.767	66					
LGEMA486	LGEMA	486	*Microcerculus*	*marginatus*	Rondonia	East Brazilian Shield	-10.173	-59.456	67					
MAR 127	MPEG	57776	*Microcerculus*	*marginatus*	Rondonia	East Brazilian Shield	-8.683	-61.400	99					
MPDS 702	MPEG	58795	*Microcerculus*	*marginatus*	Rondonia	East Brazilian Shield	-7.550	-62.550	104					
OP 086	MPEG	55072	*Microcerculus*	*marginatus*	Rondonia	East Brazilian Shield	-10.833	-64.750	105					
OP 087	MPEG	55073	*Microcerculus*	*marginatus*	Rondonia	East Brazilian Shield	-10.833	-64.750	106					
OP 088	MPEG	55074	*Microcerculus*	*marginatus*	Rondonia	East Brazilian Shield	-10.833	-64.750	107					
OP 119	MPEG	55071	*Microcerculus*	*marginatus*	Rondonia	East Brazilian Shield	-10.833	-64.750	108					
PIME 447	MPEG	69630	*Microcerculus*	*marginatus*	Rondonia	East Brazilian Shield	-5.600	-57.583	111					
TLP(A) 268	MPEG	69506	*Microcerculus*	*marginatus*	Rondonia	East Brazilian Shield	-9.400	-56.550	123					
TLP(A) 292	MPEG	69507	*Microcerculus*	*marginatus*	Rondonia	East Brazilian Shield	-9.400	-56.550	124					
TLP(B) 068	MPEG	67597	*Microcerculus*	*marginatus*	Rondonia	East Brazilian Shield	-9.467	-56.467	125					
TLP(B) 245	MPEG	69508	*Microcerculus*	*marginatus*	Rondonia	East Brazilian Shield	-9.550	-56.750	126					
TLP(C) 014	MPEG	67598	*Microcerculus*	*marginatus*	Rondonia	East Brazilian Shield	-9.382	-56.546	127					
TLP(C) 112	MPEG	69505	*Microcerculus*	*marginatus*	Rondonia	East Brazilian Shield	-9.382	-56.546	128					
TLP(C) 161	MPEG	69504	*Microcerculus*	*marginatus*	Rondonia	East Brazilian Shield	-9.382	-56.546	129					

