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**PADRÕES DE DISTRIBUIÇÃO GEOGRÁFICA DE LINHAGENS INTRA-ESPECÍFICAS E
PROCESSOS DEMOGRÁFICOS HISTÓRICOS EM AVES DA FLORESTA ATLÂNTICA**

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RESUMO

O objetivo geral da presente Tese foi estudar a evolução de organismos da Floresta Atlântica (FA), utilizando aves florestais como modelos e sob uma perspectiva filogenética – filogeográfica. As espécies estudadas foram três passeriformes: *Xiphorhynchus fuscus* (Dendrocolaptidae), *Dendrocolaptes platyrostris* (Dendrocolaptidae) e *Schiffornis virescens* (Tytyridae). Os marcadores utilizados foram seqüências do DNA mitocondrial e/ou um marcador nuclear. O trabalho foi dividido em seis capítulos. No primeiro é apresentado o problema geral e as principais hipóteses (basicamente refúgios, rios e geotectonismo) que foram exploradas nos capítulos dois a cinco. No segundo capítulo foi utilizado DNAMt para avaliar a estrutura genética populacional de *Xiphorhynchus fuscus* no sul da FA e testar algumas hipóteses de evolução das populações dessa ave. Os resultados principais do trabalho não sustentaram um modelo geotectônico como explicação para a origem da estrutura genética dessa ave, mas seriam compatíveis com o modelo de refúgios florestais. No terceiro capítulo *X. fuscus* foi novamente estudado, mas avaliamos a estrutura filogeográfica da espécie em toda sua distribuição e com dois marcadores genéticos independentes (DNAMt e o intron 5 do beta fibrinogênio). Alguns dos objetivos principais do estudo foram testar uma hipótese paleofitogeográfica da FA para a última glaciação e avaliar as implicações dos resultados genéticos para o status taxonômico das quatro subespécies e de *X. fuscus*. Os resultados genéticos sustentaram parcialmente o modelo paleofitogeográfico testado e mostraram que unicamente *X. fuscus atlanticus* poderia ser considerada uma espécie plena. O quarto capítulo descreve a variação da plumagem e a estrutura genética populacional (DNAMt) de *D. platyrostris*. Os resultados sugeriram que não existe uma continuidade recente entre as populações do domínio central da FA e as populações da diagonal aberta (Cerrado, Caatinga e Chaco). Além disso, foi estudada a relação entre a plumagem de *D. platyrostris* com a história das populações e encontramos que a coloração da plumagem não tem relação com a história das populações. O quinto capítulo apresenta um estudo preliminar da estrutura filogeográfica (DNAMt) de *S. virescens*. O estudo indicou que não há estruturação filogeográfica profunda *S. virescens* como encontrada em outros organismos da FA. Uma possível origem deste padrão poderia ser um gargalo populacional e expansão demográfica recente, além de altas taxas de fluxo gênico. Nossos resultados com as três espécies estudadas, assim como os de outros autores, sugerem a existência de um padrão filogeográfico comum para alguns organismos da FA. Além disso, foi sugerido um modelo da FA no qual a distribuição temporal

e espacial das florestas é muito dinâmica. O norte e sul da FA seriam as regiões mais instáveis devido à influência de mudanças climáticas locais e globais. Esta dinâmica florestal pode ter contribuído na evolução dos padrões filogeográficos observados, com a evolução independente de linhagens em diferentes regiões ou refúgios. Além disso, nossos resultados e os de outros autores sugerem que a atividade geotectônica não teria sido importante na evolução recente de organismos da FA, e que alguns rios do bioma (ex. rio Doce) atuariam como barreiras secundárias e não como barreiras primárias.

ABSTRACT

This dissertation explores the evolution of Atlantic Forest (AF) organisms. Specifically, we used endemic forest birds as models and a phylogenetic – phylogeographic perspective. Studied bird species are all passerines: *Xiphorhynchus fuscus* (Dendrocolaptidae), *Dendrocolaptes platyrostris* (Dendrocolaptidae) and *Schiffornis virescens* (Tyrtyridae). We used mitochondrial and nuclear DNA (mtDNA and ncDNA, respectively) sequences. The dissertation was divided in six chapters. The first one explained objectives of the dissertation and presented hypotheses and problems that were addressed in the following chapters two to five. The sixth chapter presents a general discussion. We used in the second chapter mtDNA to evaluate the phylogeographic pattern of *X. fuscus* in southern AF. The main results indicated that a geotectonic model for the evolution of local populations was not supported and that the observed genetic pattern was compatible with the theory of refuges. In the third chapter we studied again *X. fuscus*, but using samples from all the species' distribution and sequences of two independent markers (mtDNA and the intron 5 of the beta-fibrinogen gene). Some of the main objectives of the chapter were to test a published model about the distribution of AF in the last 20,000 years and to use genetic data to address the taxonomic status of the four subspecies of *X. fuscus*, particularly of the endangered *X. fuscus atlanticus*. Results supported partially the model of forest distribution in the past and to consider *X. fuscus atlanticus* as a good species. In the fourth chapter we analyzed plumage and genetic (mtDNA) variation in the woodcreeper *D. platyrostris*. Results suggested a lack of recent genetic continuity between the Atlantic Forest central domain and the open vegetation biomes Cerrado and Caatinga. Besides, we found that plumage color variation in *D. platyrostris* was not related to population history, as evaluated by neutral markers. In the fifth chapter we studied the phylogeographic pattern (mtDNA) of *S. virescens*. Results indicated that *S. virescens* lack a significant phylogeographic pattern, contrarily to what was observed in several other AF organisms. Possible explanations for this pattern may be a population bottleneck followed by a demographic expansion and current high gene flow rates. Our results, in combination with results of others, permitted to describe a common phylogeographic pattern for several AF organisms. Also, we suggested a model of the AF where forest distribution is very dynamic. Specifically, the northern and southern AF regions were proposed to be the regions with the most unstable area and continuity of forests. Perhaps, this forest dynamism contributed to the evolution of the observed common

phylogeographic pattern. Furthermore, our results, in combination with other similar studies, suggested for the studied birds that geotectonic activity at southeastern AF did not affect population evolution, and that some AF rivers (i.e., Doce river) act as secondary barriers instead of primary barriers.

CAPÍTULO 1

INTRODUÇÃO GERAL

Floresta Atlântica: limites, diversidade e conservação

A Floresta Atlântica (FA) possui 1.713.000 km² distribuídos ao longo de uma faixa no leste da América do Sul, desde o Rio Grande do Norte até o Rio Grande do Sul, sendo o limite leste a costa Atlântica e o limite oeste o sul do Estado do Mato Grosso do Sul, o leste do Paraguai e Misiones na Argentina (Galindo Leal e Câmara, 2003) (Fig. 1A). Originalmente, mais de 75% da superfície do bioma era coberto por formações arbóreas densas com estruturas e composições particulares (p. ex., floresta ombrófila mista com *Araucaria angustifolia*) e o restante possuía encaves de formações abertas ou secas (Rizzini, 1997).

A FA destaca-se pelo número de espécies e endemismos que abriga. Segundo Myers et al (2000) 8000 espécies de plantas de um total mundial de 300.000 e 567 espécies de vertebrados de um total de 27.298 são endêmicas do bioma, representando 2,7% e 2,1% das espécies do mundo, respectivamente. Dentre os vertebrados que ocorrem neste bioma, 90% dos anfíbios, 30% dos répteis, 27,9% dos mamíferos e 29,1% das aves são endêmicos.

Além disso, a FA é um dos ecossistemas mais ameaçados do mundo, já que apenas 7,4% da sua superfície original ainda persiste (Galindo Leal e Câmara, 2003). Esse território constitui atualmente de uma paisagem de áreas dedicadas à exploração agropecuária e remanescentes de florestas isolados. A maioria desses remanescentes não supera 1000 ha (Fonseca, 1985), com exceção dos contínuos florestais maiores que 1.000.000 ha compreendidos por diversas unidades de conservação na Serra do Mar e no interior do estado do Paraná junto com a província de Misiones na Argentina.

Diversificação da Floresta Atlântica

A FA é um bioma heterogêneo, por isso é difícil descrever sua origem e evolução. O conhecimento dos fenômenos evolutivos atuantes em um bioma particular é importante para fins acadêmicos e de conservação biológica (Moritz, 2002). O problema específico estudado na presente Tese é a história evolutiva de organismos da FA, no nível de populações e espécies. Ao longo do trabalho se assume que os mecanismos envolvidos na evolução de populações (geradores de estrutura genética populacional) são os

mesmos que atuam na evolução de espécies (especiação). Assim, o estudo da evolução de populações seria uma maneira de inferir fenômenos e testar hipóteses acerca de especiação (Lara et al., 2005).

Existem diversas hipóteses sobre a diversificação das biotas de florestas tropicais e subtropicais, sendo incluídas entre as mais discutidas para a FA, a teoria dos refúgios (Brown e Ab'Saber, 1979; Haffer, 1969; Haffer e Prance, 2001; Vanzolini e Williams, 1970), a hipótese dos museus (Fjeldsa, 1994; Fjeldsa e Lovett, 1997), a teoria dos rios como fator de isolamento (Patton e da Silva, 2005; Wallace, 1852), a teoria do geotectonismo (revisada em Marroig e Cerqueira, 1997) e a hipótese dos gradientes de habitat ou de diversificação parapátrida por seleção (Smith et al., 1997).

No Neotrópico, a teoria dos refúgios foi levantada inicialmente para explicar a diversificação biológica na Floresta Amazônica durante o Pleistoceno. A hipótese considera os períodos secos e frios coincidentes com as glaciações como determinantes do retrocesso das florestas até remanescentes isolados (paleorefúgios) por áreas abertas (p.ex., pampas ou caatingas). Assim, os organismos florestais isolados nos paleorefúgios por um tempo suficiente, teriam divergido formando novos táxons (divergência em alopatria). A hipótese requer a expansão de áreas abertas e a formação de paleorefúgios florestais. Segundo alguns autores esses eventos na Amazônia não teriam ocorrido (Bush, 1994; Colinvaux e De Oliveira, 2000; Colinvaux et al., 2000). Já para a FA existem trabalhos de palinologia (Behling, 1998, 2002; Behling e Lichte, 1997; Behling e Negrelle, 2001; Ledru et al., 1998) e de outras áreas (Brown e Ab'Saber, 1979; Carnaval e Moritz, 2008) que indicam que áreas abertas teriam substituído grande parte das florestas no máximo do último período glacial (25.000-18.000 anos atrás). A teoria dos refúgios é um modelo de especiação, mas estudos genéticos propuseram que a diversificação entre espécies seria mais antiga que o início do Pleistoceno e que os paleorefúgios desse período só teriam afetado as estruturas genéticas das populações (Joseph et al., 1995; Patton e da Silva, 2005; Schneider e Moritz, 1999).

A hipótese dos museus associa a especiação a locais específicos dentro de áreas de montanhas que tenham condições ambientais resilientes às mudanças climáticas (Fjeldsa et al., 1997; Fjeldsa e Lovett, 1997). Essas regiões apresentariam um número grande de espécies novas e endêmicas devido à grande diversificação que ocorreria nelas, em contraste com regiões menos estáveis e com menor complexidade topográfica. Tal hipótese se baseia na maior proporção de espécies de aves com diversificação recente nas áreas montanhosas da África Central e na base dos Andes tropicais, em comparação com as matas de baixa altitude (p.ex., terras baixas da floresta Amazônica) onde existiria maior proporção de espécies

antigas. Assim, o modelo considera que as matas de baixa altitude atuam como “museus” de espécies. Embora o modelo seja mantido na literatura como uma hipótese distinta, o mecanismo proposto (isolamento e divergência em alopatria) é relativamente semelhante ao da teoria dos refúgios.

O modelo de diversificação dos rios como barreiras (revisão em Marroig e Cerqueira, 1997; Moritz et al., 2000) tem recebido atenção no contexto da FA. Alguns rios da floresta são limites de faunas regionais; em conseqüências, poderiam atuar como barreiras ao fluxo gênico e serem importantes para a evolução da biota. Um exemplo é o rio Doce, que é limite de distribuição de muitas aves, mamíferos e borboletas (Brown, 2005; Costa et al., 2000; Silva et al., 2004). Um trabalho particular que menciona o modelo dos rios na FA é o de Pellegrino et al. (2005), que descreve filogrupos do lagarto *Gymnodactylus darwini* endêmicos de alguns interflúvios, sugerindo que os rios envolvidos são importantes na diversificação da biota Atlântica.

A atividade geotectônica do Terciário tardio e do início do Pleistoceno associada à formação e remodelamento do relevo do sudeste e sul da FA (p. ex., Serra do Mar e da Mantiqueira) (Petri e Fulfaro, 1983; Riccomini et al., 1989) foi sugerida como importante na evolução da biota local (Grazziotin et al., 2006; Lara e Patton, 2000; Leite, 2003; Mustrangi e Patton, 1997; Silva e Straube, 1996). Particularmente, foi proposto que os eventos geotectônicos teriam alterado a paisagem (biótica e abiótica) e gerado barreiras ao fluxo gênico. Por exemplo, Silva e Straube (1996) observaram que a distribuição de alguns táxons era limitada pelo vale geológico do rio Paraíba do Sul, e propuseram que a atividade tectônica que teria formado o vale haveria sido importante na separação e evolução de populações.

Finalmente, a hipótese de diversificação em parapatria propõe que em algumas situações a divergência entre organismos é mediada por diferentes pressões seletivas atuantes em ambientes diferentes e contíguos (Moritz et al., 2000; Smith et al., 1997). O modelo considera que a seleção constante sobre caracteres relacionados à aptidão biológica pode, ao longo do tempo, resultar em isolamento reprodutivo entre organismos presentes nos distintos ambientes. Como a FA abriga muitos tipos de florestas (p. ex., ombrófilas e decíduas) e diversas áreas de transição com biomas abertos (Veloso, 1991), tal hipótese é interessante para ser testada. Particularmente, o sistema de vegetação aberta formado pela Caatinga, Cerrado e Chaco, conhecido como diagonal aberta, é importante, pois vários organismos tipicamente florestais penetram nessa região usando as matas de galeria. Como as condições reinantes nessas matas de

galeria (p. ex., clima e luminosidade) são diferentes às condições das matas contínuas, diferentes pressões seletivas podem existir em cada domínio (FA e diagonal aberta).

Modelos de estudo

O presente trabalho utiliza três espécies de passeriformes de sub-bosque: *Xipborhynchus fuscus*, *Dendrocolaptes platyrostris* e *Schiffornis virecens*. Estas espécies são bons modelos para o estudo da história biogeográfica da FA pois: *i*) possuem ampla distribuição, o que permite testar efeitos de diversos elementos ou fenômenos que podem ter influenciado sua estrutura genética populacional; *ii*) não são aves ameaçadas, com exceção de *X. fuscus atlanticus* (Aleixo, 2008; IBAMA, 2003), *iii*) são dependentes de florestas, o que garante que qualquer modificação histórica na cobertura florestal poderia estar refletida na atual estrutura genética populacional de um modo mais intenso que no caso de uma espécie que também ocorre nas áreas abertas ou menos sensíveis à perturbação dessas florestas, *iv*) são aves abundantes e relativamente fáceis de serem capturadas em redes de neblina e sua manipulação para coleta de amostras de sangue é simples e não traz danos aos indivíduos (Cabanne não publicado e Ridgely e Tudor, 1996).

Xipborhynchus fuscus (Dendrocolaptidae) é um passeriforme suboscine endêmico da FA, encontrado desde o Ceará até o Rio Grande do Sul e até o leste do Paraguai e na província de Misiones da Argentina (Fig. 1B) (Ridgely and Tudor, 1996). Existem quatro subespécies descritas para *X. fuscus* (Marantz et al 2003). A subespécie *fuscus* (Vieillot 1818) habita a distribuição sul da espécie até o centro do estado de Minas Gerais (Rio Doce). No nordeste de Minas Gerais e litoral da Bahia ocorre a subespécie *tenuirostris* (Lichtenstein 1820), no interior da Bahia, *brevisrostris* (Pinto 1938) e nos estados de Pernambuco, Ceará e Alagoas, se encontra a subespécie *atlanticus* (Cory 1916). *X. fuscus atlanticus* é o único táxon ameaçado de extinção dentre todos os estudados nesta Tese (Aleixo, 2008; IBAMA, 2003). Até o presente trabalho não existia uma revisão taxonômica ou hipótese filogenética para estas quatro subespécies. *X. fuscus* é residente anual ao longo de toda sua área de ocorrência e habita os principais tipos de florestas da FA, desde altitudes próximas ao nível do mar até mais de 1000 m (Marantz et al., 2003). É uma ave abundante e habita o sub-bosque de matas primárias e secundárias tardias (Stotz et al., 1996), com preferência àquelas porções da floresta com sub-bosque denso, mas sem taquaras (Cabanne não publicado).

Dendrocolaptes platyrostris (Dendrocolaptidae) é uma espécie florestal que se distribui no domínio central da FA e nas matas de galerias da diagonal aberta até ~1300 m de altitude (Fig. 1C) (Ridgely e

Tudor, 1996). Habita florestas primárias e secundárias, podendo ser também encontrado em agroecossistemas e plantações de *Araucaria angustifolia* (Cabanne et al., 2007). É residente anual em toda a distribuição. Existem duas subespécies descritas (revisado em Marantz et al., 2003). A subespécie *platyrostris* (Spix 1824) habita o domínio central da FA e a subespécie *intermedius* (Berlepsch 1883) é associada às matas de galeria e encaves florestais no meio da Caatinga (brejos), Cerrado e leste do bioma chaco (diagonal aberta). As subespécies se diferenciam por variações de plumagem; sendo a plumagem de *platyrostris* mais escura e com estrias mais notórias, especialmente no dorso.

Schiffornis virescens (Pipridae) é uma espécie monotípica que habita o sub-bosque do centro e sul da FA (Fig. 1D) (Ridgely e Tudor, 1996). É residente anual em toda a distribuição. Ocorre desde o nível do mar até ~1200 m de altitude, como exceção das regiões de ocorrência da espécie irmã *S. turdina* (Nyari, 2007), onde se encontra limitado às regiões de maior altitude (p.ex., acima de 500 m no Espírito Santo). *S. virescens* é abundante e habita o sub-bosque de florestas primárias e secundárias tardias (Stotz et al., 1996).

Figura 1: A) Área de estudo e distribuição da Floresta Atlântica (Olson et al., 2001). B) Distribuição de *Xiphorhynchus fuscus*. C) Distribuição de *Dendrocolaptes platyrostris*. C) Distribuição de *Schiffornis virescens*. Todas as distribuições de aves são baseadas em (Ridgely e Tudor, 1996).

Objetivos

O objetivo geral desta Tese foi estudar a evolução de organismos da Floresta Atlântica, nos níveis de populações e de espécies, utilizando aves florestais como modelos e sob uma perspectiva filogenética – filogeográfica. Os objetivos específicos foram:

- 1) Descrever e comparar padrões de distribuição de linhagens intra-específicos de aves selecionadas da FA, particularmente de *Xipborhynchus fuscus*, *Dendrocolaptes platyrostris* e *Schiffornis virescens*;
- 2) Estudar os fenômenos que modelaram essas linhagens e suas distribuições;
- 3) Testar predições das principais hipóteses de diversificação da FA;
- 4) Avaliar as implicações dos resultados para a sistemática e conservação dos táxons estudados.

Síntese dos capítulos

Nesse primeiro capítulo foi apresentado o problema geral e as principais hipóteses que serão exploradas ou testadas nos capítulos dois a cinco. O segundo capítulo corresponde a um artigo publicado no qual foi utilizado DNA mitocondrial (mtDNA) para avaliar a estrutura genética populacional de *Xipborhynchus fuscus* no sul da FA e testar algumas hipóteses de evolução das populações dessa ave. Esse capítulo é a continuação do trabalho de mestrado do autor (Cabanne, 2004). O terceiro capítulo também corresponde a um artigo publicado no qual novamente foi *X. fuscus* estudado, mas se avaliou a estrutura filogeográfica da espécie em toda a distribuição por meio de dois marcadores genéticos independentes (mitocondrial e nuclear). O quarto capítulo é um manuscrito a ser submetido para publicação e descreve a variação da plumagem e a estrutura genética populacional (mtDNA) de *D. platyrostris*. O quinto capítulo é outro manuscrito a ser submetido para publicação e apresenta um estudo preliminar da estrutura filogeográfica (mtDNA) de *S. virescens*. Finalmente, o sexto capítulo é uma discussão geral e resume as conclusões de cada capítulo em particular.

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CAPÍTULO 2

PHYLOGEOGRAPHY OF *XIPHORHYNCHUS FUSCUS* (PASSERIFORMES, DENDROCOLAPTIDAE): VICARIANCE AND RECENT DEMOGRAPHIC EXPANSION IN SOUTHERN ATLANTIC FOREST

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Abstract

Knowledge of the evolutionary processes that shaped a biota is important for both academic and conservation purposes. The objective of the present study is to analyze the mitochondrial genetic variation of *Xiphorhynchus fuscus* (Aves: Dendrocolaptidae) from the southern Atlantic forest in Brazil and Argentina, and to discuss whether the results support different hypotheses regarding the local intraspecific diversification of this species. We sequenced 575 bp of the control region of 114 specimens collected in the Brazilian states of Bahia, Minas Gerais, Rio de Janeiro, São Paulo, Paraná, and Santa Catarina, and in the province of Misiones in Argentina. We studied the population genetic structure with AMOVA and the demographic history with multiple regression analysis, coalescence simulations, and demographic tests. *X. fuscus* presented a significant population genetic structure ($\Phi_{st} = 0.43$). Three mitochondrial lineages were described, one associated to *X. f. tenuirostris* and the others to *X. f. fuscus*. The data did not support the primary influence of geographic barriers or rivers in the intraspecific diversification of *X. fuscus* in the southern Atlantic forest. Instead, the data supported the influence of isolation by geographic distance, recent vicariance events, and demographic expansions apparently related to Pleistocene and Holocene forest dynamics.