Field_Numb	Museum	Tombo	Species		Lineages	Major_Area	coordenates		Map Loc	genbank accession number				
							Lat	Long		cytb	ND2	BF5	G3PDH	Myo
TUP 040	MPEG	68930	*Microcerculus*	*marginatus*	Rondonia	East Brazilian Shield	-4.083	-60.650	131					
ACTA016	MPEG	58637	*Microcerculus*	*marginatus*	Tapajos	East Brazilian Shield	-2.600	-48.333	28					
BR163-203	MPEG	59280	*Microcerculus*	*marginatus*	Tapajos	East Brazilian Shield	-6.200	-55.683	34					
BR163-204	MPEG	59281	*Microcerculus*	*marginatus*	Tapajos	East Brazilian Shield	-6.200	-55.683	35					
BR163-205	MPEG	59282	*Microcerculus*	*marginatus*	Tapajos	East Brazilian Shield	-6.200	-55.683	36					
FMNH392170	FMNH	392170	*Microcerculus*	*marginatus*	Tapajos	East Brazilian Shield	-9.876	-56.086	49					
MPDS 1234	MPEG	65239	*Microcerculus*	*marginatus*	Tapajos	East Brazilian Shield	-6.517	-57.350	102					
MPDS 1321	MPEG	65717	*Microcerculus*	*marginatus*	Tapajos	East Brazilian Shield	-6.517	-57.350	103					
PIME 105	MPEG	67650	*Microcerculus*	*marginatus*	Tapajos	East Brazilian Shield	-3.467	-54.550	109					
PIME 311	MPEG	69556	*Microcerculus*	*marginatus*	Tapajos	East Brazilian Shield	-6.583	-56.100	110					
PIME 451	MPEG	69634	*Microcerculus*	*marginatus*	Tapajos	East Brazilian Shield	-5.600	-57.583	112					
PIME 507	MPEG	69688	*Microcerculus*	*marginatus*	Tapajos	East Brazilian Shield	-7.117	-55.717	113					
FMNH391584	FMNH	391584	*Microcerculus*	*marginatus*	Xingu	East Brazilian Shield	-6.141	-50.331	46					
LGEMA10207	LGEMA	10207	*Microcerculus*	*marginatus*	Xingu	East Brazilian Shield	-2.600	-48.783	64					
MAYA 051	MPEG	61151	*Microcerculus*	*marginatus*	Xingu	East Brazilian Shield	-1.550	-50.383	100					
MAYA 053	MPEG	61150	*Microcerculus*	*marginatus*	Xingu	East Brazilian Shield	-1.550	-50.383	101					
PPBIO 051	MPEG	61945	*Microcerculus*	*marginatus*	Xingu	East Brazilian Shield	-1.950	-51.600	114					
PPBIO 173	MPEG	61946	*Microcerculus*	*marginatus*	Xingu	East Brazilian Shield	-1.950	-51.600	115					
PPBIO 215	MPEG	61949	*Microcerculus*	*marginatus*	Xingu	East Brazilian Shield	-1.950	-51.600	116					
PPBIO 289	MPEG	61948	*Microcerculus*	*marginatus*	Xingu	East Brazilian Shield	-1.950	-51.600	117					
PPBIO 309	MPEG	61947	*Microcerculus*	*marginatus*	Xingu	East Brazilian Shield	-1.950	-51.600	118					
UHE266	MPEG	55816	*Microcerculus*	*marginatus*	Xingu	East Brazilian Shield	-3.517	-51.717	136					
UHE438	MPEG	55815	*Microcerculus*	*marginatus*	Xingu	East Brazilian Shield	-3.517	-51.717	137					
UHE468	MPEG	55814	*Microcerculus*	*marginatus*	Xingu	East Brazilian Shield	-3.517	-51.717	138					

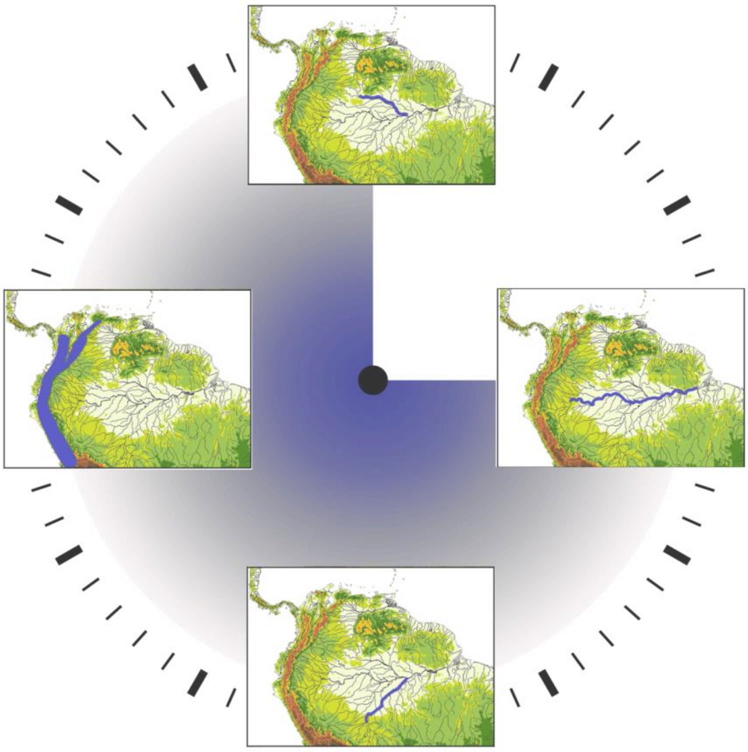
Appendix S2

Primers	Sequências (5' – 3')	Referências	Anelling Temp. [ºC]
Cytochrome *b* (cyt b)			
*L14841	AAC TGC AGT CAT CTC CGG TTT ACA AGA C	Kocher et al. (1989)	48-46[b]
*L15298	TGA GGC CAA ATA TCA TTC TGA GGG GC	Cheng et al. (1994)	
H15499	GGT TGT TTG AGC CTG ATT C	Avise et al. (1994)	
*H16065	AAC TGC AGT CAT CTC CGG TTT ACA AGA C	Sorenson et al. (1999)	
NADH Deidrogenase 2 (ND2)			
L5215	TAT CGG GCC CAT ACC CCG AAA T	Hackett (1996)	55[a]
H6313	CTC TTA TTT AAG GCT TTG AAG GC	Sorenson et al. (1999)	
β-fibrinogen intron 5 (bf5)			
S713	CGC CAT ACA GAG TAT ACT GTG ACA T	Weissbach et al. (1991)	50-48[b]
AS767	GCC ATC CTG GCG ATC TGA A	Weissbach et al. (1991)	
Glyceraldehyde-3-phospho-dehydrogenase intron 11 (G3PDH)			
G3P-13b	TCC ACC TTT GAT GCG GGT GCT GGC AT	Fjeldså et al. (2003)	60[a]
G3P-14b	AAG TCC ACA ACA CGG TTG CTG TA	Fjeldså et al. (2003)	
Myoglobin intron 2 (Myo)			
Myo2	GCC ACC AAG CAC AAG ATC CC	Slade et al. (1993)	50-48[b]
Myo3F	TTC AGC AAG GAC CTT GAT AAT GAC TT	Heslewood et al. (1998)	

(*) Primers used in sequencing
(a) Cycling parameter: 35x
(b) Touchdown protocol

Primers	**Best fit model (BIC)**	**nst**	**gamma**	**pinvar**	**-ln(L)**
Cytochrome *b* (cyt b)	TPM1uf+I	6	-	0.729	3128.2186
NADH Dehydrogenase 2 (ND2)	TIM2+I+G	6	0.644	0.484	4929.1245
mtDNA	TIM1+I+G	6	0.587	0.547	8583.5430
β-fibrinogen intron 5 (bf5)	HKY+I	2	-	0.831	1381.4660
Glyceraldehyde-3-phospho-dehydrogenase intron 11 (G3PDH)	K80+I	2	-	0.858	829.1550
Myoglobin intron 2 (Myo)	TrNef+G	6	0.197	-	1377.0869

Capitulo 3



Counterclockwise: A recurrent pattern of diversification in the Amazon

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Keywords

phylogeography, dispersal, incomplete lineage sorting, gene flow

Running head: A recurrent pattern of diversification in the Amazon

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Abstract

Until now, the topic of Neotropical biodiversity has been addressed from two main perspectives: the Pleistocene-refugial hypothesis and the Neogene tectonic hypothesis. Here, we discuss the phylogeographic structure of two passerine species complexes (*Cymbilaimus lineatus* / *sanctaemariae* and *Microcerculus bambla* / *marginatus*) that belong to two different avian families and sub-orders under the perspective that lineage-specific attributes are predicted to be the main drivers of species diversity. We compare large-scale spatio-temporal patterns of diversification between these lineages, maximizing the geographical sampling and accounting for population level phenomena such as demographic fluctuations and gene flow. Setting aside the idiosyncratic variation in spatial and temporal patterns, both taxa appear to follow the same pattern of colonization of the Brazilian Shield in southern Amazonia and Central America from ancestral areas on the Guianan shield through dispersal events, a recurrent phylogeographic pattern also observed in many other Amazonian avian lineages.

Introduction

Over the past few decades, the development of molecular phylogenetic methods has provided crucial evidence, mainly in relation to the age of extant species (Avise 2009; Knowles 2009). And, hitherto, the topic of Neotropical biodiversity has been addressed from two main perspectives: the Pleistocene-refugial hypothesis and the Neogene tectonic hypothesis (i.e., Garzón-Orduña et al. 2014; Ribas et al. 2012). On one hand, apart from a simply chronological reason, if diversification occurred during or before the Pleistocene, the principal drivers of speciation would have been very different. Nevertheless, none of these hypotheses would be applicable across all groups, and both Neogene orogeny and palaeogeographical reorganizations, and Quaternary climatic cycles seem to have been equally important drivers of the origin of Neotropical biodiversity (Rull 2015). In effect, rather than being directly linked to landscape change, the origin and maintenance of biodiversity seems likely to depend on the local of origin, timing of diversification and the ability to move through the landscape matrix (Simth et al. 2014).