Key words: coalescence simulations – isolation by distance – forest refuges - mitochondrial control region – multiple regression – neotropics – Pleistocene - riverine barriers – Valley of the Paraíba do Sul river

The Atlantic forest is distributed along eastern Brazil, eastern Paraguay, and northeastern Argentina (Gusmão Câmara 2003). Its unique biota is probably the result of a complex evolutionary history; however, few studies attempted to clarify the biogeographic processes that shaped it (Muistrangi & Patton, 1997; Costa *et al.*, 2000; Geise, Smith & Patton, 2001; Pellegrino *et al.*, 2005). The knowledge of these evolutionary processes is important for both academic and conservation purposes (Moritz, 2002). Among the several hypotheses on the diversification of rainforest biotas (reviewed by Moritz *et al.*, 2000), the evolution in paleorefuges and the influence of geographic barriers are two of the most discussed ones.

In the Neotropics, the refuge theory was originally proposed to explain speciation during the Pleistocene mainly in the Amazon basin (Haffer, 1969; Vanzolini & Williams, 1970; Brown & Ab'Sáber, 1979; Haffer & Prance, 2001). This theory proposes that during the glaciations the rainforests were reduced to refuges isolated by open areas, and that organisms isolated in these refuges could have diverged and originated new lineages. Then, in the next interglacial period, the forest expanded and the new clades would get in contact. This hypothesis requires the expansion and contraction of open areas and the formation of forest refuges. Palinological (Ledru, Salgado-Labouriau & Lorscheitter 1998; Behling & Negrelle, 2001; Behling, 2002) and other types of studies (Brown & Ab' Sáber, 1979) propose that open areas dominated the Atlantic forest's landscape during the maximum of Late Pleistocene glaciations, suggesting that the refuge theory can be important to understand the biological diversification of the biome.

Pellegrino *et al.* (2005), based on a phylogeographic study of the gecko *Gymnodactylus darwini* (Gekkonidae, Squamata), proposed that rivers play an important role in the diversification of Atlantic forest biota. Other authors proposed that the tectonic activity associated to the formation of geographic landmarks would be relevant to biodiversity modeling, as suggested by Silva & Straube (1996) who observed that the geographic range of some passerines was limited by a graben, the valley of the Paraíba do Sul river (VPSR). Those tectonic episodes would have been important for the biota in the southern Atlantic forest, where a complex relief exists with many mountain ranges and valleys, and where neotectonic activity was described (Petri & Fulfaro, 1983; Riccomini *et al.*, 1989).

Xiphorhynchus fuscus is an Atlantic forest endemic member of the Family Dendrocolaptidae (Aves) with a broad distribution. It occurs in eastern Brazil (from the state of Ceará to the state of Rio Grande do Sul), east of Paraguay and northeastern Argentina (Marantz *et al.*, 2003). There are four subspecies defined by

slight variations in color and morphological measurements (Marantz *et al.*, 2003). *X. f. tenuirostris* and *X. f. fuscus* inhabit the eastern and the southern range of the species, respectively (Fig. 1). The other subspecies inhabit the interior of the Brazilian state of Bahia (*X. f. brevirostris*) and the northeastern part of the country (*X. f. atlanticus*). The distribution limits of these subspecies are not well known. Given its geographic distribution and deep-forest dependency, *X. fuscus* is a good model to study the diversification of the Atlantic forest biota.

The objective of the present study is to analyze the mitochondrial DNA variation of populations of *Xiphorhynchus fuscus* from the southern part of the Atlantic forest and to discuss whether the results support different hypotheses regarding the local intraspecific genetic diversification of this bird.

Materials and Methods

Study area and samples

Samples (blood or muscle, n = 114) were collected between 2000 and 2004 in the Brazilian states of Bahia, Minas Gerais, Rio de Janeiro, São Paulo, Paraná, and Santa Catarina, and in the province of Misiones in Argentina (Fig. 1, Table 1). The collection localities are covered by dense ombrophilus, mixed or semideciduous forest, which are the main forest types of the Atlantic forest (Veloso, 1991). The relief in the study area is complex, especially in the eastern portion due to the presence of the Serra do Mar and the Serra da Mantiqueira coastal ridges, which can surpass 2000 m above sea level.

Blood was collected (~0.1 ml) with insulin syringes from the largest vein in the right cervical region. Muscle was obtained from specimens which were deposited at the Museu de Zoologia da Universidade de São Paulo (MZUSP). Each bird was captured with mist nets, photographed and marked with an aluminum ring. All tissue samples are deposited at the Laboratório de Genética e Evolução Molecular de Aves (Instituto de Biociências, Universidade de São Paulo, Brazil), or at the Laboratório de Biodiversidade e Evolução Molecular (Instituto de Ciências Biomédicas, Universidade Federal de Minas Gerais, Brazil).

Molecular methods

Total DNA was obtained from blood or muscle samples by a conventional proteinase K - SDS digestion, organic extraction with phenol-chloroform, and ethanol precipitation (Bruford *et al.*, 1992). To diminish the possibility of amplifying unexpected copies of non-mitochondrial origin, we amplified a ~1600 bp fragment with the primer pairs LXfB2 (5' TCAATTCCAAACAACTAGGAGG 3', present study) / HPRO (5' GCTTTGGGAGTTGGAGATAAAGG 3', present study). This amplicon contained the complete control region (total size of 1275 bp, determined in the present study). The PCR reaction (10 µl) contained 20-40 ng of total DNA, 1 X of *Taq* buffer (Pharmacia Biotech), 200 µM of each dNTP, 1 µM of each primer, and 0.1 U of *Taq* polimerase (Pharmacia Biotech). Amplifications were performed with an initial step at 95° C for 4 min and 37 cycles of 45 s at 94° C, 45 s at 53.5° C, and 110 s at 72° C, followed by a final extension of 10 min at 72° C. PCR products were purified with Shrimp Alkaline Phosphatase and Exonuclease I. Sequencing reactions were performed with Big Dye Terminator Kit ver 3.0 (Applied Biosystems Inc) using the amplification and the internal primers (HXfRC1 5'-GGGGAAAATAAACGTTTATTAAGTG-3', HXfRC2 5'-CAAGATGGACATGTTTCGACACCG-3', present study, and L537 5'-CCTCTGGTTCCTCGGTCAG-3', Sorenson *et al.*, 1999). The sequencing reactions were precipitated with isopropanol 75% and etanol 70%, and analyzed in an automatic sequencer ABI Prism 377 (Applied Biosystems Inc).

Analytical methods

The complete control region (1275 bp) and flanking genes (tRNA^{Thr} 70 bp, tRNA^{Pro} 70 bp) from one *X. f. fuscus* sample (tissue P652) were sequenced. The analysis of 1048 bp of the control region (positions 57 to 1104) from 20 individuals revealed that the most variable portion encompasses 575 bp (positions 57 to 631); thus, this segment was used in the phylogeographic analyses. Identification and simulation of the secondary structure of the tRNA sequences were carried out in the program tRNAscan-SE 1.21 (<http://www.genetics.wustl.edu/eddy/tRNAscan-SE/>, Lowe & Eddy 1997). All sequences are deposited in GenBank under the access numbers AY948386–AY948394, AY948396–AY948415, and DQ144622–DQ144636.

Sequences were aligned with the program Clustal X (Thompson *et al.*, 1997). The likelihood ratio test as implemented in the software Modeltest 3.7 (Posada & Crandall, 1998) was used to select the best fit evolutionary model HKY85 with the proportion of invariable sites (I) of 0.8404 and a discrete gamma

distribution ($\alpha = 0.6467$) (Hasegawa, Kishino & Yano, 1985). We reconstructed the phylogenies by Neighbor Joining (NJ; Saitou & Nei, 1987) with the software PAUP* (version 4.0b10, Swofford, 2001) and by Maximum Likelihood (ML) with PHYML Online (Guindon & Gascuel, 2003, Guindon *et al.* 2005). The starting tree for the ML analysis was obtained with PHYML Online. For both analyses the best fit evolutionary model was used. A sequence of *X. pardalotus* (tissue P264) was used to root the resulting trees. The support of the nodes was evaluated by non-parametric bootstrap (100 replicates; Felsenstein, 1985).

In all further population analyses we used the Tamura & Nei (1993) model of evolution which is the closest one to the HKY85 model that is available in the softwares we used. The haplotype network was constructed following Templeton, Crandall & Sing (1992) in the program TCS 1.13 (Clement, Posada & Crandall 2000). The net divergence between sets of sequences was obtained in MEGA 3.0 (Kumar, Tamura & Nei, 2004) with the formula $\delta = \delta_{xy} - [0.5 (\delta_x + \delta_y)]$, where δ_{xy} is the mean divergence between all the sequences, and δ_x and δ_y are the mean divergences within each group of sequences x and y, respectively (Wilson *et al.*, 1985). The mutation rate used to estimate the divergence time between the lineages was $\mu = 8.72 \times 10^{-8}$ changes/nucleotide, which was used by Milot, Gibbs & Hobson (2000) for the analysis of the first domain of the mtDNA control region of the passerine *Dendroica petechia*. We assumed a generation time of one year (Klicka & Zink, 1999).

To test whether the *X. f. fuscus* gene genealogy is the result of the neutral coalescence process within a panmictic population, we followed Knowles (2001) and applied a gene-tree population-tree approach using the program MESQUITE 1.02 (Maddison & Maddison, 2004). In this approach gene trees (300 replicates) were simulated by neutral coalescence under a null model of a unique panmictic *X. f. fuscus* population whose effective population size (N_e) was invariable until total coalescence. Four arbitrary N_e were tested: 100,000, 50,000, 25,000, 12,000, and 6,000. The discordance between these gene trees and the alternative model of two isolated populations (one in the north and the other in the south of the Valley of the Paraíba do Sul river, VPSR; Fig. 1) was measured using the s -statistics (Slatkin & Maddison, 1989). The discordance between the reconstructed gene tree (Neighbor Joining tree of *X. f. fuscus* sequences) and the two populations' model was also evaluated with the s -statistics and this value was compared to the expected distribution of s -values obtained from the neutral simulations. If the s -value of the reconstructed gene tree is significantly lower than the values from the simulated gene trees ($\alpha = 0.05$), the null model is

rejected. We used the mean number of pairwise differences (Π) calculated in Arlequin 2.0 (Schneider, Roessli & Excoffier, 2000) to approximate theta (Θ) and to calculate the empirical N_e according to the relationship $\Pi = \Theta = 2N_e\mu$ (Watterson, 1975; Nei & Kumar, 2000).

We estimated the nucleotide diversity (π , Nei & Kumar, 2000) for each locality with two or more individuals using Arlequin 2.0. The analysis of molecular variance (AMOVA; Excoffier, Smouse & Quattro, 1992) was used to study the population genetic structure as implemented in Arlequin 2.0. We performed global (all the localities) and regional analyses (subgroups of localities). The Φ_{ST} was obtained using corrected distances and its statistical significance was estimated by a non-parametric permutation test (Excoffier *et al.*, 1992).

We performed a partial regression analysis (Smouse, Long & Sokal, 1986) to test the effect of two independent variables (linear geographic distance among the pairs of localities and geographic location) on the average corrected genetic distances among individuals from pairs of locations. Genetic distances were estimated with the program MEGA 3.0. The matrix of geographic location was constructed as a dummy variable (Quinn & Keough, 2002) that represented whether each pair of localities were within the same main geographic region (northwestern Minas Gerais plus southern Bahia, southeastern Minas Gerais plus southern Rio de Janeiro, and south from the VPSR). The magnitude of the partial regression coefficients indicates the relative importance of the different independent variables. If genetic distance reflects simple isolation by distance, geographic distance would be the best predictor. If vicariance were the most important process, the geographic location matrix would be the best predictor of genetic distances. Each matrix was transformed to a mean of zero and a variance of one before the analyses (Smouse *et al.*, 1986). The partial regression analyses were performed in the program Fstat 2.9.3.2 (Goudet, 2002) using 10,000 permutations to obtain the probabilities values.

Demographic expansion was inferred by calculating Fu's F_s (Fu 1997) and R_2 (Ramos-Onsins & Rozas, 2002) using DnaSP 4 (Rozas & Sánchez-Del Barrio, 2003). Significantly negative F_s and low values of R_2 indicate demographic expansion. For these calculations, the three major lineages recovered in the genealogy were defined as populations, following Cheviron, Hackett & Caparella (2005). Significance was determined based on 1000 coalescent simulations under a model of population stability using empirical sample sizes and estimates of Θ . The parameters of exponential population growth (Θ_0 , Θ_1 , and τ), according to the model of Rogers & Harpending (1992), were estimated in the program Arlequin 2.0. The

expansion time was estimated according to $t = \tau/2u$ using lower and upper values of 95% confidence interval of the parameter τ , where u is the mutation rate per generation per haplotype (Rogers & Harpending 1992).

Results

Characteristics of the sequences

The sequences obtained presented several evidences of being of mitochondrial origin: *i*) the empiric base composition of the sequences used in the phylogeographic study (A 27.7%, C 25%, G 16.3%, and T 31.1%) is compatible with the expected one for avian mitochondrial DNA (Baker & Marshall, 1997), *ii*) the amplicon (1600 bp) contained the complete control region (1275 bp), flanked by a tRNA^{Thr} at the 5' end (70 bp) and a tRNA^{Pro} at the 3' end (70 bp), and this gene arrangement is the expected one for a Suboscines (Mindell, Sorenson & Dimcheff, 1998), *iii*) the inferred secondary structure and the presence of expected anticodons (UGU and UGG) suggested that these tRNA genes are functional (data not shown), and (5) the amplicon analyzed is longer than the average size of translocated copies of mitochondrial genes in an avian nuclear genome (Pereira & Baker, 2004). The 575 bp of the control region of 114 individuals of *X. fuscus* presented 32 haplotypes (Table 1, Fig. 2 and 3), with 31 polymorphic sites (5.39% of all positions), 30 transitions and 2 transversions, and no indels.

Genealogy, divergence, and geographic distribution of X. fuscus mitochondrial lineages

The haplotype network presented three major clades (Fig. 2): one associated with *X. f. tenuirostris* and the others with *X. f. fuscus* (northern lineage [N] and southern lineage [S]). The *X. f. tenuirostris* lineage occurred in samples from northeastern Minas Gerais and southern Bahia. Haplotypes of the *X. f. fuscus* northern lineage only occurred in southeastern Minas Gerais, southern Rio de Janeiro and northeastern São Paulo; whereas sequences of the southern lineage occurred in all localities in southern and central São Paulo, Paraná, Santa Catarina, and Misiones. Based on coalescence principles, the TCS algorithm (Crandall & Templeton, 1996) selected the haplotype S4 (*X. f. fuscus* lineage S) as the most likely ancestral one. The phylogenetic analysis by Neighbor Joining (NJ) and Maximum Likelihood (ML) resulted in similar

topologies and recovered the same three main lineages. Thus, we only present the NJ tree with the bootstrap supports obtained by both methods (Fig. 3).

Mean Tamura & Nei (1993) distance between *X. pardalotus* and *X. fuscus* was $12.366\% \pm 1.481\%$ ($\pm$ SD). Mean distance between *X. f. fuscus* and *X. f. tenuirostris* was $2.368\% \pm 0.561\%$, and the mean distance between *X. fuscus* lineages N and S was $1.493\% \pm 0.400\%$. The net divergence between the two *X. f. fuscus* lineages was $1.228\% \pm 0.432\%$, which corresponds to $73,500 \pm 24,500$ years of divergence. The low number of samples of *X. f. tenuirostris* did not allow us to estimate the net divergence between this clade and *X. f. fuscus*; thus the estimated divergence time between these clades ($135,000 \pm 32,000$ years) was based on the mean distance between groups ($2.368\% \pm 0.561\%$).

Coalescence simulations

Sequences within *X. f. fuscus* grouped in two main lineages that were strongly associated to specific geographic regions, suggesting that each lineage possibly evolved in allopatry. However, given that the divergence between lineages is low and that the stochastic nature of the coalescence process can produce many different genealogies, we applied a gene-tree population-tree analysis to test if a single historical population could have originated these results. The *s*-value (nine) of the reconstructed genealogy was significantly lower than the values obtained by the simulated gene trees, regardless of the N_e used ($P < 0.01$); thus, the null model of a single population was rejected ($P < 0.01$). Also, the empirical N_e was 52,000 and it is compatible with the range of N_e (6000-100,000) used in the simulations.

Population genetic structure

A global AMOVA indicated that 57.7% ($\Phi_{ST} = 0.577$, $P < 0.01$) of the genetic variation in the samples of *X. fuscus* is allocated among the geographic regions where the main lineages occur (northeastern Minas Gerais plus southern Bahia, southeastern Minas Gerais plus southern Rio de Janeiro, and south of the VPSR). A regional AMOVA without the samples from the *X. f. tenuirostris* lineage (northeastern Minas Gerais and southern Bahia) was performed subdividing each main region in two sub-regions. The sub-regions of the first region are Minas Gerais and Rio de Janeiro, whereas the sub-regions of the second region are São Paulo and south of São Paulo. This analysis indicated that 30.84 % ($P < 0.01$) of the genetic diversity within *X. f. fuscus* was found among regions, that 12.93% ($P < 0.01$) occurred among the sub-

regions within regions, and that 56.23% ($P < 0.01$) was allocated within sub-regions. The Φ_{ST} of this analysis was 0.43.

Relationships between genetic distances and predictor variables

The partial regression analysis indicated that 40.5 % of the variation in genetic distances can be predicted by the linear geographic distance among pairs of localities (partial regression coefficient = 0.585, $P < 0.01$) and the geographic location (partial regression coefficient = 0.251, $P < 0.01$).

Historical demography

The F_s value for *X. f. tenuirostris* is non-significant, suggesting that there was demographic stability (Table 2). The F_s values are significant and negative for both *X. f. fuscus* lineages, suggesting the presence of past demographic expansion. Since the R_2 test is more suitable for the analysis of small samples than the F_s test (Ramos-Onsins & Rozas, 2002), we analyzed *X. f. tenuirostris* ($n = 12$) with the R_2 test. The result was non-significant ($R_2 = 0.1800$, $P = 0.6960$), indicating that this taxa presented past demographic stability. Based on the estimations of τ , the demographic expansions of the northern and southern lineages of *X. f. fuscus* started around 57,000 and 19,000 years ago, respectively.

Discussion

Phylogeographic structure of X. fuscus in the southeastern Atlantic forest

The study of the control region of *X. fuscus* revealed a significant population genetic structure, which is in accordance with other Neotropical birds (Bates, 2000, 2002; Aleixo, 2004; Cheviron *et al.*, 2005), and contrasts with the patterns observed with the same genetic marker in Palearctic passerine populations separated by thousands of kilometers (Merilä, Björklund & Baker 1997, Kvist *et al.*, 1998; Kvist *et al.*, 1999a, b; Uimaniemi *et al.*, 2003).

Three main mitochondrial lineages were revealed in the *X. fuscus* populations studied. One lineage was associated to the subspecies *X. f. tenuirostris* and the others to the subspecies *X. f. fuscus* (Fig. 1, 2 and 3). Haplotypes of *X. f. fuscus* northern lineage were found from the Doce river basin in Minas Gerais to northeastern São Paulo. The other *X. f. fuscus* lineage was distributed from northern São Paulo to Santa

Catarina and Misiones in Argentina. The two subspecies clades seem to have diverged in the late Pleistocene, around 130,000 years ago. While the separation of the two *X. fuscus fuscus* clades was estimated to have occurred around 70,000 years ago. A contact zone between the two clades of *X. f. fuscus* was located to the southwest of VPSR (Fig. 1). We hypothesize that a contact zone between the *X. f. tenuirostris* clade and the northern *X. f. fuscus* lineage can occur along the Doce river in Espírito Santo and somewhere between the basins of the Jequitinhonha and the Doce rivers in the interior of Minas Gerais, as suggested by the distribution of these two subspecies (Fig. 1). Further studies are needed to test whether the Doce river can be a barrier to gene flow between the two subspecies. Another interesting result is that all southern localities (Misiones and Santa Catarina) present only three haplotypes (S1, S2, and S3). These haplotypes are grouped in the network and in the NJ tree and were only present in this region and in northern Paraná (Table 1). This result suggests that there was a past range fragmentation somewhere between São Paulo and northern Paraná, and this should be tested in future studies with a more detailed sampling.

Even though studies on Atlantic forest organisms are scant, the biogeographic pattern observed in *X. fuscus* with three phylogeographic groups that seem to be limited by geographic landmarks or specific geographic regions, such as the VPSR and the transition between the basins of the Doce and the Jequitinhonha rivers, is compatible with patterns observed in other organisms. For example, small mammals present biogeographic divergence among northeastern Minas Gerais, southeastern Minas Gerais, and southwards regions (Mustang & Patton, 1997; Costa *et al.*, 2000). Pellegrino *et al.* (2005) also found in the gecko *G. darwini* (sampled in the same geographic region as the present study) three main mitochondrial lineages. Geckos from southern Minas Gerais are divergent from those from São Paulo and southwards regions, and both clades are also separated from geckos from the Jequitinhonha river basin. Thus, *X. fuscus* and *G. darwini* present similar phylogeographic patterns. However, even considering that coalescence times present high variance and estimated divergence times may not be reliably compared, the difference between the splitting times among clades in the gecko (youngest divergence at least 0.9 Myr) and those among the bird clades (oldest divergence 130,000 years ago) does not support a common temporal origin of these patterns. Furthermore, Pellegrino *et al.* (2005) suggested that rivers were barriers that generated the phylogeographic pattern observed in the gecko. Some predictions for the hypothesis of rivers as primary gene flow barriers are that sister clades should occur across major rivers rather than

along the same river bank and should not present signals of demographic expansion, that would favor secondary contact (Moritz *et al.*, 2000). The samples we had available did not permit us to test these predictions in all the main river systems of the study area. However, for the area around the Paraíba do Sul river, the number of samples allowed us to start to explore this issue. As the two *X. fuscus fuscus* lineages presented signals of demographic expansion (Table 2) and the northern lineage occurs on both sides and southwards of the VPSR (Fig. 1), this river does not seem to be a primary barrier for the gene flow of *X. fuscus*. Nevertheless, the limits of the distribution of the subspecies *X. fuscus tenuirostris* and *X. fuscus fuscus* seem to coincide with the Doce river (Fig. 1), especially in Espírito Santo, suggesting that this river could be a barrier. It is necessary to add more samples from key locations to test these hypotheses.