Because of most Neotropical diversification models show allopatric speciation as the primary mode of differentiation, strongly supported by geographical delimited populations separated by rivers and mountains (Ribas et al. 2012; Fernades et al. 2013; Thom and Aleixo 2015), vicariance is widely invoked to explain lineage or species diversification. Although these apparent spatial commonality (i.e., areas of endemism), substantial variation in the timing and spatial sequence of diversification associated with barriers have been demonstrated (Smith et al. 2014). Furthermore, modes of geographic divergence other than vicariance, such as dispersal-mediated allopatry, maybe important. Nevertheless, even in cases where a phylogenetic study included taxa composing a suite of habitats – to provide a more robust test of diversification mechanisms – the analysis did not include ancestral reconstructions (Ribas et al. 2012; Fernades et al. 2013; Thom and Aleixo 2015); and inferring historical biogeographic patterns requires methods for reconstructing ancestral ranges on phylogenetic trees.

Here, we discuss the phylogeographic structure of two passerine species complex (*Cymbilaimus lineatus* / *sanctaemariae* and *Microcerculusbambla* / *marginatus*) that belong to two different avian families showing that temporal patterns of genetic differentiation in Neotropical birds are discordant across lineages, despite some spatial congruence. Beyond, they are not compatible with a model linking speciation solely to landscape change but to a greater extent by dispersal events.

Models and Results

Both species complex are relatively common insectivorous birds with a wide distribution in lowland and foothill humid forests ranging throughout the entire Amazon Basin and Central America, west to the Andes. However, *Cymbilaimus* (Suboscines; Thamnophilidae) consists of birds inhabiting the mid-story canopy, mainly in dense forest cover but also near treefall gaps and along forest edge (Zimmer & Isler 2003); in contrast, *Microcerculus* (Oscines; Troglodytidae) are very terrestrial birds (skulking on or near the ground), found mainly in forest with dense tangles, rich in rotting logs, and near treefall gaps (Kroodsma & Brewer, 2005). Altogether both lineages present ecological characteristics that are associated with the ability of crossing gaps in habitat and consequently can be interpreted as an indirect measure of dispersal capacity (Stotz et al. 1996).

We found strong phylogeographic structure in both species complexes, which presented breaks in approximately the same areas. Phylogeographic patterns were identified based on a multilocus species tree topology, and supported by haplotype networks and Bayesian clustering analyzes (see Miranda et al. *in prep*; cap. 1 and cap. 2). For *Cymbilaimus* we found five well-supported phylogroups and, although the Bayesian clustering analysis of the nDNA loci recovered three slightly structured groups, with the most common alleles spread across the entire *C. lineatus* range, but others mostly restricted to specific areas similar to the ranges of the recovered mtDNA lineages. Furthermore, our analyses detected evidence of paraphyletic and unidirectional gene flow between pairs of phylogroups. The spatio-temporal pattern suggests the most likely center of origin for *Cymbilaimus* as northwestern Amazonia (equivalent to the Negro-Solimões rivers interfluve) and a diversification time frame of 0.8 My (see Miranda et al. *in prep*; cap. 1). For *Microcerculus* 10 reciprocally monophyletic mtDNA lineages were recovered mostly in accordance with the nuclear-based network and Bayesian clustering analyses. Our inferences favored scenarios of very low levels of asymmetrical gene flow among five out of seven lineages pairs potentially in contact. The time frame for the diversification of *Microcerculus* was ca. 2.4 Mya, with a likely origin in northwestern Amazonia before dispersing into both the Guiana and Brazilian shields, as well as Central America (see Miranda et al. *in prep*; cap. 2).

Comparative Spatio-temporal Pattern

The patterns observed are spatially varied in some aspects, indicating evolutionary histories shaped by species-specific historical and ecological variables, such as center of origin and dispersal ability (Fig. 1). *Cymbilaimus* show a wider range ancestral distribution that included West and North of Amazon; whereas *Microcerculus* ancestral was inferred to occurs in Western Amazon. Comparisons of divergence time estimates demonstrated that first splits events also occurred in different times: *Microcerculus* at

2.4 Mya and *Cymbilaimus* at 0.8 Mya. Although the dissimilarities among temporal patterns, mainly in relation to the amount of time the taxa occupies the landscape (*Microcerculus*: ~2.4 Mya and *Cymbilaimus* ~0.8 Mya), we found striking concordance for the Brazilian Shield lineages diversification, for which in both cases was estimated to occur around 0.2 Mya (see Fig. 3 cap.1 and Fig. 3 cap. 2 in Miranda et al. *in prep*). Yet, it is noteworthy to mention that the most pronounced difference is the timing of diversification of Central America lineages (Fig. 1), which for *Microcerculus* corresponds to one of the earliest diversification (around 1.4 Mya) while for *Cymbimlaimus* to one of the latest (around 0.2 Mya). Nevertheless, setting aside the idiosyncratic variation in spatial and temporal patterns both appear to follow the same pattern of colonization of the Brazilian Shield and Central America from the ancestral area in West Amazon and/or Guyana by dispersal events.

Concluding Remarks

We gathered and analyzed an unprecedented amount of data for 2 understudied birds species from pan-Amazonia region. As far as we are aware, this study is the first to compare spatio-temporal pattern of Neotropical diversification in such a large scale that maximizes the geographical sampling to avoid underestimate cryptic diversity and that also account for others population level phenomena such as demographic fluctuations and gene flow. When compared to other Amazon avian lineages which were also shown phylogeographical breaks in the same areas, the order in which different barriers accounted for splits of lineages of the *Cymbilaimus* and *Microcerculus* complexes resembles significantly from most of them.