On the origin of the phylogeographic structure of X. fuscus

The partial regression analysis suggested that isolation by geographic distance and the history of vicariance (geographic location) were important in shaping the population genetic structure of *X. fuscus*. The coalescence simulations supported that *X. f. fuscus* lineages evolved in two populations instead of in a single one. At least two main vicariant phenomena were detected: one that separated the two subspecies lineages and the other which resulted in the divergence between the two *X. f. fuscus* phylogroups. The following discussion will focus on the latter divergence.

Evolution in isolation and secondary contact provides a possible explanation for the phylogeographic structure of *X. f. fuscus*. Two vicariant events could have separated the ancestral population: the geologic episodes that formed the VPSR or natural forest fragmentation.

The VPSR is a 173 km long and up to 2 km deep graben that separates the Serra da Mantiqueira and the northern Serra do Mar (Petri & Fulfaro, 1983) (Fig. 1). It is a contact area for birds (i. e. *Lepidocolaptes squamatus* and *L. falcinellus*, Silva & Straube 1996; *Heliobletus contaminatus contaminatus* and *H. c. camargoi*, Silva & Stotz 1992) and marsupials (i. e. *Marmosops paulensis* and *M. incanus*, Mustrangi & Patton, 1997), and it separates mitochondrial lineages of the gecko *G. darwini* (Pellegrino *et al.*, 2005). Also, morphologically distinct populations of the passerine *Scytalopus speluncae* (Giovanni, 2005) get in contact in the same geographic sector where the two *X. f. fuscus* lineages meet. Silva & Straube (1996) postulated that the tectonic process that opened the valley and the consequent alteration of the forest cover could have been a significant vicariant event for forest species. Assuming that this proposition is correct, the two

lineages of *X. f. fuscus* that meet near to the valley could have evolved in isolation after the formation of the valley, and when the conditions of dispersion improved, they came into contact. Predictions of this hypothesis are that populations at each side of the valley are differentiated, and that the separation time between the lineages is compatible with the valley's age. The first prediction is supported by morphological characters that diagnose each of the bird populations from Minas Gerais and São Paulo (Albuquerque 1996). However, the prediction regarding the age of the valley is not accepted. According to Petri & Fulfaro (1983) the formation of the valley started in the Miocene-Pliocene (c. 15 millions of years ago) and lasted until the Early Pleistocene. The estimated divergence time of the two *X. f. fuscus* lineages was 50,000 – 100,000 years ago, which is too recent to match the formation of the valley. Thus, our data does not support the formation of the current VPSR as the vicariant event that produced the two *X. f. fuscus* lineages.

Forest fragmentation related to historical climatic oscillations was discarded as an explanation for the distribution pattern of *L. falcinellus* and *L. squamatus* (Silva & Straube, 1996), because Brown & Ab'Saber (1979) suggested that the forest cover along the contact area of these species (the VPSR) was not discontinued in the last glacial period. However, Clapperton (1993) suggested that in the last glacial maximum there were two main forest refuges in this area: one in the northern Serra do Mar and the other in the Serra da Mantiqueira and part of the Serra do Espinhaço in Minas Gerais; and these refuges were separated by a grassland area coincident with the VPSR. Under this scenario, the paleoreference hypothesis could explain the diversification of clades in *X. f. fuscus*, and thus the current contact area of the *X. f. fuscus* lineages should be secondary (Moritz *et al.*, 2000). A prediction of this hypothesis is that clades involved in the secondary contact should exhibit evidence of range expansion (Hewit 2000; Moritz *et al.*, 2000; Cheviron *et al.*, 2005) and concomitant demographic expansion. The demographic analysis supports this prediction for the two *X. f. fuscus* lineages. Furthermore, the contact area between *X. fuscus fuscus* lineages has a high proportion of derived haplotypes, especially from the northern lineage (i.e.: haplotypes N1, N4, and N5; see Table 1 and Fig. 2), which is also compatible with population expansion and secondary contact (Templeton, Routman & Phillips, 1995).

According to Behling (1998; 2002), grassland dominated the southern and southeastern Brazilian landscape during the Late Pleistocene, where diverse forest ecosystems exist today. The current southern limit of the Atlantic forest biome contacts the grasslands biome of southern Brazil at latitude 28° - 27° S in

the north of Rio Grande do Sul, and Behling (2002) concluded that during the Late Pleistocene this limit extended 750 km northwards, at least to latitude 20° S (southern Minas Gerais and northern São Paulo). Behling (2002) also proposes that modern forest cover of south and southeast Brazil was established only in the Late Holocene. Under this historic scenario of dynamic forest cover, it is expected that forest dependent organisms presented strong demographic expansion in response to the forest advance in the Late Holocene, especially in regions southwards to the Late Pleistocene northern limit of grassland. Thus, we suggest that the demographic expansion of *X. f. fuscus* lineages is related to their geographic expansion that followed the forest advance in the Holocene, as this is compatible with the estimated expansion dates. The southern lineage possibly expanded from the Serra do Mar mountain range or from forest refuges along the Paraná river (Ledru *et al.*, 1998). On the other hand, *X. f. tenuirostris* presented no demographic expansion, possibly because their forest habitat was less affected by Pleistocene climatic alterations (Behling, 1998; 2002).

The hypothesis of refuges, the influence of geography, and river barriers are among the most discussed models in the study of Neotropical diversification. In conclusion, our data did not support the primary influence of geographic barriers (VPSR) or rivers in the divergence between the two main mitochondrial lineages of *X. f. fuscus* of the southeastern Atlantic forest. Instead, the data supported the influence of isolation by geographic distance, recent vicariance events, and demographic expansions in shaping the *X. fuscus* phylogeographic structure. These vicariance and expansions events seem to be related to recent natural forest landscape dynamics. There are more than 1,000 bird species in the Atlantic forest, many of them distributed along the same geographic range of *X. fuscus* and with comparable ecological requirements. Then, it is plausible that the same forest dynamics that seem to have modeled the phylogeographic structure of *X. fuscus* could have also affected other forest-dependent birds. More studies are needed to understand the importance of those evolutionary and demographic factors in shaping the Atlantic forest biota.

Conservation implications

This study revealed a strong phylogeographic structure in *X. f. fuscus* that defined two divergent populations in different geographic areas with dissimilar histories in the southern Atlantic forest: *i*) southeastern Minas Gerais plus southern Rio de Janeiro and *ii*) São Paulo plus southwards regions. As

indicated by AMOVA, genetic differences between the two regions explained c. 31% of the mitochondrial variation of *X. f. fuscus*. Unfortunately, current taxonomy does not recognize this diversity within *X. f. fuscus*, and even though the taxon is not threatened, this data is important for local conservation. It is possible that other forest birds present a similar condition. Thus, the importance of characterization of the distribution of the genetic diversity of threatened species is further reinforced by the present data, as divergences not yet detected may be ignored in their conservation plans.

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Table 1: Localities, sample sizes (n), haplotypes, and nucleotide diversity (π) in percentage.

	Locality	n^a	Haplotypes ⁱ	$\pi^0\%$ (S. D.%)
1	Minas Gerais (MG), Jequitinhonha, left bank of the Rio Jequitinhonha, 16° 20'S, 41° 00'W	5	T2 ¹ , T4 ¹ , T6 ¹ , T7 ²	0.5186 (0.2198)
2	MG, Salto da Divisa, left bank of the Rio Jequitinhonha, 16° 05'S, 40° 02'W	6	T1 ⁴ , T5 ¹ , T7 ¹	0.4606 (0.1785)
3	Bahia, Vila Nova, 17° 22'S, 40° 17'W	1 ^b	T3 ¹	-
4	MG, Nova Lima, 19° 59'S, 43° 49'W	1	N8 ¹	-
5	MG, Marliéira, 19° 43'S, 42° 44'W	1	N9 ¹	-
6	MG, Caratinga, 20° 50'S, 42° 05'W	1	N12 ¹	-
7	MG, Simonésia, 20° 07'S, 42° 00'W	1	N13 ¹	-
8	MG, Araponga, 20° 40'S, 42° 31'W	3	N2 ¹ , N10 ¹ , N11 ¹	0.4710 (0.2374)
9	Rio de Janeiro, Itatiaia National Park, 22° 25'S, 44° 36'W	10	N2 ⁶ , N6 ¹ , N14 ¹ , N15 ²	0.2274 (0.1239)
10	São Paulo (SP), Bananal State Park, 22° 41'S, 44° 19'W	2 ^c	N3 ¹ , N6 ¹	0.7109 (0.3368)
11	SP, Picinguaba, 22° 31'S, 44° 50'W	4	N2 ⁴	0.0
12	SP, Caraguatatuba, 23° 37'S, 42° 26'W	2	N2 ¹ , N14 ¹	0.1760 (0.1771)
13	SP, Morro do Diabo State Park, 22° 30'S, 52° 18'W	1	S1 ¹	-
14	Paraná (PR), Ortigueira, 24° 12'S, 50° 55'W	1	S8 ¹	-
15	PR, Wenceslau Braz, 22° 51'S, 49° 47'W	7 ^d	S1 ⁵ , S8 ²	0.1677 (0.1237)
16	SP, Barreiro Rico, 22° 38'S, 48° 13'W	3 ^e	N2 ² , S8 ¹	0.9479 (0.3195)
17	SP, Itaberá State Park, 23° 51'S, 49° 08'W	7	S4 ³ , S6 ³ , S10 ¹	0.2014 (0.1154)
18	SP, Burí State Park, 23° 39'S, 48° 32'W	9 ^f	S4 ⁴ , S7 ¹ , S8 ³ , S9 ¹	0.1760 (0.1098)
19	SP, Caboclos, 24° 28'S, 48° 35'W	8	S4 ⁵ , S8 ³	0.0943 (0.0949)
20	SP, São Roque, 23° 34'S, 47° 09'W	9	N1 ³ , N2 ¹ , S4 ⁵	0.9199 (0.2815)
21	SP, Morro Grande State Park, 23° 42'S, 46° 59'W	14	N1 ⁵ , N2 ³ , S5 ¹ , S6 ² , S7 ³	0.8830 (0.2589)
22	SP, Juituba, 23° 53'S, 47° 00'W	7 ^g	N1 ¹ , N4 ² , S4 ¹ , S6 ¹ , S7 ¹ , S8 ¹	1.0172 (0.3025)
23	SP, Serra do Mar State Park, Station Curucutú, 23° 58'S, 46° 44'W	4 ^h	N2 ¹ , N4 ¹ , N5 ¹ , N7 ¹	0.4424 (0.1858)
24	Argentina, Misiones, Campo San Juan State Park, 27° 22'S, 55° 39'W	3	S1 ¹ , S2 ²	0.1173 (0.1065)
25	Misiones, Yabotí Biosphere Reserve, 26° 48'S, 53° 55'W	3	S1 ² , S3 ¹	0.1173 (0.1181)
26	Santa Catarina, Botuverá, 27° 13'S, 49° 03'W	1 ⁱ	S1 ¹	-

^a- samples with museum voucher or specimen field number (f) are identified: ^b- Museu Paraense Emílio Goeldi, fAA568; ^c- Museu de Zoologia da Universidade de São Paulo (MZUSP) f76, f91; ^d- MZUSP fITA257, fITA302, fITA283; ^e- MZUSP f31, f59, f60, ^f- MUZUSP 76134, MZUSP 75588; ^g- MZUSP fITA141, fITA171, fITA182, fITA152, fITA172, fITA157, MZUSP 75565; ^h- f9; ⁱ- MZUSP 75032; ^j- numbers in superscript: numbers of individuals with the corresponding haplotype.

Table 2: Historical demographic analysis and expansion dates in years ago.

Clade	n	Fu's F_s	P(Fu's F_s)	Θ_0	Θ_1	Expansion dates
<i>X. f. tenuirostris</i>	12	-1.400	0.160	n.a.	n.a.	n.a.
<i>X. f. fuscus</i> N	46	-7.080	0.001	0.0010	7.0460	10,130-57,370
<i>X. f. fuscus</i> S	56	-3.789	0.015	0.0000	3415	4,690-19,460

n- sample size; P(Fu's F_s)- probabilities of the F_s statistic value being lower than the observed one based on 1000 coalescent simulations; Θ_0 and Θ_1 - parameters of the model of exponential growth of Rogers and Harpending (1992). n.a.: not applicable.

Fig. 1. Study area, sampling localities, and distribution of *X. fuscus* subspecies and mtDNA lineages.

Locality numbers correspond to Table 1. BA: Bahia, ES: Espírito Santo, GO: Goiás, MG: Minas Gerais, MI: Misiones, MT: Mato Grosso do Sul, PR: Paraná, RJ: Rio de Janeiro, RS: Rio Grande do Sul, SC: Santa Catarina, SP: São Paulo. Approximate distribution limits of the two subspecies indicated by broken lines (Zimmer, 1947, Marantz et al., 2003): A: *X. fuscus tenuirostris*, B: *X. fuscus fuscus*.

Fig. 2. Haplotype network of *X. fuscus* based on 575 bp of the control region. The size of each figure is proportional to the frequency of the corresponding haplotype in the total sample. Haplotype identification: T1 to T7- *X. f. tenuirostris* lineage, N1 to N15- *X. f. fuscus* northern lineage, and S1 to S10- *X. f. fuscus* southern lineage.

Fig. 3. Neighbor Joining (NJ) tree based on 575 bp of the control region of *X. fuscus*. The numbers at nodes show NJ and Maximum Likelihood bootstrap values above 50%, respectively. *X. pardalotus* was used to root the tree. Haplotype identification: T1 to T7- *X. f. tenuirostris* lineage, N1 to N15- *X. f. fuscus* northern lineage, and S1 to S10- *X. f. fuscus* southern lineage.

CAPÍTULO 3

NUCLEAR AND MITOCHONDRIAL PHYLOGEOGRAPHY OF THE ATLANTIC FOREST

ENDEMIC *XIPHORHYNCHUS FUSCUS* (AVES: DENDROCOLAPTIDAE):

BIOGEOGRAPHY AND SYSTEMATICS IMPLICATIONS

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Abstract

We studied the intraspecific evolutionary history of the South American Atlantic forest endemic *Xiphorhynchus fuscus* (Aves: Dendrocolaptidae) to address questions such as: Was the diversification of this bird's populations associated to areas of avian endemism? Which models of speciation (i.e.: refuges, river as barriers or geotectonism) explain the diversification within *X. fuscus*? Does the genetic data support subspecies as independent evolutionary units (species)? We used mitochondrial (n=34) and nuclear (n=68) DNA sequences of *X. fuscus* to study temporal and spatial relationships within and between populations. We described four main monophyletic lineages that diverged during the Pleistocene. The subspecies taxonomy did not match all the evolutionary lineages; subspecies *atlanticus* was the only one that represented a monophyletic and isolated lineage. The distribution of these lineages coincided with some areas of endemism for passerines, suggesting that those areas could be regions of biotic differentiation. The ancestor of *X. fuscus* diverged ~3 million years ago from Amazonian taxa and the phylogeographic pattern suggested that *X. fuscus* radiated from northeastern Brazil. Neither the riverine nor the geotectonic vicariance models are supported as the primary cause for diversification of geographic lineages, but rainforest contractions and expansions (ecological vicariance) can explain most of the spatial divergence observed in this species. Finally, analyses of gene flow and divergence time estimates suggest that the endangered subspecies *atlanticus* (from northeastern Brazil) can be considered a full species under the general lineage species concept.

Keywords: Atlantic Forest, Caatinga, phylogeography, nuclear markers, woodcreepers, areas of endemism, *Xiphorhynchus*.

1. Introduction

The Atlantic forest (AF) (Fig. 1A) is the second richest Neotropical biome and one of the most endangered in the world (Galindo Leal and Câmara, 2003; Myers et al., 2000; Tabarelli et al., 2005).

According to a model of forest stability during the last 21,000 years (Carnaval and Moritz, 2008) and to geological and palinological studies (i.e.: Behling, 2002; Behling and Negrelle, 2001; Brown, 2005; Brown and Ab'Saber, 1979; Ledru et al., 2005; Ledru et al., 1998), three historically different regions can be defined in the AF (Fig. 1B): (i) northern AF (NAF), with high forest stability in the eastern coastal region and short periods of rainforest expansions in areas currently covered by a type of dry forest (caatingas) (Velloso, 1991); (ii) central region (CAF), characterized by stability in the east and short periods of instability in the west, and (iii) southern AF (SAF), distinguished by strong instability (regression and fragmentation, and expansion at the mid and late Holocene).

According to the ecological vicariance model of diversification instability of forests can be important for speciation (reviewed in Marroig and Cerqueira, 1997; Moritz et al., 2000). Rainforest taxa may evolve in allopatry within rainforest relicts (refuges) that are caused and isolated by expansion of open areas (i.e.: grasslands, savannas or dry forest). Predominant evolutionary forces are drift and divergent selection. In the Neotropics, this model was initially known as the theory of the Pleistocene forest refuges (Brown and Ab'Saber, 1979; Haffer, 1969; Haffer and Prance, 2001; Vanzolini and Williams, 1970). Specifically, the refuges' theory considers that speciation was driven by forest fragmentation that occurred during the maximum of the global Pleistocene glaciations. However, the ecological vicariance model may also be considered for interglacial periods, such as the Holocene. For example, caatingas currently cover most of northeastern Brazil, from Ceará to northern Minas Gerais (Velloso, 1991), and rainforest organisms are restricted to small rainforest relicts (*brejos*), or to coastal forests, which are likely to be remnants of a past continuous rainforest that was fragmented with the advance of caatingas (Ab'Saber, 1977; Carnaval and Bates, 2007; Carnaval and Moritz, 2008; Oliveira et al., 1999; Wang et al., 2004). Therefore, these *brejos* are today's forest refuges and isolation by dry landscapes could have contributed to the evolution of the noteworthy endemic biota that characterizes the NAF (Silva and Casteleti, 2003).

The riverine barriers model of diversification (reviewed in Marroig and Cerqueira, 1997; Moritz et al., 2000) has also received attention to explain AF evolution. Some AF rivers limit regional faunas and therefore they may represent gene flow barriers and be important to model the biota distribution. This is

the case of Doce river, which limits the range of many birds (Silva et al., 2004), mammals (Costa et al., 2000) and butterflies (Brown, 2005). Also, Lacerda et al. (2007) presented genetic data that suggests a role of the Jequitinhonha river for separating populations of the passerine *Thamnophilus ambiguus*. Finally, Pellegrino et al. (2005) found that some phylogroups of the gecko *Gymnodactylus darwini* are endemic to specific inter basins and proposed that rivers played an important role in the diversification of the AF biota.

The late Tertiary and early Pleistocene geotectonic activity associated to the formation and remodeling of geological landmarks of SAF (i.e.: Serra do Mar mountain range) (Petri and Fulfaro, 1983; Riccomini et al., 1989) was suggested to be important for the evolution of the AF biota (Grazziotin et al., 2006; Lara and Patton, 2000; Leite, 2003; Mustrangi and Patton, 1997; Silva and Straube, 1996). For example, Silva and Straube (1996) observed that the range of some passerines were apparently limited by the geologic valley of the Paraíba do Sul river (Fig. 1) and proposed that the tectonic activity that opened the valley was important to split populations. However, our previous study of the mitochondrial DNA (mtDNA) of the passerine *Xiphorhynchus fuscus* did not support this geotectonic valley as a primary barrier (Cabanne et al., 2007).

The aforementioned hypotheses try to explain the origin of a highly diverse and heterogeneous AF (Silva and Casteleti, 2003). AF birds are good models for addressing these hypotheses given their high level of endemism (199 endemics, Stotz et al., 1996) and also because three different sets of endemic bird areas have been proposed (Fig. 1C). Areas of endemism are important to generate hypotheses about the history of geographical units and their biotas (Cracraft, 1985). These areas may be common regions of biotic differentiation (historical entities of a biome, Cracraft, 1985; Haffer, 1985), representing areas with past stability (refuges), regions isolated by rivers or geotectonic events, areas with different types of forest that define divergent selection, etc. The three groups of AF endemic bird areas are not totally congruent (Fig. 1C) and it is not known which one could best represent the AF biogeographic history. Regardless the process that lead to diversification of diagnostic taxa of each area of endemism, if areas of endemism were common regions of biotic differentiation, monophyletic populations from widespread and humid forest taxa are expected to be associated to those areas. And, phylogenetic relationship among these populations can help to understand the evolution of the areas' biotas. Alternatively, if areas of endemism only

represent fortuitous assemblages of taxa without any historical significance, monophyletic lineages of widespread rainforest taxa should not be associated to these areas.

X. fuscus (Aves: Dendrocolaptidae) is a good model for biogeographic studies because it occurs in well preserved forest of most of the AF's range, from sea level up to 1200 m a.s.l. (Ridgely and Tudor, 1996) (Fig. 1D). Four subspecies (*atlanticus*, *tenuirostris*, *brevirostris* and *fuscus*) are described according to plumage variation and overall body size (Fig. 1D) (reviewed in Marantz et al., 2003). It is not clear if these subspecies represent real natural entities as no obvious diagnosis exist when series of specimens were compared, except for *atlanticus* (Cabanne, unpubl.; Marantz et al., 2003). Subspecies *atlanticus* inhabits the most endangered forests of the biome (Galindo Leal and Câmara, 2003): coastal NAF, north to the São Francisco river, and some of the brejos of Ceará (Baturité and Ibiapaba) (Fig. 1D). Those forests are naturally isolated from southern portions of the biome by caatingas and therefore we hypothesize that *atlanticus* is genetically isolated. The subspecies *atlanticus* is diagnosed by plumage, overall size and song (Cabanne, unpubl.; Marantz et al., 2003) and since it is endangered (IBAMA, 2003), a genetic analysis is a priority study to help defining its evolutionary and taxonomic status.

We have previously described the mtDNA phylogeographic structure of *X. fuscus* at SAF (Cabanne et al., 2007). Now, our main goal is to study *X. fuscus*' populations in the entire AF to address the following questions: (i) Is the mtDNA phylogeographic pattern congruent with the nuclear DNA pattern?, (ii) Was the intraspecific diversification of *X. fuscus* associated to any area of avian endemism? Which are those areas?, (iii) does the genetic structure of *X. fuscus* support the forest stability model of Fig. 1B?, (iv) Which models of speciation (i.e.: refuges, river as barrier, etc) can explain the diversification within *X. fuscus*?, and (v) Does the genetic data support subspecies as independent evolutionary units (species)? In order to achieve these goals and to test predictions for some models of diversification (reviewed in Lara et al., 2005; Marroig and Cerqueira, 1997; Moritz et al., 2000; Patton and da Silva, 2005), we first analyzed the monophyly of *X. fuscus* based on mtDNA and then addressed evolutionary and spatial relationships among the *X. fuscus*' lineages by using mtDNA and nuclear sequences.

2. Materials and methods

2.1. Samples and sequences for the monophyly study (dataset 1)

We followed the taxonomic arrangement of Marantz et al. (2003). We tested the monophyly of *X. fuscus* using 29 terminals: 10 *X. fuscus*, 16 other taxa of the genus *Xiphorhynchus*, one *Lepidocolaptes angustirostris*, one *Campiloramphus trochilirostris* and, to root the phylogeny, *Sittasomus griseicapillus*. The sequences for this dataset were: ~1000 bp of Cytochrome B (CytB), ~1000 bp of NADH dehydrogenase subunit 2 (ND2) and ~450 bp of NADH dehydrogenase subunit 3 (ND3). Some sequences other than of *X. fuscus* were obtained from GenBank (Aleixo, 2002). See appendices A and B for details of the origin of sequences.

2.2. Samples and sequences for the intraspecific study (dataset 2)

For the intraspecific phylogeny we used 34 samples of *X. fuscus* collected in 22 localities from all over the species' range (Fig. 1D and Appendix B) and samples of six closely related outgroups (*X. spixii*, *X. ocellatus* and *X. pardalotus*). *X. guttatus* was used to root the intraspecific tree. This group of samples, without the outgroup taxa, was also used to obtain the sequence networks and for the population genetic analyses (section 2.6). The DNA sequences obtained were: mtDNA (same genes used for dataset 1) and 547 bp of the intron 5 of the β -Fibrinogen gene (FIB5). See appendixes A and B for details on origin of the samples, type of tissue, GenBank access, etc. From some specimens of *X. fuscus* we only collected blood because the species was either locally not abundant or endangered (i.e.: subspecies *atlanticus*).

2.3. DNA sequencing, neutrality tests and recombination

Total DNA was obtained from blood or muscle following Bruford et al. (1992). The following primers were used for amplification and sequencing: for CytB –primers L14841 and H16065 (Lougheed et al., 2000), for ND2 – primers LMET (Ribas et al., 2005) and H6313 (Sorenson et al., 1999), for ND3 – primers L10755 and H11151 (Chesser, 1999), and for FIB5 –primers FIB5 and FIB6 (Marini and Hackett, 2002). The sequences were obtained in sequencers ABI Prism 377 (Applied Biosystems) or MegaBACE 1000 (Amersham-Biosciences).

To identify haplotypes of heterozygous we used a Bayesian approach implemented in the program PHASE 2.0 (Stephens and Donnelly, 2003; Stephens et al., 2001). Heterozygous nucleotide positions were identified by double peaks in the electropherograms. Heterozygous indels positions were identified by an abrupt transition in the electropherogram from clean to unintelligible or a series of double peaks. All resolved positions received a posterior probability of one in the phasing analyses.

We used the PHI test in the program SPLITSTREE (Bruen et al., 2006; Huson and Bryant, 2006) to check for recombination in the FIB5. The PHI test is powerful for detecting recombination and has been shown to be less sensitive to recurrent mutation and homoplasy than other traditional tests (i. e.: Hudson and Kaplan, 1985). The neutrality tests of Tajima (1989) and of McDonald and Kreitman (1991) (MK test) were performed in DNASP 4.0. The MK test used *X. spixii* as outgroup.

2.4. Phylogenetic and genealogical analyses

Two different phylogenetic analyses were performed using maximum parsimony (MP) and maximum likelihood (ML) approaches. Firstly, we evaluated whether *X. fuscus* was monophyletic using mtDNA sequences (dataset 1). Secondly, we studied relationships among the intraspecific *X. fuscus*' lineages using a total evidence approach (concatenated dataset 2, mtDNA plus FIB5). Only for the second phylogeny, polymorphic positions in FIB5 were coded using the IUPAC ambiguity codes.

MP heuristic searches were performed in PAUP* 4.0 b10 (Swofford, 2001) with 100 replicates of random stepwise addition and a limit of 1,000,000 rearrangements of TBR branch swapping per replicate. We summarized MP results in a majority rule consensus tree. ML heuristic searches were performed in PHYML 2.4.4. (Guindon and Gascuel, 2003). The likelihood-ratio test was used as implemented in MODELTEST 3.7 (Posada and Crandall, 1998) to select the best-fit model of molecular evolution to be used in ML analyses. We did 500 MP bootstrap replicates in PAUP* and 500 ML bootstrap replicates in PHYML. The partition homogeneity test (Farris et al., 1994) was performed in PAUP* using 1000 replicates and tested for incongruence between mtDNA and FIB5 data.

We also explored relationships among haplotypes of dataset 2 using median joining networks (Bandelt et al., 1999) obtained in NETWORK 4.1.0.8 (<http://www.fluxus-engineering.com>). These analyses included all *X. fuscus* (n = 34) and one outgroup (*X. spixii*). Summary statistics for sequences were obtained using ARLEQUIN 3.1 (Excoffier et al., 2006).

2.5. Shimodaira and Hasegawa (1999) test

We used the Shimodaira and Hasegawa (1999) test (SH test) to assess whether a specific phylogenetic tree obtained in a constrained heuristic search and a non-constrained tree are equally good explanations of the data (Goldman et al., 2000). The constraints for the heuristic searches were derived from the three set of

areas of endemism for AF's birds (Fig. 1C). If areas of endemism were historical units in the evolution of *X. fuscus* (i.e.: refuges), we predicted to find reciprocally monophyletic populations in each of them. Thus, constraints forced monophyly (backbone option) of samples associated to each area of endemism depicted in Fig. 1C. Constraints did not force any specific relationship among areas, except in one specific case for the areas of Silva et al. (2004) [-(PE, ((CB, SM), COB))- see areas in Fig. 1]. Backbones only included samples collected in localities mapped within areas of endemism. Appendix B shows the assignment of samples to corresponding areas. Because *X. fuscus atlanticus* is relatively differentiated from the other subspecies, we also used the SH analysis to test the null hypothesis of paraphyly of *X. fuscus*, with *atlanticus* forced to group with other species of *Xipborhynchus*. SH tests were performed in PAUP* 4.0 b10 using the intraspecific phylogeny of *X. fuscus* obtained with dataset 2 (see Fig. 4), and applying the resampling estimated log-likelihood (RELL) option and 1000 replicates of non-parametric bootstrap to obtain the distribution of the statistics of the test.

2.6. Population analyses, divergence times and gene flow

We used the likelihood ratio test, as implemented in MODELTEST, to test the null hypothesis of constancy of evolutionary rates. This was done by comparing the log likelihood values of maximum likelihood trees with and without enforcing a molecular clock.

Rates of nucleotide change (sensu Ho et al., 2005) show variation depending on the date used to calibrate it (age of calibration); the relationship is not linear because rates tend to be faster when calibration points are recent. Ho et. al (2005) analyzed several calibrations of mtDNA molecular clock rates and developed a mathematical approach to directly relate DNA sequence distances to divergence dates. To obtain divergence dates (in million years, MYr) from mtDNA distances, we used the model of for protein-coding sequences of the avian mtDNA and resolved numerically the equation [7] of the Ho et al.'s paper. To approximate a mtDNA rate of change to be used for the isolation migration analyses, we transformed the observed mtDNA divergence (p-distance) to absolute time by the aforementioned method and then related both distance and time to obtain a rate of change. The mtDNA calibrations that resulted after applying this method were 2.46% of divergence per MYr for the study of the divergence between AF and the Amazon (dataset 1), and 4% to 4.6% per MYr for the population study (dataset 2).

The substitution rate adopted for FIB5 was 0.72% divergence /MYr, according to a specific calibration for other intron in chicken (see Axelsson et al., 2004).

We used the isolation-migration (IM) model (Hey and Nielsen, 2004; Nielsen and Wakeley, 2001), implemented in the program IM (ver. April 21, 2008), to estimate gene flow rates and final divergence times between lineages of *X. fuscus*. IM estimates the effective population size parameters of two populations (θ_1 and θ_2 , theta per marker) that diverged from an ancestral population (θ_a) t time ago in the presence of gene flow (m). Units of analysis (Table 1) were selected according to specific phylogenetic clades with geographic and or morphological correspondence, and not strictly following the subspecies taxonomy. For all analyses we used the complete sequences of mtDNA and FIB5 together (dataset 2). We used an inheritance scale of 0.25 for the mtDNA, the HKY model of evolution and assumed $\theta_1 = \theta_2 = \theta_a$ and $m_1 = m_2$. For testing whether a model of isolation without gene flow fitted to the data better than a model with gene flow, we repeated the former analysis constraining $m = 0$ and used the Akaike information criterion to compare models according to Nielsen and Wakeley (2001). For each analysis, we measured the distribution of the average time of migration events. Inspecting the posterior distribution of migration times allows a qualitative evaluation of the gene flow scenario. A high concentration of migration events near the present is consistent with secondary contact and posterior gene flow after allopatric divergence, while a broad distribution of migration times is consistent with recurrent gene flow since the time of population splitting (Niemiller et al., 2008; Won and Hey, 2005). We made preliminary runs to adjust priors and at least five subsequent analyses with different starting points to check for parameter convergence. We used 500,000 iterations for burn-in and analyses were stopped when the smallest effective sample sizes (ESS) were higher than 50 and parameter trend lines stabilized after four to 30 million iterations. For parameters in which the complete posterior distribution was completely estimated, we presented the highest posterior density interval. We transformed the time parameter t into time in years using the formula $t = t/u$, where u is the geometric mean of mutation rates per marker and generation (Hey and Nielsen, 2004), and the migration parameter m into the effective number of diploid individuals migrants per generation using the formula $M = (\theta m)/4$. We assumed a generation time of one year for *X. fuscus*.

To estimate migration rates and genetic diversity (Θ , theta per site) for each independent marker (mtDNA and FIB5) and to further evaluate recombination at the FIB5 we used the maximum likelihood

coalescent method implemented in LAMARC 2.1.2b (Kuhner, 2006). Unlike IM, LAMARC assumes migration equilibrium. In order to compare mtDNA and nuclear (FIB5) migration, we corrected the difference between the markers' effective size by applying an mtDNA effective size scalar equal to 4. We also used LAMARC to evaluate the demographic signature of *X. fuscus*' lineages by estimating the exponential population growth rate g ($\Theta_t = \Theta_{\text{now}} e^{-gt}$, where Θ_{now} is the current Θ and Θ_t is the value of the parameter t time ago) using mtDNA and FIB5 together. Positive g -values indicate population growth and negative values, population decline. All final LAMARC analyses used the F84 model of sequence evolution with empirical base frequencies and transition/transversion ratios and ran 10 short chains (500 sampled trees every 50 iterations and burn-in of 1000 trees) followed by two long chains (20,000 sampled trees every 50 iterations and burn-in of 1000 trees).

Finally, we compared the migration rates obtained by IM and LAMARC with those obtained by assuming the island model of Wright (1940). Global Φ_{st} and its 95% bootstrap confidence interval (c.i. 20,000 replicates) for each marker were obtained by AMOVA (Excoffier et al., 1992) in ARLEQUIN and gene flow estimations were obtained using the formula $M = (1 - \Phi_{st})/2\Phi_{st}$ (Hedrick, 2000).

3. Results

3.1. Molecular variation

A mtDNA sequence matrix of 2471 bp [dataset 1: 785 variable positions, 522 (66.5%) informative for parsimony] was obtained for the *X. fuscus* monophyly study. The alignment for the total evidence of intraspecific study resulted in 3047 bp [dataset 2: 2499 bp of mtDNA: 445 variable positions, 303 (68%) informative for parsimony; and 547 bp of FIB5: 37 variable positions, 23 (62.1%) informative for parsimony]. Summary statistics for all the *X. fuscus*' sequences (dataset 2) are presented in Table 1. The MK test for the *X. fuscus*' mtDNA (2499 bp, $n = 34$) was marginally significant (G test with William's correction, $P = 0.046$) due to the existence of an excess of non-synonymous polymorphism. This result is expected for mtDNA (Nachman, 1998) and since Tajima's D test (Tajima, 1989) was non-significant (Table 1) we interpreted this pattern as a result of demographic history. FIB5 sequences of *X. fuscus* did not present evidence of recombination, according to the PHI test ($P = 0.848$) and LAMARC (recombination parameter $r \ll 0.0001$).

3.2. Phylogenetic and genealogical analyses

The topologies obtained by MP and ML were similar, and only ML trees are shown. *X. fuscus* samples grouped in a well supported monophyletic clade in the mtDNA analysis (Fig. 2). Subspecies *atlanticus* was monophyletic, and the other three subspecies formed another monophyletic clade, with *tenuirostris* and *brevirostris* grouped in one well-supported lineage, and *fuscus* forming two separate lineages. The median joining network of *X. fuscus* mtDNA (Fig. 3A) resulted in four main lineages congruent with the phylogenetic analysis of Fig. 2 and confirmed that *atlanticus* is monophyletic and the most divergent lineage. FIB5 network did not corroborate all the mtDNA lineages (Fig. 3B). The total evidence topology (mtDNA + FIB5, Fig. 4A) did not differ from the one obtained with mtDNA alone (Fig. 2) and was in accordance with the mtDNA network (Fig. 3). The null hypothesis of *X. fuscus* paraphyly was rejected by the SH test (Table 2, line 1). The incongruence between mtDNA and FIB5 suggested by the networks (Fig. 3) was confirmed by the partition homogeneity test ($P = 0.005$). This result was expected because the dataset have a high number of sequences from the same species, and it is not surprising to find incongruence between loci at the intraspecific level, especially when one of the markers (FIB5) presents higher effective size and lower evolutionary rate. This was confirmed when the number of *X. fuscus* sequences in dataset 2 was reduced and the partition homogeneity test was not significant ($P < 0.01$), indicating congruence between mtDNA and FIB5 among the species of *Xipborhynchus* analyzed (data not shown).

The SH tests showed that monophyletic intraspecific lineages of *X. fuscus* were only associated to some of the areas endemism of Silva et al. (2004) ($P > 0.01$, Table 2), rejecting the hypotheses that the other areas of endemism would represent centers of diversification.

In summary, four well-supported main lineages with geographic correspondence were revealed by both mtDNA only (dataset 1, Fig. 2) and combined markers (dataset 2, Fig. 4): *atlanticus*, the clade of *tenuirostris* and *brevirostris*, and the two lineages of *fuscus* (N and S, for north and south). These four lineages, or subsamples of them, were used as units of analysis for the population genetic studies (Table 1).

3.3. Mitochondrial genetic distances

The null assumption of constancy of molecular evolution rates for the mtDNA phylogeny data (dataset 1) was not rejected ($P>0.05$). The mtDNA corrected genetic distance between *X. fuscus* and its sister clade containing the Amazonian *X. spixii*, *X. elegans*, *X. ocellatus* and *X. pardalotus* (Fig. 2), was 0.0836 (SD = 0.006) and according to the method of Ho et al. (2005) the distance corresponded to 3.07 (SD = 0.13) MYr of divergence. Table 3 presents uncorrected mtDNA distances between intraspecific lineages of *X. fuscus* (dataset 2).

3.4. Population divergence, gene flow and demography

Divergence among *X. fuscus*' lineages obtained by IM ranged from 0.5 to 1.5 MYr (Table 4). The Akaike information test rejected the model of divergence without gene flow for all the comparisons (data not shown). Migration rates obtained by IM were slightly lower than those obtained by LAMARC. However, both methods showed a similar scenario. In general, migration rates between *atlanticus* and any other lineage, and between Ceará and Alagoas, were the lowest. Distribution of migration events obtained by IM peaked between 0.3 MYr to 0.15 MYr and suggested that migration was not concentrated at the end of the divergence as would be expected in a model of complete allopatric divergence followed by a secondary contact, except for the pair *atlanticus* x *tenuirostris*/*brevirostris*, which also presented recent gene flow (Fig. 5).

The nuclear marker presented higher migration rates than the mitochondrial marker in all the comparisons with LAMARC (Table 4). However, when mtDNA migration estimations considered the difference between markers' effective size, the confiability intervals overlapped and therefore the disparities were not important. The population genetic structure among the four lineages of *X. fuscus* was smaller with the FIB5 than with the mtDNA ($\Phi_{st_{FIB5}} = 0.147$, c.i.: 0.08-0.215, $P < 0.01$, and $\Phi_{st_{mtDNA}} = 0.916$, c.i.: 0.88-0.93, $P < 0.01$). Based on the island model of Wright (1940), the overall M_{FIB5} was 2.9 (c.i.: 1.82 – 5.75) and the M_{mtDNA} was 0.045 (c.i.: 0.037 - 0.07). Migration rates obtained by the Wright (1940) island model are slightly higher from those obtained by the IM model (average $M = 0.39$, $n=6$) but similar to those obtained for the FIB5 by LAMARC (average $M = 2.92$, $n = 6$).

The highest levels of genetic diversity (Θ -values) obtained in LAMARC were found at CAF, while Θ -values at NAF and SAF were similar (Fig. 4C). The exponential growth rates (g) indicated signatures of demographic growth in all the samples other than Ceará, which showed decline (Fig. 4C).

Notwithstanding some confiability intervals of g included stability ($g = 0$), all the demographic tendencies were clear as stability was a very marginal situation.

4. Discussion

The analyses showed that *X. fuscus* is monophyletic (Figs. 2 and 4). Even though FIB5 and mtDNA sequences were congruent at the level of the genus *Xiphorhynchus*, FIB5 sequences did not evidence a strong phylogeographic structure as shown by the mtDNA (Fig. 3). This result is not contradictory because nuclear markers are less variable and have longer coalescence times than the mtDNA. Thus, and also because mtDNA patterns are robust indicators of population history (Zink and Barrowclough, 2008), we consider our results unbiased and reliable.

The differences between migration values obtained by LAMARC and IM were not unexpected. IM does not consider migration equilibrium and is able to distinguish between shared polymorphism originated by gene flow and incomplete lineage sorting (Nielsen and Wakeley, 2001; Pinho et al., 2008). On the other hand, both LAMARC and the F_{ST} -based methods may retrieve higher migration values because they interpret shared polymorphism as gene flow; thus we considered M-values obtained by those approaches as overestimations. A mtDNA phylogeographic pattern more structured than the FIB5 pattern, as observed in *X. fuscus*, may be caused by diminished female gene flow. However, LAMARC gene flow analyses did not support this scenario and therefore the incongruence between markers (Fig. 3) at the population level could be explained as consequence of the difference between the markers' effective sizes.

4.1 Congruence between genetic structure and subspecies

The subspecies taxonomy did not match all the evolutionary lineages of *X. fuscus* (Fig. 3 and 4). *X. f. atlanticus* was the only subspecies that represented a monophyletic and isolated lineage. A completely resolved relationship among all *X. fuscus*' lineages was not obtained, possibly due to a rapid and recent diversification (Fig. 4). IM analyses indicated divergence times greater than zero between all the lineages (Table 4). These results, the geographic partition of lineages and the congruence of genetic and morphological differences between *atlanticus* and all the other lineages indicated altogether that the results

evidenced organismal lineages rather than clades, which are consequence of stochastic coalescence of sequences (Dolman and Moritz, 2006; Irwin, 2002). Since the subspecies other than *atlanticus* lack an obvious diagnosis (genetic or morphological), a detailed study is needed to reevaluate the geographic variation of external characters and to understand the correspondence of such variation with the genetic pattern.

4.2. Contact between the Amazon and the Atlantic forest

Several evidences support a historical contact between the Amazon and the Atlantic forest (i.e.: Bates et al., 1998; Behling et al., 2000; Costa, 2003; Oliveira et al., 1999; Silva, 1996; Wang et al., 2004) and our results provided additional data to better understand about this contact. The ancestral *X. fuscus* is likely to be Amazonian, as almost all species of *Xipborhynchus* are restricted to this region or to Central America. The oldest split within *X. fuscus* separated NAF from other regions (Fig. 4 and Table 4) and the most basal FIB5 haplotype is from NAF (Fig. 3B), suggesting that the ancestor of *X. fuscus* arrived in the AF at northeastern Brazil, perhaps through extinct rainforests in the coast or in the currently dry inland. A southern dispersion scenario, for example through the Paraguay river basin (Nores, 1992), is less parsimonious as it implies a long distance dispersion from SAF towards NAF before the first intraspecific divergence occurred.