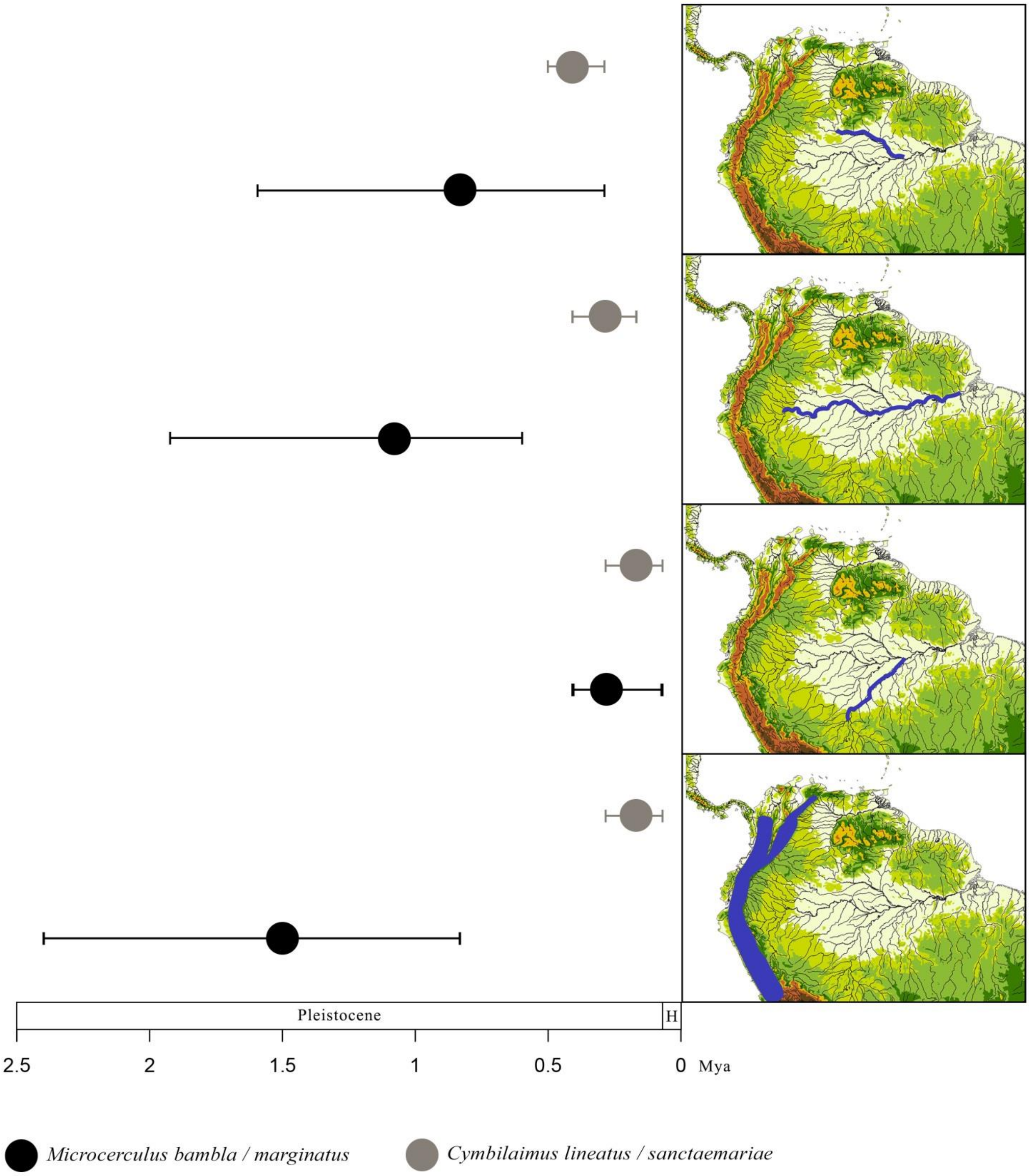

Figure 1 – Divergence times for two taxon pairs across barriers: Negro, Amazonas-Solimões and Madeira Rivers, and Andes. Estimates were derived from multilocus species tree inference assuming a relaxed log-normal 2.1% mitochondrial clock in the program BEAST. Circles represent mean estimates and bars represent the 95% highest posterior density.

A recurrent phylogeographic pattern in the Amazon is that where earliest splits usually involve lineages in Western Amazon or Guyana shield and the most recent splits encompass the east of Madeira River lineages (Fernandes et al., 2013; Ribas et al., 2012; Carneiro et al., 2012; Batista et al., 2013; d'Horta et al., 2013; Rodrigues et al., 2013; Sousa-Neves et al., 2013; Aleixo et al. 2014).

Based on the idea that species with overlapping ranges also share common histories, one would expect concordant histories among co-distributed lineages under the model of landscape changing. Conversely, our results show that two unrelated bird species, displaying different natural histories but inhabiting the same areas, presented some spatial pseudocongruence in the phylogeographic structure with each species showing breaks in roughly the same areas despite different time (Fig. 1). According to some estimates, the modern pan-Amazon landscape was already in place about 2.5 Mya (Campbell et al. 2006) and, once all lineages we identified originated during the Pleistocene, there appears to be no signature of large-scale landscape changes having had a pronounced effect. Instead, indicates that distinct lineages respond differently to the process of landscape evolution in Amazonia.

An alternative, recently proposed model posits that lineage-specific attributes (dispersal abilities) are predicted to be the main drivers of species diversity within lineages (Smith et al. 2014). In accordance with this model, the longer a lineage occupies the landscape higher the likelihood of dispersing across geographical barriers and diversifying (i.e., *Microcerculus*); as well as, lineages with higher dispersal ability are expected to reach wide range more quickly than less dispersive lineages and accrue genetic differentiation between populations at a relatively lower rates (i.e., *Cymbilaimus*). Moreover, beyond he amount of time a lineage has persisted in the landscape, the differences in the relative order and timing in which different barriers account for splits throughout the Amazon are related to the geographic origins of the ancestral populations of these lineages.

Finally, our finds do not exclude the role of the barriers in restrain the movement of individuals across them, neither the effects of climatic oscillation in promoting geographical isolation, speciation and expansion. After all, as verified by the phylogeographic studies conducted so far, *trans*-and *cis*-Andes, and modern interfluves in Amazonia harbor distinct evolutionary lineages, but with little gene flow among them.

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