Few studies addressed the timing of the connection between the two forests. Most of the palinological and geological studies only cover the last 200,000 years and evidence several short periods of forest expansion that could have linked the two biomes (i.e.: Behling et al., 2000; Oliveira et al., 1999; Wang et al., 2004). The divergence between *X. fuscus* and its Amazonian sister clade (~3 MYr) also suggests older connections (Pliocene), which is in accordance with other studies (i.e.: Costa, 2003; Ribas and Miyaki, 2004). The radiation of the clade of *X. fuscus*, compared to its Amazonian sister clade, showed shorter branches (Fig. 2) and lower genetic distances among terminals (data not shown). This pattern suggests lower diversification rates in AF, and could be product of higher speciation rates in Amazon forest or higher extinction rates in AF (Ricklefs, 2007). More studies are necessary to address this issue.

4.3. Areas of endemism

Areas of endemism are important to generate hypotheses about the history of geographical units and their biotas. The areas of endemism for AF passerines described by Silva et al. (2004) were not rejected as regions of differentiation for *X. fuscus* (Table 2). However, test significances were low because two of the propositions of Silva et al. (2004) were not supported: (i) coastal Bahia plus northern Espírito Santo as an independent area from inland Bahia plus part of northeastern Minas Gerais (Figs. 1C, Fig. 4), and (ii) the region south to the Doce river (area SM) as a homogeneous biogeographic unit. Since organisms can react idiosyncratically to specific biogeographic events (Prum, 1988) other patterns could emerge from other organisms. Notwithstanding our results are among the first steps for understanding the AF evolution, they suggest that the areas of Silva et al. (2004) may represent a good hypothesis about biogeographical units of the biome. However, only corroboration of our results with other taxa would show if these areas have a general biogeographic significance.

4.4. Intraspecific pattern of *X. fuscus* and its evolution: geotectonism and rivers

The subspecies *X. fuscus atlanticus* is differentiated and isolated in the NAF while the other lineages are connected by moderate migration rates. Interestingly, even though the ancestral *X. fuscus* seems to be pre-Pleistocenic, the current lineages only diverged in a relatively short period at the mid - late Pleistocene (Table 4).

The proposition of Silva and Straube (1996) about the primary biogeographic role of the valley of the Paraíba do Sul river (Fig. 1D) was not supported for *X. fuscus* in our previous study (Cabanne et al., 2007). In that study we only analyzed mtDNA data and discussed whether the tectonic formation of the valley initiated the divergence of lineages *fuscus* N and S, which get into contact near the valley (Fig. 1D). Even though divergence times obtained in the present study (Table 4) are slightly older than the first estimates (probably because migration equilibrium was not assumed now) they confirm previous conclusions. The geotectonic valley is not supported as a primary barrier for the divergence between *X. fuscus* lineages because the valley's age (>15 MYr; Souza et al., 2005) does not match the divergence between lineages (~0.4 MYr) and because the lineages' contact is outside and perpendicular to the valley (Fig.4). See Cabanne et al. (2007) for a detailed discussion on this issue.

Rivers and their valleys can be primary or secondary barriers to gene flow (Moritz et al., 2000; Patton and da Silva, 2005). A primary barrier is one that imposed itself on an existing taxon range and separated populations that became sister clades. A secondary barrier is the encounter point of clades that evolved elsewhere. A river can also act as a barrier when episodes of overwater dispersal occurred, which can be recognized when haplotypes from each bank present a paraphyletic relationship (see Patton and da Silva, 2005). Lineages *brevirostris* / *tenuirostris* and *fuscus* N meet at the lower portion of the Doce river in Espírito Santo (Fig. 4), thus the river could have been a significant barrier for gene flow. Assuming that the divergence time between *X. fuscus* and its Amazonian sister clade (Pliocene) is the earliest date of arrival of the ancestral *X. fuscus* at the AF and considering that the current fluvial system of eastern Brazil was already established at that period (Dominguez et al., 1987; Lundberg et al., 1998; Potter, 1997; Ribeiro, 2006), the Doce river appears to be a secondary barrier. The relationships among the three lineages at eastern Brazil are not well resolved (Fig. 4), possibly as a consequence of rapid diversification. This lack of resolution did not allow us to distinguish between a secondary contact and overwater dispersion (Patton and da Silva, 2005). However, the signal of population expansion for the lineages (Fig. 4C) that meet at the Doce river is congruent with a secondary barrier (Cheviron et al., 2005). An alternative view related to a sort of primary barrier effect of the Doce river proposes that birds could have dispersed along the river from its headwaters in the Espinhaço mountain range in central Minas Gerais and reached the mouth of the stream. Later, downstream populations in each margin may have diverged due to the isolation by the river. This model is compatible with the population expansion signals that we have found (this work and Cabanne et al., 2007). However, it implies that the headwaters of the Doce river are not an effective barrier and we would expect to find at the springs intermediary haplotypes between the two lineages currently separated by the river. This prediction was not corroborated by the mtDNA data since the lineage *fuscus* N is found at the two margins of the river in its springs (Fig. 4 and Cabanne et al., 2007).

The São Francisco is the largest river in the study area and could have isolated populations of *X. fuscus*. However, this river runs mostly in the middle of a dry landscape (Caatinga) unsuitable for rainforest birds. Thus, it is difficult to detach the effect of the river from the effect of caatingas in the isolation of forest organisms. Also, São Francisco river is ancient and runs over the tectonically stable Brazilian shield (Lundberg et al., 1998; Potter, 1997; Ribeiro, 2006), thus if it acted as barrier it should be secondary.

4.5. Evolution of the intraspecific pattern of *X. fuscus*: ecological vicariance

Part of the controversy related to the evolution in refuges lies on whether past climatic cycles fragmented forests significantly (i.e.: Colinvaux et al., 2000). Main predictions for the hypothesis of refuges are demographic expansions and secondary contacts of forest taxa (Moritz et al., 2000). According to these predictions and to the model of Fig. 1B, the following general pattern is expected: (i) low genetic diversity and signatures of stability and or demographic decline in NAF, especially in interior regions where rainforest regressed during the Holocene; (ii) high genetic diversity and signature of stability or weak expansion in CAF (high forest stability), and (iii) low genetic diversity and strong signatures of demographic expansion in populations of SAF (low forest stability).

Overall genetic diversity levels are in accordance with the predictions mentioned (Fig. 4C). For both markers, the higher genetic diversity was found in CAF, suggesting that this region maintained a larger population of *X. fuscus* than the other regions (NAF and SAF). Despite the fact that SAF presents today (before human impact) a similar or even larger range of rainforest than the CAF (Fig. 1A), the difference in genetic diversity levels between both regions suggest that SAF has had smaller populations of *X. fuscus* over the long term. This comparison showed the impact of the forest history on the genetic diversity of organisms. The demographic signatures suggest population growth for all the samples, except for Ceará where decline was evidenced (Fig. 4C). The decline at Ceará is compatible with the existence of a past large rainforest in the interior of the current Caatinga and thus supported the model depicted in Fig. 1B. The signature of expansion in Alagoas, the other sample from NAF, contrasts with the one of Ceará and with our expectations. Even though the coastal region of Alagoas and Pernambuco, known as the Pernambuco refugia (Carnaval and Moritz, 2008), is likely to have maintained forest over the long term (Fig. 1B), the results suggest that this refugia was smaller than the current distribution of rain forest in the region. A signature of demographic growth in CAF similar to the ones observed at SAF (lineages *fuscus* N and *fuscus* S) was not expected (Fig. 4C). However, even though sample sizes of each lineage other than *fuscus* S are relatively similar (Table 1), the confiability interval of the CAF g-value is smaller than the other intervals. This result may indicate a violation of the LAMARC model (constant growth) in regions other than CAF (M. Kuhner pers. com.), which would be compatible with the notion of high forest instability at those regions. Fluctuation in the forest range and connectivity related to global glacial cycles could have

alternated short periods of growth with periods of stability or population decline in NAF and in SAF, producing the observed demographic patterns. Since CAF forest are likely to have been more stable during the late Pleistocene climatic cycles (Carnaval and Moritz, 2008), any demographic trend could have been maintained longer than in NAF and SAF. However, as the behavior of methods to study historical demography (e.g.: LAMARC or the ones based on summary statistics, Ramos-Onsins and Rozas, 2002) are not well understood when the constant population trend is violated (M. Kuhner pers. com.), our comparative discussion of the demography of *X. fuscus* should be interpreted with caution.

Secondary contacts also evidence demographic fluctuations compatible with refuges. Our previous study of *X. fuscus* mtDNA describes a secondary contact between lineages *fuscus* N and S along no evident barrier to gene flow in central São Paulo (Cabanne et al., 2007). However, the migration-time graphic did not evidence recent gene flow between these lineages (Fig. 5), perhaps because the contact is very recent or the analysis lacked statistical power. Interestingly, phylogeographic gaps in other vertebrates also occur in central and eastern São Paulo (Grazziotin et al., 2006; Mustrangi and Patton, 1997; Pessoa et al., 2006), supporting the idea that common events affected a great proportion of the biota, as could have happened during past global climatic fluctuations. Even though populations are currently isolated, the migration-time graph showed recent gene flow for the pair *atlanticus* - *tenreirostris* / *brevirostris* (Fig. 5). Gene flow is also suggested by the FIB5 network (Fig. 3), which showed one common haplotype shared between interior CAF (*brevirostris*) and NAF, a pattern likely to be originated by population contact (Omeland et al., 2006). These results supported the model of Fig. 1 B, where short rainforest expansions connected in the past NAF with CAF, and suggested a stronger biogeographic link between interior CAF and NAF than between coastal CAF and NAF.

NAF and CAF populations of *X. fuscus* diverged ~0.5 MYr ago while Ceará and Alagoas populations diverged more than 0.1 MYr ago (Table 4). Even though rainforests are likely to have expanded historically in the interior of the continent and to have survived at the NAF coastal region during the last 21,000 years (Fig 1B), our results suggest that NAF has not been totally covered by rainforest during the recent past (i.e.: the last global glaciation). We believe that this spatial and temporal isolation may be responsible for the extraordinary endemism observed at NAF (Galindo Leal and Câmara, 2003). For example, the southern range border of *atlanticus* coincides with a strip of caatingas along the northern

basin of the São Francisco river (Fig. 1D) and this region could have acted as a gene flow barrier and isolated the ancestral of *atlanticus*.

Additionally, divergence at NAF may be currently ongoing. This appears to be evidenced by the sample from Ceará that presented only one mtDNA haplotype, but regular diversity at the nuclear marker, different from the ones at Alagoas (Figs. 3 and 4). This genetic diversity pattern was not observed in any other sample of comparable size (i.e.: *atlanticus* from Alagoas, or *fuscus* S) and may have been caused by strong genetic drift, a major factor occurring in allopatric divergence and expected to be strong in small and isolated populations such as the one at Ceará (Serra de Baturité, ~125 Km²). Our study agrees with the report of Carnaval and Bates (2007) on the phylogeography of two frogs from northeastern Brazil. They found monophyletic populations with low genetic diversity at brejos of Ceará that diverged from coastal Pernambuco and Alagoas more than 0.1 MYr ago. Furthermore, our results and those from others (Carnaval and Bates, 2007; Leite, 2003) support the view that the formation of northernmost forest enclaves (i.e.: Serra de Baturité, Fig. 1D) could have been a consequence of the early expansion of caatingas (mid Pleistocene) (Ab'Saber, 1977) and that, in opposition to southern relicts of Ceará (i.e.: Serra de Araripe), where *X. fuscus* does not occur, were not extinct at the end of the Pleistocene.

Contrarily to NAF, isolation by caatingas did not result in a phylogeographic gap between the Chapada Diamantina rainforest relict and coastal rainforest in CAF (Fig. 1D), possibly because the relict is large and recent (Table 4). Samples from the Chapada Diamantina grouped with birds from coastal Bahia and Espírito Santo (Fig. 4), a pattern that supports a recent forest connection between both regions and is in accordance with the model of Fig 1B. Our results show the magnitude of the dynamism of the ecotone rainforest-caatinga (Oliveira et al., 1999; Wang et al., 2004). In some regions, such as interior Bahia (CAF), this dynamism allowed sporadic connection of forests and prevented significant divergence, but in other regions, such as NAF, this dynamism did not allow reconnection of forests and homogenization in the short term.

Our genetic diversity and demographic results (Fig. 4C) matched the general predictions of the model of Carnaval and Moritz (2008). However, the existence of *X. fuscus*' lineages, as well of other taxa or phylogroups (Brown, 2005; Costa et al., 2000; Grazziotin et al., 2006; Pessoa et al., 2006; Silva et al., 2004), endemic to SAF suggests that the model failed to predict large areas with stable rainforest at SAF. It might be proposed that those *X. fuscus*' lineages (*fuscus* N and S) dispersed from the stable CAF after forests

expanded in SAF. Under this scenario, the SAF lineages should be derived phylogenetically from the CAF lineage or be present in CAF, but none of these predictions are supported (Fig. 4). Perhaps a small range of stable forest, as predicted for the western SAF (Fig. 1B), might explain the *X. fuscus* lineages endemic to SAF. However, the high number of other taxa endemic to the eastern SAF (i.e.: in Serra do Mar at eastern São Paulo, Paraná and Santa Catarina, Costa et al., 2000; Silva et al., 2004) cannot be explained by these small forests. We believe that the persistence of forest blocks has been essential for the evolution of endemic taxa and phylogroups at eastern SAF.

The IM analyses and the Akaike information criteria (Nielsen and Wakeley, 2001) ruled out fully allopatric divergence. Allopatric models are often used to explain biogeographic patterns, especially those with reciprocal monophyly. However, isolation with gene flow can also result in monophyletic clades (Hey and Nielsen, 2004; Nielsen and Wakeley, 2001; Nosil, 2008). If isolation-migration models were more frequently considered, minor geographic features that are traditionally considered inefficient barriers would become important to explain biogeographic patterns. From this standpoint, climatic changes could be central since they would modulate the effect of gene flow barriers. For example, a river may have a stable course but changes in its water flow induced by climatic alterations could modify its effect as a barrier, or a mountain range could be geologically stable but an alteration of its vegetation cover could also modulate its effect as a barrier. The transition of *X. fuscus*' lineages at eastern São Paulo occurs in proximity of the Paraíba do Sul valley and the mountain ranges of Serra da Mantiqueira and Serra do Mar (Fig. 4B) (Cabanne et al., 2007). This region is topographically and phytogeographically complex. There are steep altitudinal (>1000 m) and climatic transitions and the vegetation types are various, such as tropical evergreen forest, semideciduous forests, enclaves of Cerrado (South American Savanna, Veloso, 1991) and grasslands at mountains' tops. Besides, palynological studies suggest the existence during the late Pleistocene and early Holocene of a larger range of cerrados and grasslands in southeastern Brazil (Behling, 1998); therefore indicating that local past landscapes were complex as well. Thus, these landscape characteristics may present a partial barrier effect for the gene flow of forest organisms that could have been modulated by climatic oscillations. Hot and dry periods could have expanded cerrados and cold periods could have narrowed forest corridors at hillsides by modifying the grassland – forest line at mountains. This partial barrier effect may be considered together with more widespread changes in the

forest distribution during glaciations (Fig. 1B) to explain divergence and transitions of lineages in SAF (Fig. 4).

4.6. Systematics and conservation of *X. fuscus atlanticus*

Based on our results and on the distinctiveness of the subspecies (Cabanne, unpubl.; Marantz et al., 2003), we followed the general lineage species concept (de Queiroz, 1998) and propose to recognize *X. fuscus atlanticus* as a full species. The mtDNA p-distance between *atlanticus* and any other genetic lineage ($>2.6\%$, Table 3) is compatible with mtDNA divergences found between well-recognized species of the genus *Xiphorhynchus* (Aleixo, 2002). Gene flow between *atlanticus* and other lineages was generally low ($M < 1$) and likely not enough to avoid allele fixation and strong divergence (Hedrick, 2000; Wang, 2004; Wright, 1931). This gene flow level supports the condition of independent evolutionary lineage for *atlanticus*. Besides, *atlanticus* is diagnosed by five aminoacidic changes at the mtDNA, whereas the other lineages are only characterized by synonymous or at most one non-synonymous change (Fig. 3). *X. fuscus atlanticus* is monophyletic at the mtDNA, but it shares one FIB5 haplotype with interior Bahia birds (Fig. 2 and 3). This condition is not unexpected for a nuclear marker and should not be overvalued for taxonomical discussions. Particularly in this case, the specific population is isolated, diagnosable by external characters and monophyletic at mtDNA. All isolated populations will eventually acquire reciprocal monophyly, as stated by the coalescence theory (Hudson, 1990). However, as not all genetic markers fixate new mutations at the same rate, it is expected to find intermediate states of polyphyly in recently isolated populations (Baker et al., 2003; Omland et al., 2006; Zink and Barrowclough, 2008). Our data did not support the other three lineages (*tenuirostris/brevirostris*, *fuscus* N and *fuscus* S) as independent evolutionary entities. Those lineages are differentiated at the mtDNA, but gene flow among populations was relatively high ($M > 1$) and diagnosis by external characters is not obvious.

If *atlanticus* is recognized as a species it will add a new globally threatened taxon to the region, since the population is already locally endangered. Notwithstanding the shallow genetic divergence (i.e. mtDNA p-distance 0.0012), our results indicated that the two populations of *atlanticus* studied (Alagoas and Ceará), should be considered different evolutionary units (Moritz, 1995) and treated as different management units for conservation purposes. Gene flow between Ceará and Alagoas was very low (Table 4), which indicated low genetic exchangeability between them (Crandall et al., 2000). This result was

expected as the two regions are isolated by c. 500 km of caatinga and no stepping stone populations are known.

4.7. Conclusions

Some monophyletic lineages of *X. fuscus* were associated to the areas of endemic passerines described by Silva et al. (2004). Since the genetic data of *X. fuscus* supported in some degree the palaeobotanic model of the AF (Fig. 1B), those endemic bird areas appear to be reflecting zones with long term forest stability (see also Carnaval and Moritz, 2008).

According to its biological composition and late Pleistocene and Holocene history, the AF can be divided in at least three areas: NAF and SAF, which are instable in terms of range and connectivity, and the more stable CAF. Perhaps, the stability is one of the reasons why CAF shares a considerable amount of taxa with lowland Amazon forest (i.e.: Bates et al., 1998; Ridgely and Tudor, 1996) that appears to be more stable in the long term (Colinvaux et al., 2000). The main forces driving instability at AF are apparently the climatic alterations related to global glacial cycles (Carnaval and Moritz, 2008). A traditional view about diversification driven by glacial cycles is that it occurs in pulses and creates topologically symmetric phylogenies (Marroig and Cerqueira, 1997). However, there would not be such pulses of diversification in the AF as a whole because instability at NAF and at SAF is not completely synchronic. Forest fragmentation at NAF is maximum during interglacial periods (i.e.: Holocene), while fragmentation at SAF is maximum during the peaks of glaciations. Therefore, if ecological vicariance is important for the diversification of the AF biota, these asynchronic cycles of fragmentation would create a constant rhythm of diversification and phylogenies that may not be symmetric. To support this hypothesis, we would expect to find more signatures of population decline and divergence in isolated forest at NAF and additional secondary contact regions at SAF will help to address this hypothesis.

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Fig. 1. Distribution of the Atlantic forest (AF), endemic bird areas, sampling locations and distribution of *Xiphorhynchus fuscus* and its subspecies. A) Distribution of AF biome. NAF: northern AF; CAF: central AF; SAF: southern AF. B) Historically stable areas for the AF during the last 21,000 years. Modified from Carnaval and Moritz (2008). The darker the area the higher the forest stability; i.e.: black areas presents the maximum likelihood to have maintained forest. The circle indicates the area of highest forest stability (Bahia refuge). C) Areas of endemism of birds in the AF. SMC: Serra do Mar

Center, PRC: Paraná Center, AAP: Atlantic slope of Alagoas and Pernambuco, DB: Deciduous forest of Bahia, DMGG: Deciduous forest of Minas Gerais and Goiás, AL: AF Lowlands, AM: AF Mountains, Pe: Pernambuco, CB: Central Bahia, CoB: Coastal Bahia, SM: Serra do Mar. D) Approximate distribution of *X. fuscus* (gray line) and approximate core distribution of subspecies shown by gray areas (Ridgley and Tudor 1996; Marantz et al., 2003; Cabanne unpubl.). Collection localities indicated by numbers (see Appendix B for details). States, PI: Piauí, CE: Ceará, RN: Rio Grande do Norte, PA: Paraíba, AL: Alagoas, PE: Pernambuco, BA: Bahia, ES: Espírito Santo, MG: Minas Gerais, RJ: Rio de Janeiro, SP: São Paulo, PR: Paraná, SC: Santa Catarina, RS: Rio Grande do Sul, Mi: Misiones (Argentina).

Fig. 2. Maximum likelihood (ML) tree based on 2471 bp of the CytB, ND2 and ND3 (mtDNA, dataset 1). Model: Tamura & Nei (1993) + I (I = 0.6036) + G (α = 2.1493). Estimated base frequencies: A = 0.32, C = 0.33, G = 0.11, T = 0.24. Bootstrap values (percentage of 500 replicates) at the nodes: ML / maximum parsimony. The maximum parsimony analysis resulted in eight most parsimonious trees with 1807 steps.

Fig. 3. Median joining networks based on A) mitochondrial DNA of *X. fuscus* (2499 bp of concatenated CytB, ND2 and ND3, dataset 2, n = 34 sequences), and on B) FIB5 of *X. fuscus* (547 bp, dataset 2, n = 68 sequences). Number of aminoacidic changes are shown *X. fuscus*. See Table 1 for summary statistics of all the mtDNA and FIB5 data set.

Fig. 4. Phylogenetic relationships, genetic diversity and demography of intraspecific lineages of *X. fuscus*. A) Maximum likelihood (ML) tree based on a total evidence analysis of mtDNA and FIB5 (dataset 2, total alignment of 3047 bp, CytB, ND2, ND3 and FIB5). Model of evolution: Tamura & Nei (1993) + I (I = 0.501) + G ($\alpha = 0.8132$). Estimated base frequencies: A = 0.31, C = 0.29, G = 0.13, T = 0.27. Bootstrap values (percentage of 500 replicates) at the nodes: ML / maximum parsimony. The maximum parsimony tree had 639 steps. Numbers within parentheses indicate sampling locality (see Fig. 1 and Appendix B). B) Distribution of the main lineages of *X. fuscus*. Based on the present work and in Cabanne et al. (2007). C) Estimation of genetic diversity (Θ) and demographic signature (g) of samples of *X. fuscus*.

Fig. 5. Distributions of migration events for each pairwise comparison between geographically contiguous lineages of *X. fuscus* obtained from the IM program (Won & Hey 2005), averaged across markers.

Table 1

Summary statistics of the mtDNA (concatenated CytB, ND2 and ND3) and FIB5 sequences of the complete dataset and intraspecific lineages of *X. fuscus*.

	<i>X. fuscus</i> , complete dataset 2		Lineages (geographic region)							
			<i>atlanticus</i> (NAF)		<i>brevirostris</i> / <i>tenuirostris</i> (CAF)		<i>fuscus</i> N (SAF)		<i>fuscus</i> S (SAF)	
	mtDNA (2499 bp)	FIB5 (547 bp)	mtDNA	FIB5	mtDNA	FIB5	mtDNA	FIB5	mtDNA	FIB5
N	34	68	10	20	11	22	8	16	5	10
K	23	28	6	8	8	15	6	7	3	3
$\pi\%$	1.51	0.693	0.0961	0.612	0.2778	0.735	0.0837	0.61	0.1436	0.4233
D	1,019 ^{NS}	-1,249 ^{NS}	-0.856 ^{NS}	0.841 ^{NS}	-0.541 ^{NS}	-0.977 ^{NS}	-0.632 ^{NS}	0.039 ^{NS}	-0.807 ^{NS}	0.370 ^{NS}

n: number of individuals, N: number of sequences, K: number of haplotypes, $\pi\%$: nucleotide diversity (Nei and Kumar, 2000) in percentage based on p-distance, D: Tajima's D (1989). NS: Non Significant ($P > 0.05$). NAF: northern AF; CAF: central AF; SAF: southern AF.

Table 2

Results of Shimodaira and Hasegawa (1999) test based on a total evidence tree (Fig. 4) to evaluate the monophyly of *X. fuscus* and to test if monophyletic clades are associated to Atlantic forest endemic bird areas. -ln L of the unconstrained tree is 7648.95470.

Hypothesis	-ln L constrained tree	delta -ln L	<i>P</i>
<i>X. fuscus</i> paraphyletic	7701.48898	52.53429	0.000
Cracraft (1985)	7696.28494	47.33024	0.000
Stattersfield et al. (1998)	7770.7244	113.26381	0.000
Silva et al. (2004)	7657.45863	8.50392	0.085
Silva et al. (2004) with hierarchy *	7684.90740	27.44877	0.018

*: (PE, ((CB, SM), COB)), see areas in Fig. 1.

Table 3

Uncorrected genetic distances between intraspecific lineages of *X. fuscus* based on 2499 bp of the mtDNA (concatenated Cytb, ND2 and ND3) above the diagonal and standard errors below the diagonal. Geographic regions indicated between parentheses.

	<i>atlanticus</i> (NAF)	<i>brevirostris</i> / <i>tenuirostris</i> (CAF)	<i>fuscus</i> N (SAF)	<i>fuscus</i> S (SAF)
<i>atlanticus</i> (NAF)		0.026	0.025	0.026
<i>brevirostris</i> / <i>tenuirostris</i> (CAF)	0.003		0.011	0.013
<i>fuscus</i> N (SAF)	0.003	0.002		0.012
<i>fuscus</i> S (SAF)	0.003	0.002	0.002	

NAF: northern Atlantic Forest; CAF: central Atlantic forest; SAF: southern Atlantic forest.

Table 4

Population divergence times and migration between populations of *X. fuscus* based on the isolation-migration model (Hey and Nielsen (2004) and the model of LAMARC 2.0 (Kuhner 2006). Confiability intervals (c.i.) are shown between parentheses. Divergence times (t) in million years (MYr) and migration (M) in effective number of individuals per generation.

Estimation	Divergence with gene flow, IM (90% c.i.)			Migration per marker, LAMARC (95% c.i.)		
	θ	t (MYr)	M	M _{FIB5}	M _{mtDNA}	M _{mtDNA-4Ne}
Inter lineages (AF region)						
1 [<i>atlanticus</i>] x [<i>brevirostris</i> / <i>tenuirostris</i>] (NAF x CAF)	12.31 (8.62-18.28)	0.47 (>0.25) ‡	0.15 (0.01-0.65)	1.25 (0.16-6)	0.08 (0.00-0.89)	0.33 (0.01-3)
2 [<i>atlanticus</i>] x [<i>fuscus</i> N] (NAF x SAF)	7.11 (4.64-11.37)	0.79 (>0.45) ‡	0.08 (0.00-0.39)	0.64 (0.06-3.0)	0.06 (0.00-0.75)	0.23 (0.01-3.57)
3 [<i>atlanticus</i>] x [<i>fuscus</i> S] (NAF x SAF)	6.58 (3.96-10.42)	0.79 (>0.44) ‡	0.03 (0.00-0.32)	0.46 (0.01-3.66)	0.06 (0.00-0.69)	0.24 (0.01-2.66)
4 [<i>brevirostris</i> / <i>tenuirostris</i>] x [<i>fuscus</i> N] (CAF x SAF)	11.55 (8.17-17.52)	0.43 (0.08-1.66)	0.65 (0.11-3.12)	6.18 (1.48-10.71)	0.14 (0.01-0.70)	0.58 (0.02-6.12)
5 [<i>brevirostris</i> / <i>tenuirostris</i>] x [<i>fuscus</i> S] (CAF x SAF)	12.77 (8.62-19.25)	0.44 (0.13-1.34)	0.30 (0.03-1.88)	4.44 (1.35-7.98)	0.14 (0.00-1.50)	0.55 (0.02-6)
6 [<i>fuscus</i> N] x [<i>fuscus</i> S] (SAF)	5.53 (3.30-8.94)	0.39 (>0.25) ‡	0.40 (0.04-2.58)	4.59 (0.8-16.8)	0.10 (0.00-0.91)	0.39 (0.01-5.09)
Intra lineages *						
7 <i>atlanticus</i> [CE] x [AL]	2.73 (1.31-5.16)	0.11 (>0.04) ‡	0.21 (0.00-1.78)	0.56 (0.02-5.25)	0.38 (0.01-5.40)	1.52 (0.02-21.6)
8 <i>brevirostris</i> / <i>tenuirostris</i> [CH] x [COB]	9.53 (5.96-16.22)	0.13 (0.05-0.61)	0.36 (0.00-5.67)	3.09 (0.57-16.2)	0.49 (0.02-6)	2 (0.08-24)

θ : theta value for all the markers in the IM model.

M, M_{FIB5}, M_{mtDNA}: migration rates for all the markers in the IM model, and for FIB5 and mtDNA in LAMARC, respectively. M_{mtDNA-4Ne}, migration value obtained with the mtDNA and forcing a fourfold effective size.

*: CE and AL, populations of *atlanticus* of Ceará and Alagoas, respectively (see Fig. 1D). CH and COB, populations of *brevirostris* / *tenuirostris* of the Chapada Diamantina range (see Fig. 1A) and of the coastal region of Bahia plus northeastern Minas Gerais, respectively. NAF: northern AF; CAF: central AF; SAF: southern AF. ‡: maximum value of the interval not presented because the likelihood distribution was flat at the end.

Appendix A

GenBank accession numbers of sequences other than those of *Xiphorhynchus fuscus* used for phylogenetic analyses (see Appendix B for *X. fuscus* sequences). Underlined accession numbers indicate sequences obtained from GenBank. All samples are muscle.

Taxon	CytB	ND2	ND3	FIB5	Reference [sample ID]
<i>Sittasomus griseicapillus</i>	<u>AY089796</u>	<u>AY089834</u>	<u>AY089894</u>	-	Aleixo (2002)
<i>Lepidocolaptes angustirostris</i>	<u>AY089811</u>	<u>AY089838</u>	<u>AY089881</u>	-	Aleixo (2002)
<i>Campyloramphus trochilirostris</i>	<u>AY089822</u>	<u>AY089857</u>	<u>AY089906</u>	-	Aleixo (2002)
<i>Xiphorhynchus flavigaster flavigaster</i>	<u>AY089828</u>	<u>AY089849</u>	<u>AY089896</u>	-	Aleixo (2002)
<i>X. guttatus guttatus</i>	<u>AY089808</u>	<u>AY089869</u>	<u>AY089908</u>	EF190698	Aleixo (2002), this work [LGEMA P238 = LSU35582] ^{*1}
<i>X. g. eytoni</i>	<u>AY089794</u>	<u>AY089845</u>	<u>AY089884</u>	-	Aleixo (2002)
<i>X. g. guttatoides</i>	<u>AY089791</u>	<u>AY089866</u>	<u>AY089882</u>	-	Aleixo (2002)
<i>X. obsoletus obsoletus</i>	<u>AY089823</u>	<u>AY089868</u>	<u>AY089913</u>	-	Aleixo (2002)
<i>X. chunchotambo chunchotambo</i>	<u>AY089815</u>	<u>AY089844</u>	<u>AY089915</u>	-	Aleixo (2002)
<i>X. ch. Brevirostris</i>	<u>AY089793</u>	<u>AY089846</u>	<u>AY089885</u>	-	Aleixo (2002)
<i>X. ocellatus ocellatus</i>	<u>AY089804</u>	<u>AY089861</u>	<u>AY089909</u>	EF190699	Aleixo (2002), this work [LGEMA P249 = LSUB35600] ^{*1}
<i>X. o. weddellii</i>	<u>AY089820</u>	<u>AY089859</u>	<u>AY089878</u>	-	Aleixo (2002)
<i>X. pardalotus pardalotus</i>	<u>AY089831</u>	<u>AY089848</u>	<u>AY089910</u>	EF190701	Aleixo (2002), this work [LGEMA P265 = LSUB35635] ^{*1}
<i>X. p. pardalotus</i>	EF190607	EF190632	EF190669	EF190700	This work [LGEMA P264 = LSUB35634] ^{*1}
<i>X. elegans elegans</i>	<u>AY089805</u>	<u>AY089852</u>	<u>AY089899</u>	-	Aleixo (2002)
<i>X. e. juruanus</i>	<u>AY089824</u>	<u>AY089874</u>	<u>AY089883</u>	-	Aleixo (2002)
<i>X. e. ornatus</i>	<u>AY089812</u>	<u>AY089841</u>	<u>AY089889</u>	-	Aleixo (2002)
<i>X. susurrans</i>	<u>AY089800</u>	<u>AY089863</u>	<u>AY089914</u>	-	Aleixo (2002)
<i>X. triangularis bangsi</i>	<u>AY089826</u>	<u>AY089864</u>	<u>AY089918</u>	-	Aleixo (2002)
<i>X. spixii</i>	EF190605	EF190630	EF190667	EF190696	This work [FMNH 391312]
<i>X. spixii</i>	EF190606	EF190631	EF190668	EF190697	This work [FMNH 391313]

CytB: cytochrome B, ND2: NADH dehydrogenase subunit 2, ND3: NADH dehydrogenase subunit 3, FIB5: intron 5 of the β -fibrinogen gene. ^{*1}- sample deposited in two institutions. Tissue collections: LGEMA – Laboratório de Genética e Evolução Molecular de Aves, Universidade de São Paulo, São Paulo; LSU-Louisiana State University Museum of Natural Science, Baton Rouge; FMNH - Field Museum of Natural History, Chicago.

Appendix B

Collection localities (all in Brazil, except when indicated) of *Xiphorhynchus fuscus* and areas of endemism (Cracraft, 1985; Stattersfield et al., 1998; Silva et al., 2004) to which samples are associated, tissue identification and GenBank access numbers. FIB5 heterozygous individuals present two GenBank accesses, one for each chromosome.

Locality	Subspecies	N	Cracraft (1985)*	Stattersfield et al. (1998)*	Silva et al. (2004)*	Sample ID ^{TISSUE}	CytB	ND2	ND3	FIB5
1	Mulungú, Serra de Baturité, Ceará (CE), 4° 17' 45' S, 38° 55' W, 724m.	3	-	-	-	LGEMA10391 ^B	EU073025	EU073030	EU073035	EU073040 EU073041
			-	-	-	LGEMA10392 ^B	EU073026	EU073031	EU073036	EU073042
			-	-	-	LGEMA10395 ^B	EU073027	EU073032	EU073037	EU073043
2	Guaramiranga, Serra de Baturité, CE, 4° 14' S, 38° 56' W, 724m.	2	-	-	-	LGEMA10406 ^B	EU073028	EU073033	EU073038	EU073044
			-	-	-	LGEMA10417 ^B	EU073029	EU073034	EU073039	EU073045
										EU073046
3	Ibateguara, Alagoas, 8°59'S, 35°52'W, 200 m	5	SMC	AAP	Pe	FMNH399195 ^M	EF190601	EF190608	EF190645	EF190670
						FMNH399196 ^M	EF190583	EF190609	EF190646	EF190670
										EF190683
						FMNH399197 ^M	EF190584	EF190610	EF190647	EF190671
						FMNH399198 ^M	EF190585	EF190611	EF190648	EF190684
						FMNH399199 ^M	EF190586	EF190612	EF190649	EF190672
										EF190685
4	Bonito, Bahia (BA). 11°54'S, 41°12'W, 945 m.	1	-	DB	CB	LGEMAP2267 ^M	EF190597	EF190623	EF190660	EF190678
5	Lençóis, BA. 12°25'S, 41°21'W, 520 m.	3	-	DB	CB	LGEMAP2245 ^M	EF190594	EF190620	EF190657	EF190689
						LGEMAP2249 ^M	EF190595	EF190621	EF190658	EF190671
						LGEMAP2258 ^M	EF190596	EF190622	EF190659	EF190686
										EF190687
6	Porto Seguro, BA. 17°22'S, 40°17'W, 50 m.	1	SMC	AL	CoB	LGEMAP330 ^M	AY089819* ₁	AY089851* ₁	AY089904* ₁	EF190692
										EF190693
7	Salto Divisa, Minas Gerais (MG). 16°5'S, 40°2'W, 100 m.	2	SMC	AL	CoB	B0822 ^B	EF190577	EF190633	EF190639	EF190675
						B0918 ^B	EF190582	EF190638	EF190644	EF190673
										EF190679

8	Jequitinhonha (Mata Escura), MG. 16°20'S, 41°0'W, 900 m.	Af. <i>brevirostris</i>	2	SMC	DB	CB	B0913 ^B	EF190581	EF190637	EF190643	EF190682 EF190694
9	Caratinga, MG. 20°50'S, 42°5'W, 55 m.	<i>fuscus</i>	1	-	-	-	B0911 ^B B0908 ^B	EF190580 EF190578	EF190636 EF190634	EF190642 EF190640	EF190678 EF190674
10	Nova Lima, MG. 19°59'S, 43°49'W, 900 m.	<i>fuscus</i>	1	-	-	-	B0909 ^B	EF190579	EF190635	EF190641	EF190692
11	Sooretama, Espírito Santo (ES). 19°3'S, 39°55'W, 50 m.	<i>fuscus</i>	2	SMC	AL	CoB	LGEMAP2876 _M LGEMAP2877 _M	EF190602	EF190629	EF190664	EF190695 EF190689 EF190691
12	Santa Teresa, ES. 19°56'S, 40°34'W, 650 m	<i>fuscus</i>	1	SMC	AL	SM	LGEMAP1644 ^B	EF190592	EF190618	EF190655	EF190676 EF190680
13	Cantagalo, Rio de Janeiro (RJ). 22°S0'W, 42°33'W, 400 m.	<i>fuscus</i>	1	SMC	AL	SM	LGEMAP2878 _M	EF190604	EF190628	EF190666	EF190689 EF190690
14	Itatiaia, RJ. 22°24'S, 44°38'W, 800 m.	<i>fuscus</i>	1	SMC	AM	SM	LGEMAP1321 ^B	EF190589	EF190615	EF190652	EF190681
15	Bananal, São Paulo (SP). 22°41'S, 44°19'W, 500 m.	<i>fuscus</i>	1	SMC	AL	SM	LGEMAP1171 _M	EF190588	EF190614	EF190651	EF190692
16	Barreiro Rico, SP. 22°38'S, 48°13'W, 550 m.	<i>fuscus</i>	1	-	AL	SM	LGEMAP1544 _M	EF190591	EF190617	EF190654	EF190678
17	São Roque, SP. 23°34'S, 47°09'W, 960 m.	<i>fuscus</i>	1	SMC	AM	SM	LGEMAP1024 ^B	EF190587	EF190613	EF190650	EF190692
18	Morro Grande, SP. 23°42'S, 46°59'W, 800 m.	<i>fuscus</i>	1	SMC	AM	SM	LGEMAP652 ^B	EF190598	EF190624	EF190661	EF190692
19	Morro do Diabo State Park, SP. 22°30'S, 52°18'W, 300 m.	<i>fuscus</i>	1	-	-	-	LGEMAP1379 ^B	EF190590	EF190616	EF190653	EF190688
10	Wenceslau Braz, Paraná (PR). 22°51'S, 49°47'W, 800 m.	<i>fuscus</i>	1	PRC	AM	SM	LGEMAP937 ^M	EF190599	EF190625	EF190662	EF190681
21	Campo San Juan, Misiones, Argentina. 27°22'S, 55°39'W, 150 m.	<i>fuscus</i>	1	PRC	AM	-	LGEMAP994 ^B	EF190600	EF190626	EF190663	EF190692
22	Rancho Queimado, Santa	<i>fuscus</i>	1	PRC	AM	SM	LGEMAP1771	EF190593	EF190619	EF190656	EF190692

Catarina, Br. 27°41'S,
49°2'W, 850 m.

M

CytB: cytochrome B, ND2: NADH dehydrogenase subunit 2, ND3: NADH dehydrogenase subunit 3, FIB5: intron 5 of the β -fibrinogen gene.

*SMC: Serra do Mar Center, PRC: Paraná Center. AAP: Atlantic slope of Alagoas and Pernambuco, DB: Deciduous forest of Bahia, AL: AF Lowland, AM: AF Mountains. Pe: Pernambuco, CB: Central Bahia, CoB: Coastal Bahia, SM: Serra do Mar. Ba: Bahia, RJ: Rio de Janeiro, SC: Santa Catarina. -; not present in any area of endemism. ^{TISSUE}: Tissue type, ^M: muscle; ^B: blood. *1: from Aleixo (2002). Tissue collections: LGEMA - Laboratório de Genética e Evolução Molecular de Aves, Universidade de São Paulo, São Paulo. FMNH - Field Museum of Natural History, Chicago. B - Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Belo Horizonte.

CAPÍTULO 4
PATTERNS OF PLUMAGE AND GENETIC VARIATION IN *DENDROCOLAPTES*
***PLATYROSTRIS* (AVES: DENDROCOLAPTIDAE): RECENT DISCONTINUITY**
BETWEEN THE SOUTH AMERICAN OPEN VEGETATION CORRIDOR AND THE
ATLANTIC FOREST

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Abstract

Although dominated by savannas or dry forests, the open vegetation corridor of South America (biomas Caatinga, Cerrado and Chaco) contains humid forests in the forms of galleries and relicts. Those humid forests are or were linked to the Atlantic and Amazon Forests. Thus, this current/historical connection seems to have allowed humid forest organisms to circulate unrestrictedly between domains (i.e., Atlantic Forest to Cerrado through gallery forests) and therefore it is believed that the open vegetation corridor is not an independent biogeographic region for humid forest organisms. We addressed this hypothesis by studying the plumage and mitochondrial DNA sequences of the woodcreeper *Dendrocolaptes platyrostris* (Dendrocolaptidae). Our results for *D. platyrostris* did not support both regions as biogeographically similar in the short term; divergence driven by different ecological conditions at each domain could explain this result. Divergence between the two domains' populations was very recent (<0.1 MYr). If the problem of the open vegetation corridor – Atlantic Forest biogeographic continuity was considered from a phylogenetic standpoint (long term approach) both regions appeared as continuous, as observed in other taxa. The majority of the plumage variation of *D. platyrostris* followed biomes' transition instead of population history, suggesting that selection may be directly related to the evolution of the species' plumage. Also, our results suggest the hypothesis that *D. platyrostris* at the open vegetation corridor disperse longer distances than birds in the continuous AF, and we propose that this differential pattern may be an adaptation to live in the OVC.

The Amazonian and Andean forests are separated from the Atlantic forest (AF) by three domains (the Caatinga, Cerrado and Chaco) that form an open vegetation corridor (OVC) at central South America (Fig. 1) (Olson et al., 2001). Although dominated by savannas or semideciduous/dry forest, the OVC contains humid forests along rivers (gallery forest) and isolated humid forest relicts (*brejos*) (Veloso, 1991). Those humid forests at the OVC are physical extensions of the AF and the Amazon central domains, or were part of those domains when larger humid forests existed during global glaciations at the OVC (Ledru, 1992, 1993; Ledru et al., 2006). Thus, this current/historical pattern seems to have allowed humid forest organisms to circulate unrestrictedly between domains (i.e., AF to Cerrado through gallery forests) and therefore it is believed that the OVC is not an independent biogeographic region for humid forest organisms, despite both OVC and AF are differentiated in terms of landscape characteristics, history and climate. Examples of studies supporting this idea are Silva (1996), Costa (2003) and Redford (1986). Under this hypothesis of OVC-AF continuity, it is expected to find for humid forest organisms: (1) no genetic divergence between forest and OVC contiguous populations and (2) phylogeographic structures within forest domains which are mirrored in the OVC (Fig. 2A). Alternatively, if both domains are independent biogeographic regions, the pattern shown in Figure 2B should be observed.

Some organisms present different morphotypes associated to each AF and OVC domain and this does not seem to be in accordance with the aforementioned hypothesis of inter domain continuity. Gallery forests at the OVC are significantly differentiated from the continuous AF in several aspects, such as climate (Veloso, 1991) and luminosity levels, and different regimes of natural selection on adaptive traits (e.g., plumage color) are expected to occur in each region. As strong selection on adaptive traits may derive in morphologically differentiated populations in each domain (Moritz et al., 2000; Smith et al., 2005a; Smith et al., 2005b), despite the presence of gene flow (parapatric divergence), the existence of morphotypes associated to each domain does not necessarily rejects the idea of inter domain historical continuity. To test this hypothesis we selected the woodcreeper *Dendrocolaptes platyrostris* (Dendrocolaptidae). This is a forest dependent bird with two subspecies described according to plumage variation (reviewed in Marantz et al., 2003). Each subspecies is associated to either the OVC or the AF central domain, thus the species is a good model to address the problem of the continuity between the two domains. Subspecies *platyrostris* inhabits the AF core domain. Subspecies *intermedius* is associated to humid forest of the OVC and has a paler and more cinnamon overall plumage. Even though there is not

a clear gap between the subspecies' geographic ranges -there are intermediates at the AF-OVC ecotone (Marantz et al., 2003)-, the distribution of both subspecies suggests a discontinuity between domains that deserves to be studied. This discontinuity may be historical or ecological. If selection instead of population history (isolation between domains and drift) was responsible for plumage variation in *D. platyrostris*, it is expected that phylogeographic gaps within each biome do not match significant changes in plumage characters. Then, for similar levels of genetic divergence (divergence between localities, or individuals, evaluated by neutral markers), it is expected to find a larger plumage divergence in inter domain comparisons than in within domain comparisons (Fig 2 C)(Moritz et al., 2000; Norman et al., 2007; Smith et al., 2005a; Smith et al., 2005b). Alternatively, if plumage variation accompanies population structure and not domain transitions, the pattern shown in Figure 1D should be found.

Our objective was to describe plumage and genetic variation of *D. platyrostris* to address the following questions: (1) Is there any evidence of genetic continuity between *D. platyrostris*' populations at the OVC and the AF? (2) Is plumage variation in *D. platyrostris* congruent with its population genetic structure, as evaluated by a neutral marker (mtDNA)? or (3) is it related to biome factors and independent of population genetic structure? To address these questions we studied five plumage characters and mtDNA variation and discussed whether data supported predictions from Figure 2. We also discuss briefly the systematic implication of results.

Materials and Methods

Plumage analyses

In order to describe plumage stability geographic regions and to obtain plumage divergence estimations we examined 510 specimens (180 females, 302 males and 28 undetermined) from the following museums: Museu Paraense Emílio Goeldi (MPEG), Brazil; Museu de Zoologia da Universidade de São Paulo (MZUSP), Brazil; and American Museum of Natural History (AMNH), U.S.A. These specimens represented 172 localities that encompassed a large portion of the *D. platyrostris*' range (Fig. 1). Localities were taken from specimen labels and coordinates were obtained from ornithological gazetteers (i.e., Paynter & Traylor 1991; Vanzolini 1992) or from the specimen labels.

We recorded five plumage colour/pattern characters from each study skin. We established for each portion of the plumage to be compared (character) a scoring system subdividing the total range of

observed color/pattern variation into visually equivalent states. Specimens were scored by only one observer (F.M. Horta) by comparison to reference specimens (ID follows “ref”). Colors are described according to the Munsell Soil Color Charts (2000): their names, and between parenthesis hue, value/chroma. The studied characters and their states are (1) HEAD (characteristic of contour feathers of the crown and nape): state 0, external (ext.) portion of the vane dark yellowish brown (10YR 3/3) and internal (int.) portion of the vane pale yellow (2,5Y 8/4) –ref. MZUSP 41640-; state 2, ext. vane very dark grayish brown (10YR 3/2) and int. vane pale yellow (10YR 8/2) –ref. MZUSP 72052-; state 4, ext. vane very dark gray (10YR 3/1) and int. vane white (2,5Y 8/8)- ref. MZUSP 72374-; and 6, ext. vane black (2,5Y 8/1) and int. vane as state 4 –ref. MZUSP 35465-. Heads with state 0 did not present strong streaks while state 6 represented the most streaked heads. (2) TAIL: state 0, yellowish red (5YR 4/6) – ref. MZUSP 41640-; state 3, dark reddish brown (5YR 3/4) -MZUSP 72374-; and state 6, dark reddish brown (5YR 3/2) –ref. MZUSP 35469-. (3) UPPERTAIL (uppertail-coverts): state 0, yellowish red (5YR 5/8) – ref. MZUSP 41.640); state 2, yellowish red (5YR 4/6) –ref. MZUSP 72.374-; state 4, background dark yellowish brown (10YR 3/6) with three to four bars black (2,5Y 8/1) –ref. MZUSP 73.767-; state 6, unbarred with background dark brown (7,5YR 3/4) and a black patch at the subterminal portion of feathers –ref. MZUSP 70.204-. (4) BACK (mantle and back contours): state 0, no streaks, dark yellow brown (10YR 4/4) –ref. MZUSP 41.640-; state 3, no streaks evident, olive brown (2,5Y 4/4) –ref. MZUSP 51.859-; state 6, streaked, ext. vane dark brown (2,5Y 3/3) and int. vane white (2,5Y 8/8). Also, black mark at distal portion of internal vane –ref. MZUSP 35.469-. (5) VENT (belly and flank contours), varied from slightly barred to strongly barred: state 0, background olive yellow (2,5Y 6/6) and fine bars dark olive brown (2,5Y 3/3) –ref. MZUSP 41.064-; state 2, background light yellowish brown (2,5Y 6/4) and bars very dark grayish brown (2,5Y 3/2) –ref. MZUSP 72.374-; state 4, background light olive brown (2,5Y 5/3) and bars very dark grayish brown (2,5Y 3/2) –ref. MZUSP 35469-; state 6, color as state 4, but bars bolder (thicker than 2 mm) –ref. AMNH 794072-.

Following Cracraft (1989), we first analyzed the variation of plumage characters to identify and delimit the smallest cluster of populations that are diagnosably distinct from other clusters. As a preliminary study did not show plumage sexual dimorphism (F. M. Horta unpublished), analyses considered sexes together. Specimens were organized into localities (N = 172) for the analysis of geographic variation in plumage (Figure 1). The unit of plumage analysis was the population sample (N=

38), which consisted in a locality with at least five specimens or in a set of localities grouped with the nearest one to form a new population sample with at least five specimens. For each population sample we obtained the median of each character scores and a total score by summing medians of individual character scores. The geographic variation in medians of plumage character was mapped not considering any previous taxonomic arrangement (i.e., subspecies). This allowed us to identify geographical areas with little or no change in states of a character (zones of phenotypic stability) and areas with abrupt changes (transition zones). Individual character maps were then overlapped to identify populations that can be diagnosed by a unique combination of plumage character states (congruent zones of phenotypic stability). Those populations are interpreted as evolutionary units (Cracraft, 1989). For taxonomic discussions we adopted the general lineage species concept (de Queiroz, 1998) and followed Aleixo (2007) for operational criteria.

Genetic datasets

To test the monophyly of *D. platyrostris* we used 983 bp of the cytochrome b (*cytb*) from 33 *D. platyrostris* (one from GenBank and the others sequenced in the present study), four *D. picumnus*, one *D. certhia* and one *D. sanctithome*. We used *Xiphocolaptes promeropirhynchus* as the outgroup. Procedures for DNA purification and for obtaining *cytb* sequences followed Bruford (1992) and Cabanne et al. (2008). For the population genetic study we used 584 bp of the first domain of the control region of the mtDNA (CR) of 43 samples from 19 localities. We selected CR for this set of analyses because it is more variable than the *cytb*. Amplification and sequencing of the CR of *D. platyrostris* followed Cabanne (2007). Sampling localities, vouchers and GenBank accession numbers are presented in Table 1.

Genetic data exploration

We used maximum likelihood and Bayesian analyses in PHYML 2.4.4 (Guindon and Gascuel, 2003) and MRBAYES 3.1 (Ronquist and Huelsenbeck, 2003), respectively, to obtain phylogenetic hypotheses for the species studied and the intraspecific *D. platyrostris*' clades. Molecular evolution models were selected in MODELTEST 3.7 (Posada and Crandall, 1998). The neutrality tests of Tajima (1989), Fu (1997) and of McDonald and Kreitman (1991) (MK test) were performed in DNASP 4.0 (Rozas et al., 2003). The MK test used *D. picumnus* as outgroup. Visualization of relationships among CR unique sequences was done by

constructing a median joining network in NETWORK 4.1.0.8 (<http://www.fluxus-engineering.com>). Analyses of molecular variance (AMOVA) were done in ARLEQUIN 3.1 (Excoffier et al., 2006). We performed two AMOVA analyses. The first one (AMOVA I) estimated a Φ_{st} value for the entire study region and used localities grouped in three populations according to mtDNA lineages (OVC, CAF and SAF). The second analysis (AMOVA II) estimated two Φ_{st} values, one for each domain (OVC and AF). In each domain localities were organized in two groups (OVC north and south, and CAF and SAF) according to a configuration that maximized Φ_{st} values. Columns AMOVA I and AMOVA II of Table 1 present the organization of samples in populations for the AMOVA analyses.

Genetic landscape shape

We used a visualization method to obtain a graphical representation of the pattern of genetic distance between individuals across the study area. The procedure followed Miller (2006) and was done in the program AIS 1.0. We performed analyses using p-distances –no other distance allowed by the soft AIS– between individuals and using residual genetic distances derived from a regression between genetic and geographic distances. As the results of both analyses were the same we only present those obtained with genetic distances.

Genetic and plumage divergence analyses

As an approach to study genetic divergence we performed a partial correlation analysis (partial Mantel tests) using average Tamura and Nei (1993) (TN 93) genetic distances between individuals from pairs of localities (variable DGEN) as a dependent variable and two independent variables. Geographic distance between localities (variable DGEO) was the first independent variable, and an indicator variable (DLAND) that represented whether comparisons were made within the same domain (within OVC or AF, DLAND = 0) or between domains (OVC vs AF, DLAND = 1) was the second independent variable. See Quinn and Keoug (2002) for an explanation on indicator variables. If genetic distances are only affected by isolation by geographic distances, we expected DGEO to be the only predictor of DGEN. But, if biome transition was also related to genetic divergence, we also expected a significant correlation between DGEN and DLAND. We used FSTAT (Goudet, 2002) to perform partial Mantel tests with 20,000 replicates to check for significance.

To evaluate whether plumage variation is related to population history (addressed by the genetic distances at the mtDNA) and to domain transition (AF vs OVC), we performed two analyses. First, we made a partial Mantel test to address whether plumage variation (variable DPLUM: plumage dissimilarity between localities with associated genetic data- Table 1-; not dissimilarity between population samples used as units of the plumage geographic variation study) was related to two independent variables, namely genetic distances (variable DGEN: average TN 93 distances between localities, used as a proxy of population history) and to domain transition (variable DLAND, see previous paragraph). Plumage character states for localities were defined as the most frequent state in the location. We used two variants of plumage distances. DPLUM 1 represented the number of characters without shared states between two localities. It varied from zero (all characters with the same state) to five (all characters with different states). DPLUM 2 represented character state changes summed across the five characters. This distance considered ordered character states and was obtained by the difference between total plumage scores between pairs of localities. Plumage scores for locations were obtained by summing medians of individual character scores. For those localities without museum specimens available (Table 1) we assumed characters states from the nearest locality. We used FSTAT to perform all partial Mantel tests (20,000 replicates). A second analysis evaluated the relationship between plumage divergence (DPLUM) and genetic divergence (DGEN) within and between domains (AF or OVC) to test predictions (Fig. 2C and 2D).

The two former analyses that used DPLUM as dependent variable excluded two localities (Felixlândia and Bocaiuva), which were located in an area of transition between the OVC and the AF and did not have their own plumage evidence (Table 1) nor close localities to extrapolate plumage states. Because analyses performed either with DPLUM 1 or DPLUM 2 returned similar results (data not shown), we only reported results obtained with DPLUM 2.

Divergence, gene flow and historical demography

To obtain a rate of change for the *cytb* we used the method of Ho et al. (2005) as implemented in Cabanne et al. (2008). Ho et. al (2005) analyzed several calibrations of mtDNA molecular clock rates and developed a mathematical approach to directly relate DNA sequence distances to divergence dates. Briefly, to obtain preliminary divergence dates (in million years, MYr) from *cytb* distances, we used the

model for protein-coding sequences of the avian mtDNA and resolved numerically the equation [7] of the Ho et al.'s paper. To approximate a *cytb* rate of change, we transformed the observed maximum *cytb* divergence to absolute time by the aforementioned method and then related both distance and time to obtain a rate of change. The *cytb* calibration that resulted after applying this method was 4.5% of divergence per MYr, which is in accordance with other calibrations obtained for closely related *cytb* sequences (i.e., Warren et al., 2003). For obtaining a rate of change for the CR we scaled the *cytb*-rate of change with a ratio between the markers' population parameter (or marker's variability) $\Theta_{CR}/\Theta_{cytB}$, where Θ_{CR} is the Θ -value of the CR and Θ_{cytB} is the Θ -value of the *cytb*. For this calibration Θ -values of each marker were obtained from a sample of 31 individuals (see below). The CR calibration that resulted after applying this method was 30.42 % per MYr (see results for details). A published example of this approach to obtain a calibration rate for CR sequences can be found in Toon et al (2007).

We used CR sequences and the isolation-migration (IM) model (Hey and Nielsen, 2004; Nielsen and Wakeley, 2001), implemented in the program IM (ver. April 21, 2008), to estimate gene flow rates and final divergence times between populations of *D. platyrostria*. We applied an inheritance scale of 0.25, the HKY model of evolution and assumed $\theta_1 = \theta_2 = \theta_a$ and $m_1 = m_2$. Analyses used 500,000 iterations for burn-in and analyses were stopped when the smallest effective sample sizes (ESS) were higher than 50 and parameter trend lines stabilized after 30 to 100 million iterations. For parameters with posterior distribution completely estimated, we presented the highest posterior density interval. We transformed the time parameter t into time in years using the formula $t = t/u$, where u is the mutation rate of per marker (CR) and generation, and the migration parameter m into effective number of migrant genes (=females) per generation using the formula $M = (\theta m)/2$ (Hey and Nielsen, 2004). We assumed a generation time of one year.

To estimate the parameter Θ we used the maximum likelihood and coalescent method implemented in LAMARC 2.1.2b (Kuhner, 2006). We used a relative effective size of one. We also used LAMARC to evaluate the demographic signature of lineages recovered in the genealogy by estimating the exponential population growth rate g ($\Theta_t = \Theta_{now}e^{-gt}$, where Θ_{now} is the current Θ and Θ_t is the value of the parameter t time ago). Positive g -values indicate population growth and negative values, population decline. Final runs used the F84 model of sequence evolution with empirical base frequencies and transition/transversion ratios, and 10 short chains (500 sampled trees every 50 iterations and burn-in of

1000 trees) followed by two to ten long chains (20,000 sampled trees every 20 iterations and burn-in of 5000 trees).

We also evaluated demography by calculating the R_2 (Ramos-Onsins and Rozas, 2002) statistic using DNASP 4.0. Significant and low values of R_2 suggest demographic expansion when neutrality is not rejected. Significance was determined based on 1000 coalescent simulations under a model of population stability using empirical sample sizes and estimates of Θ .

Results and Discussion

Plumage variation

According to the characters geographic variation and the congruence among regions of character stability, three regions of phenotypic stability (populations) were found (Fig. 3). The core range of population I is at the Cerrado and the population's diagnosis is: HEAD very dark grayish brown with streaks pale yellow (state 2), TAIL dark reddish brown (state 3), UPPERTAIL-coverts yellowish red (state 2), BACK dark yellow brown without streaks (state 0) and VENT light yellowish brown with thin bars very dark grayish brown (state 2). Population II is centered at southern Atlantic Forest and is diagnosed by: HEAD black with white streaks (state 6), TAIL dark reddish brown (state 6), UPPERTAIL-coverts dark brown with a black patch at the subterminal portion of feathers (state 6), BACK dark brown streaked white (state 6) and VENT light olive brown with bold bars dark grayish brown (state 6). The third phenotypic stability area is small and closely related to the population II (Fig. 3F, inset), geographically and phenotypically. This small population is only differentiated from population II by one state change at the VENT (state 4 vs state 6); therefore both populations will be considered as a single one (Population II) in following discussions. Overall, birds from population I are lighter, less streaked, and associated to subspecies *intermedius*; but, the phenotypic stability area is smaller than the subspecies' range (Marantz 2003). Birds from population II are the darkest of the species and are associated to the nominal subspecies. As suggested by the subspecific taxonomy, our results on plumage variation of *D. platyrostris* does not support the idea of continuity between the OVC and the AF because each main morph (populations I and II) are associated to each domain. This is also visualized in single character variation (i.e., TAIL and VENT), where many states change across the AF-OVC transition (Fig. 3).

Genetic pattern, general aspects

For the monophyly study we obtained a *cytb*-alignment of 33 *D. platyrostris* and seven other dendrocolaptids [983 bp, 173 variable positions, 93 (53.7%) informative for parsimony]. Neutrality of the *cytb* from *D. platyrostris* was not rejected by the MK test (G-test $P > 0.05$), by the test of Tajima (1989) (Tajima's $D = -0.092$, $P > 0.1$) and by the test of Fu (1997) (Fu's $F_s = -1.97$, $P > 0.22$). For the population analyses the CR-alignment had 43 *D. platyrostris* (584 bp, 41 variable positions, 33 (80.5%) informative for parsimony]. The tests of Tajima (1989) and the test of Fu (1997) did not reject neutrality for this data set (Tajima's $D = -0.03438$, $P > 0.1$; and Fu's F_s statistic = -7.455 , $P > 0.02$).

We evaluated the relative diversity of *cytb* and of CR in a subsample of 31 individuals. We estimated Θ for CR ($\Theta_{CR} = 0.0395$, 95 % c.i.: 0.025-0.063) and for *cytb* ($\Theta_{cytb} = 0.005843$, 95 % c.i.: 0.003-0.0105) and obtained the ratio $\Theta_{RC} / \Theta_{cytb} = 6.76$. As we used the same individuals for this analysis the ratio $\Theta_{RC} / \Theta_{cytb}$ suggested that the mutation rate at the CR of *D. platyrostris* is ~ 6.7 times higher than the *cytb*'s rate, which is in accordance with observations made in other birds (Ruokonen and Kvist, 2002). A similar result is obtained if nucleotide diversities are compared ($\pi_{RC} = 0.01827$, $SD = 0.00127$; $\pi_{cytb} = 0.00448$, $SD = 0.00026$; $\pi_{RC} / \pi_{cytb} = 3.8$). We used the ratio of Θ values (6.7) as a scalar for the *cytb*'s rate of change (4.5% of divergence / MYr, see Methods) and obtained a CR's rate of change equal to 148.13×10^{-6} changes seq./year (30.42 % of divergence / MYr).

Even though we were not able to sample all species from genus *Dendrocolaptes* -we sampled four of a total of five species (Marantz et al., 2003)-, our analysis suggested that *D. platyrostris* is monophyletic. The *cytb* phylogeny grouped *D. platyrostris* and *D. picumnus* as sister taxa (Fig. 4A), in accordance with a phylogeny obtained with morphological data (Raikow, 1994). *D. platyrostris* appears to be a relatively young species, as inferred by the combined moderate intraspecific differentiation (maximum TN 93+G *cytb* distance of 0.47%, ~ 0.1 MYA) and the close proximity to *D. picumnus* (TN 93+G *cytb* distance of 3.35%, ~ 0.9 MYA), in contrast with the pattern observed in other AF passerines with a similar widespread distribution (Cabanne et al., 2008; D'Horta, 2009; Pessoa et al., 2006). The low divergences amongst the intraspecific clades suggest that *D. platyrostris* may not become paraphyletic even if a larger sample of *D. picumnus*, or any other member of the genus, was added to the study. Some intraspecific nodes of the *cytb* tree are weakly supported. However, this result was expected because the phylogenetic hypothesis was

obtained with approaches (ML and Bayesian) developed to reconstruct interspecies relationships and many of the assumptions of these methods are violated by intraspecific datasets, and this may result in low phylogenetic resolution (Crandall and Templeton, 1996). To evaluate phylogenetic relationships by haplotype networks is more suitable for intraspecific datasets because this procedure cope with population-level phenomena (i.e., ancestral and derived sequences present in the sample) that traditional species tree methods do not. The CR presented 26 haplotypes and the network corroborated the existence of three main mtDNA lineages in *D. platyrostris* (Fig. 4B). One lineage is associated to the OVC, to the population I and to subspecies *intermedius*. The other two lineages are associated to the nominal subspecies and to the core domain of the AF. Within the AF, one lineage is associated to central AF (CAF) and the other to southern AF (SAF). Moreover, the lineage SAF is associated to the population II (Fig. 4C), suggesting that the phenotypic stability area is monophyletic. The CR network also denoted derived haplotypes of the CAF lineage that occur in the ecotone OVC-AF (e.g.: localities 3 and 6, Fig. 4B). Those derived haplotypes may indicate a past range expansion of forest birds toward the OVC with posterior restriction of gene flow and divergence, situation known as neotypy (Omeland et al., 2006). An AMOVA analysis indicated that the population genetic structure amongst regions defined by the three lineages is weak (AMOVA I Φ_{st} = 0.2647, c.i.: 0.18 - 0.33, $P < 0.01$). Since IM analyses indicated divergence times greater than zero (Table 3) and as clades are geographically partitioned, we consider that the results indicated that the clades of Figure 4 represent organismal lineages rather than clades that resulted from stochastic coalescence of sequences within a panmictic population (Dolman and Moritz, 2006; Irwin, 2002).

Genetic pattern, OVC-AF transition

According to some authors, gallery forests at central Brazil serve as corridors between the Amazon and the AF forest (e.g.:Costa, 2003; Silva, 1996). Under this model we expected to find no genetic divergence between contiguous AF and OVC populations, and that phylogeographic structures within the AF were mirrored in the OVC (Fig. 2A). Neither of the two predictions is supported by *D. platyrostris*. Although the divergence between the OVC and the AF seems to be small and gene flow between regions moderated (Table 2), there is a phylogeographic gap between the OVC and the AF (Fig. 5). This pattern was also evidenced by a partial Mantel test that indicated that biome transitions, as well as geographic distances, are

significantly correlated to genetic differentiation in *D. platyrostris* (partial $r_{\text{DGEN-DGEO}} = 0.421$, $P = 0.0003$; partial $r_{\text{DGEN-LAND}} = 0.171$, $P = 0.026$; $R^2 = 0.20$). Under the tested model of AF-OVC continuity we expected to find no significant correlation between genetic distances and the biome transition (not significant $r_{\text{DGEN-LAND}}$).

There are two likely explanations for the existence of a phylogeographic gap between the AF and the OVC: a) vicariance and posterior secondary contact, and b) divergence with gene flow. As the transition between OVC and AF is very gradual and occurs over an axis of thousand of kilometers (Fig. 1), and since there is not any particular geographic landmark (i.e., valleys, rivers or a large mountain range) that could have acted as a gene flow barrier associated to this transition, we believe it is unlikely that the complete system of gallery forests in the OVC was isolated from the core of the AF in the past (vicariance option). However, one scenario that would make vicariance plausible (ecological vicariance) states that a small population could have been isolated in the system of gallery forests, diverged and then expanded to all the OVC range. This is a model known in the literature as the vanishing refuges (Vanzolini and Williams, 1981). However, this hypothesis to be accepted requires signals of population expansion at the OVC lineage, signals that we have not found. Specifically, the R_2 test was not significant, a large proportion of the confidence interval of the OVC's g -value is negative (Table 3) and no star-like genealogy was observed (Fig. 4B). On the other hand, divergence with gene flow between the OVC and the AF seems to be plausible since the biomes' transition might be a partial ecological barrier if populations had acquired adaptations for their corresponding landscapes. Even though the OVC and AF populations are physically continuous, the two domains differ significantly (Veloso, 1991) and thus birds at both regions may have acquired different adaptive features (i.e., plumage coloration). It is possible that characters favored at the OVC could be negatively selected at the AF, and vice versa, and this event could have caused a partial barrier along the domains' transition that in the long term would be reflected at neutral markers such as the mtDNA (Norman et al., 2007).

Discrimination between vicariance and divergence with gene flow across the OVC-AF transition may be explored by studying multiple independent markers and the distribution of migration events in time using the isolation migration model of Hey and Nielsen (2004). Currently, lineages CAF and OVC meet at the domains' transition and, if this pattern was generated by vicariance followed by secondary contact, high density of recent gene flow should be observed (Cabanne et al., 2008; Niemiller et al., 2008;

Nosil, 2008; Won and Hey, 2005). Otherwise, if the pattern evolved in parapatry, migration should be distributed homogeneously during the divergence period.

Genetic pattern, intra AF history

D. platyrostris presented within the AF a mtDNA phylogeographic pattern similar than the one described for several other AF organisms (Batalha, 2008; Cabanne et al., 2008; D'Horta, 2009; Grazziotin et al., 2006; Mustrangi and Patton, 1997; Pessoa et al., 2006). Specifically, the common pattern consists in the existence of two main mtDNA clades (CAF and SAF) southwards the Doce river, in central Minas Gerais, that get into contact along no evident gene flow barrier in central-southern São Paulo. Some species present a third lineage northward the Doce river, but samples used in this study are not enough to demonstrate this feature in *D. platyrostris*. Another characteristic of the common pattern is that the corresponding southern clades present evidences of population expansion. In *D. platyrostris*, the SAF clade was the only one with a significant R_2 statistic, presented the lowest genetic diversity and a star-like genealogy (Table 3 and Fig. 4), all results indicative of population expansion (Avice, 2000; Ramos-Onsins and Rozas, 2002). The exponential growth parameter g of the *D. platyrostris*' SAF clade is positive and the highest one (Table 3), also suggesting an important population expansion. However, the estimation of g has a wide confidence interval and must be interpreted carefully. Interestingly, when compared organisms that share this common phylogeographic pattern there seems to be not congruence of temporal divergence among different pairs of CAF and SAF clades. For example, the diversification within *D. platyrostris* occurred very recently (last 0.1 MYr, Fig. 4 and Table 3), while the CAF and SAF clades of the passerines *Xiphorhynchus fuscus* and *Sclerurus scansor* diverged more than 0.25 MYr ago (Cabanne et al., 2008; D'Horta, 2009). Other similar example is the viper *Bothrops jararaca*, whose CAF and SAF clades diverged more than 1 MYr ago (Grazziotin et al., 2006). Therefore, the congruence amongst the phylogeographic patterns of several AF organisms at the geographic and genealogical level, but not at the temporal level, indicate that similar and repeated biogeographic events may have affected the evolution of the AF's organisms.

Cabanne (2007) and D'Horta (2009) discussed the origin of the phylogeographic structure of two passerines (*X. fuscus* and *S. scansor*, respectively) that presented a phylogeographic pattern similar than the one of *D. platyrostris* within the AF. As *D. platyrostris* is ecologically and phylogenetically related to those

passerines (Ridgely and Tudor, 1996), we followed the rationale of Cabanne (2007) and D'Horta (2009) to explain our results within the AF. Briefly, considering that the origin and geographic distribution of lineages CAF and SAF did not have correspondence with the origin and location of major rivers and other physiographic features of the AF, as the graben Paraíba do Sul and the mountain ranges Serra do Mar or Serra da Mantiqueira (Petri and Fulfaro, 1983), we propose that the phylogeographic pattern of *D. platyrostris* in the AF was not affected by the location or origin of those landmark features (rivers and geotectonic barriers). On the other hand, and based on the phylogeographic predictions for the effects of global glaciations on forest organisms (reviewed in Lara et al., 2005; Marroig and Cerqueira, 1997; Moritz et al., 2000), on palinology (i.e., Behling, 2002; Behling and Negrelle, 2001) and on simulation of forest distribution (Carnaval and Moritz, 2008), we suggest that the *D. platyrostris*' phylogeographic pattern within the AF could have been significantly affected by changes in forest distribution (ecological vicariance), perhaps changes related to global glaciations. Specifically, the southern AF was the region of the biome most affected by fragmentation and retraction of humid forest during global glaciations (Behling, 2002; Behling and Negrelle, 2001; Carnaval and Moritz, 2008; Whitmore and Prance, 1987), therefore it is expected that its organisms presented today genetic signs of those changes, such as low genetic diversity and signs of population expansions associated to forest advance during post-glaciation periods (i.e., Holocene). All these features were found in *D. platyrostris*. Furthermore, lineages CAF and SAF of *D. platyrostris* meet in a region that presented in the past different landscapes than today. Palynological studies indicate that this contact region was covered by savannas (cerrados) and grasslands (Behling, 1998) during the late Pleistocene and early Holocene (Behling, 1998). Thus, the initial divergence of lineages may have been triggered by those landscapes changes.

It was proposed that the southern AF landscape during maximum of glaciations was dominated by grasslands. Palinological evidences suggest that the grassland-forest limit changed near 750 km northward, from northern Rio Grande do Sul (latitude 27°S) to southern Minas Gerais (latitude 20°S) (Fig. 1) (Behling, 2002). However, phylogeographic studies on AF organisms shows the existence of endemic lineages to SAF (i.e., this study, Batalha, 2008; Cabanne et al., 2008; D'Horta, 2009; Grazziotin et al., 2006; Mustrangi and Patton, 1997; Pessoa et al., 2006) and suggest that this region maintained enough forest during maximum of glaciations to preserve viable populations of forest organisms. Interestingly, many of these organisms are strongly dependent on good forests, such as the passerines *Xiphorhynchus fuscus* and

Sclerurus scansor (Cabanne et al., 2008; D'Horta, 2009). Then, we believe that our current results and those from others indicate that the SAF was significantly affected by forest regressions during the past (apparently linked to global glaciations), but that those events did not locally extinct most organisms as occurred on other regions of the globe where biome forest were significantly affected by global glaciations (Hewitt, 2000).

Evolution of plumage color: population history vs. biome transition

One of the questions we addressed here is whether plumage variation in *D. platyrostris* matches populations history (drift) or if it is affected by other factors such as selection associated to biomes' differences. We studied the correlation of plumage divergence with population history (based on mtDNA genetic distances) and with biomes transition by a partial Mantel test and results indicated that population history did not affect plumage divergence and that biomes transition is significantly correlated to plumage variation in *D. platyrostris* (partial $r_{\text{DPLUM 2-DGEN}} = 0.357$, $P = 0.973$; partial $r_{\text{DPLUM 2-DLAND}} = 0.701$, $P = 0.0000$; total model $R^2 = 0.62$). Also, we studied the relationship between plumage and genetic divergence within and between domains to test predictions of Figure 2C and D. We found that for a specific genetic divergence comparisons between domains always resulted in larger plumage divergences than in comparisons within biomes (Fig. 6).

Our results are in accordance with the expectations of parapatric divergence (Moritz et al., 2000; Smith et al., 2005b). We believe that the two morphotypes of *D. platyrostris* evolved by different selection regimes in each biome instead of by population isolation and drift (allopatric model). For instance, one of the main phylogeographic gaps is located within the AF (CAF vs SAF, Fig. 5) but the main plumage transition is located at the ecotone AF-OVC (Fig. 3F). These results propose that factors associated to biome transition, instead of historical regimes of gene flow within the same biome, generated the observed phenotype pattern. The contact area between lineages CAF and SAF match the transition between a cline zone for some plumage characters and an area of phenotype stability (Fig. 3F and 5). It is not clear whether the phenotypic stability at the southern range is maintained by selection, or it is consequence of a recent expansion of the population with an initial low phenotypic diversity. The last possibility is plausible because the SAF has a significant R_2 statistic, the lowest genetic diversity (Θ), and because the exponential growth parameter (g) suggest a demographic expansion (Table 3).

Notwithstanding results of the plumage versus genetic divergence study may not be considered robust at the quantitative level because we inferred plumage states for some localities, differently to other studies (i.e., Ogden and Thorpe, 2002; Schneider et al., 1999; Smith et al., 2005b), we consider our results reliable at the qualitative level. The observation that factors associated to biome transition, instead of historical patterns of gene flow within the same biome, likely generated the observed phenotype pattern can also be obtained by inspecting data in a qualitatively way. On one hand, the main genetic gap is located within the AF (Fig. 5 and 4B, nine mutational steps at the CR between CAF and SAF), across which plumage variation is minimal (Fig. 3). Note that only two characters changed states (UPPEPRTAIL and VENT) at that region. On the other hand, the larger plumage variation (all characters changed states) occurred at the OVC-AF limit, where genetic gap is smaller than the one occurring within the AF (Fig. 5 and 4B, four steps at CR between lineages CAF and OVC).

We believe the two *D. platyrosris*' plumages may have evolved by differential selection regimes on each biome (ecological divergence) instead of by isolation and drift (allopatric divergence). Birds at the OVC are lighter than birds at the AF, which can be an adaptation to each landscape. The system of dry forests and gallery forest within savannas of central South America is contiguous and intermingled to the Atlantic and Amazonian forests, and it is possible to found other pairs of bird populations/taxa that could have diverged by adaptation to each domain. Putative pairs are *Hylophilus poecilotis* (AF) and *H. amaurocephallus* (cerrado scrubs) and *Thamnophilus ruficapillus* (lower growth, borders and secondary growth at AF and the Andes forest) and *T. torquatus* (scrubs and lower growth at Cerrado and Caatinga). Interestingly, those species of *Thamnophilus* are sister taxa (Brumfield and Edwards, 2007). Ecological diversification is important in generating phenotypic diversity elsewhere (Chaves et al., 2007; Moritz et al., 2000; Ogden and Thorpe, 2002; Schneider et al., 1999; Smith et al., 2005b; Smith et al., 1997), and we encourage biogeographers and evolutionary biologists to direct more attention to the open vegetation corridor of South America and its ecotone with contiguous forests to address the possible role of this system on Neotropical taxa diversification. See Vanzolini and Williams (1981) for an early discussion on this issue.

Forests at the OVC form an intrinsically fragmented system (isolated forest relicts) or can be a sort of unidimensional habitat (gallery forests: narrow stripes following rivers). Intuitively, it may be expected that forest animals had lower dispersion (and gene flow) within the OVC than at the continuous AF. Genetic versus geographic distance relationships showed for *D. platyrostris* a differential pattern between the OVC and the AF (Fig. 7). In the AF, genetic distances increased with geographic distances while at the OVC the relationship was negative and significance borderline. We believe that these results may be revealing different demographic processes in each domain. The AF pattern suggests that dispersion is restricted over long distances (Hutchison and Templeton, 1999). Such a pattern could be originated because dispersion may be limited to neighboring populations, or because environmental (i.e., current habitat gaps) or historical factors (i.e., historical habitat gaps) may have affected gene flow between populations at extremes of the range. Absence of a clear positive correlation between genetic and geographic distances, such as in the OVC, can be observed in systems out of drift-gene flow equilibrium (Hutchison and Templeton, 1999). The OVC's pattern has three potential explanations: a) recent population expansion or range colonization (Excoffier and Ray, 2008), b) very low gene flow rates (Hutchison and Templeton, 1999), c) very high gene flow rates (Hutchison and Templeton, 1999). Recent expansion in the OVC is not supported because of the lack of a star phylogeny of the OVC clade (Fig. 3B), the non-significant R_2 statistic and because the coalescent exponential growth rate g did not indicate a clear expansion (Table 3). Very low gene flow also seems to be unlikely to explain the pattern because there is a substantial genetic admixture at the OVC (Fig. 4). For instance, it is possible to note that some *cytb* sequences from the extremes of the range are very similar (Fig. 3A; e.g., sequence 11443 from Ceará, locality 1; and sequence Y442990 from Paraguay, locality 20). This pattern can also be observed by an exploratory AMOVA (AMOVA II in Table 1) within each domain, where structure within the OVC ($\Phi_{st} = 0.329$, 95% c.i. 0.297- 0.357, $P < 0.01$) is smaller than the structure within the AF ($\Phi_{st} = 0.752$, 95% c.i. 0.58 - 0.857, $P < 0.01$). Thus, high gene flow instead of extremely low gene flow seems to be a more plausible explanation for the lack of correlation between genetic and geographic distances at the OVC. Interestingly, our results also suggest that gene flow rates are in average higher at the OVC than at the continuous AF.

It might appear implausible that a forest bird presented in a landscape such as the OVC higher gene flow rates than in the continuous AF. However, our hypothesis makes sense in light of recent studies

on dispersion of forest birds in landscapes fragmented by human action (Hansbauer et al., 2008; Moore et al., 2008; Van Houtan et al., 2007). As forest fragmentation reduces resource availability and quality, some forest birds respond to habitat fragmentation enlarging their home ranges to include several habitat fragments and in turn to be able to find enough food, shelter and nesting places (Hansbauer et al., 2008). Forest birds that inhabit gallery forests and natural forest relicts at the OVC may have to act accordingly, and a first outcome of this behavior could be larger home ranges at the OVC than at AF. *D. platyrostris* is a forest dependent species but persists well in moderately fragmented forests (Stotz et al., 1996), thus it is expected a response such as the mentioned before. Larger home ranges at the OVC than at the AF may have as consequence higher dispersion and gene flow rates at the OVC than at the AF (Walters, 2000).

Our results are congruent with the few studies on population genetic structure of Neotropical birds from open areas (Bates et al., 2003; D'Horta et al., 2008; Nodari, 2008), which suggest that those birds have low genetic differentiation among populations in comparison to forest birds (Aleixo, 2004; Cabanne et al., 2008; Cheviron et al., 2005; Nyari, 2007; Pessoa et al., 2006). However, our study is the first one suggesting for a specific taxon differential genetic patterns between the OVC and the core domain of the AF.

No matter the process that generated the differential pattern between the OVC and the AF (Fig. 7), we believe it reflects different life histories or processes that may be linked to adaptation to each landscape. For example, if *D. platyrostris* presented higher dispersion in the OVC than in AF it is expected to find longer wings at the open vegetation landscape, as observed in African birds that occur in both forests and forest-savanna ecotones (Smith et al., 2005b). Long wing feathers are associated to an improved flight capacity in open or semi-open spaces. To test our hypothesis on differential dispersion of *D. platyrostris* in each natural landscape, it is necessary to collect additional samples and to study nuclear markers to obtain robust gene flow rates.

Systematic implications

Do populations I and II deserve the status of species? Even though both populations are diagnosable (Fig. 3F), and this is an important condition for considering a populations as a full species (Aleixo, 2007; de Queiroz, 1998; Helbig et al., 2002), our genetic analysis sampled phenotypic stable populations only marginally and therefore did not demonstrate robustly that these populations are reciprocally

monophyletic (Fig. 4C). Reciprocal monophyly is another condition for considering populations as full species (Aleixo, 2007; Helbig et al., 2002). Besides, both populations are connected by a large area of plumage transition (Fig. 3F), throughout the OVC – AF ecotone and a large track of AF (from São Paulo to Bahia), indicating that they are evolutionary closely related. This close relationship is further indicated by the moderate gene flow rates obtained between regions (Table 2). Thus, our results do not support populations I and II as full species because it is unlikely that they represent independent evolutionary lineages.

An alternative view might consider both populations as full species connected by a large hybrid zone (i.e., D'Horta et al., 2008). However, the region outside the range of populations I and II is a zone of character transition (cline) instead of a hybrid zone. All individuals of that region have intermediary plumage instead of parental and intermediary morphs (Fig. 3)(d'Horta unpublished, Marantz et al., 2003), which is not compatible with two independent evolutionary lineages (full species) which are hybridizing (Aleixo, 2007).

Conclusions

Even though the OVC and the AF are today physically continuous, our results for *D. platyrostris* did not support both regions as biogeographically similar in the short term; different ecological conditions at each domain could explain this result. Divergence between the two domains' populations was very recent (<0.1 MYA) and it is not known whether *D. platyrostris* is an exception or a rule amongst birds that inhabit both the AF and the OVC. If the problem of OVC –AF biogeographic continuity was considered from a phylogenetic standpoint (long term approach) both regions appeared as continuous, as observed in other taxa (Costa, 2003). The majority of the plumage variation of *D. platyrostris* followed biomes' transition instead of population history, suggesting that selection may be directly related to the evolution of the species' plumage. Also, our results suggest the hypothesis that *D. platyrostris* at the OVC disperse longer distances than birds in the continuous AF, and we propose that this differential pattern may be an adaptation to live in the OVC. Further studies should incorporate additional samples and independent markers to test our hypotheses.

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Table 1: Collection localities of *Dendrocolaptes platyrostris* and of other taxa, sample identification, source of plumage data for the plumage vs genetic analysis, organization of samples and population structure considered for analyses of molecular variance (AMOVA I and AMOVA II) and GenBank accession numbers. OVC: open vegetation corridor, CAF: central Atlantic forest, SAF: southern Atlantic forest

Taxa	Locality (biome)	Sample ID ^{TISSUE}	Voucher	Locality and sample size of plumage vs genetic analyses ^{*1}	AMOVA I	AMOVA II	GenBank [cytb] (RC) and author (if not sequenced in this work)
<i>D. platyrostris</i>	1- Ubajara, Ceará (CE). 3° 51'S, 40° 56'W (Caatinga)	LGEMA11443 ^B	Photo	Baturité, CE. 4° 17'S, 38° 55'W, n = 6	OVC	OVC north	[cytb] (RC)
	2- National Park (NP) Serra das Confusões, Piauí (PI). 9° 40'S, 44° 8'W (Caatinga)	All LGEMA: P1853 ^M , P2277 ^M , P2278 ^M , P2329 ^M , P2379 ^M , P2431 ^M	MZUSP...	NP Serra das Confusões, n = 7	OVC	OVC north	[cytb] (RC)
	3- Bonito, Bahia (BA). 11° 56'S, 41° 15'W (Caatinga)	LGEMAP2272 ^B	Photo	Fazenda Mocambo e Iracema (Chapada Diamantina), BA. 12° 25'S, 41° 21'W. n = 3	CAF	OVC north	[cytb] (RC)
	4-Brasilândia de Minas, Minas Gerais (MG). 16° 59'S, 46° 0'W (Cerrado)	B1718 ^B , B2055 ^B , B2058 ^B , B2064 ^B , B2083 ^B , B2278 ^B		Arinus, MG. 15° 55'S, 46° 4'W, n = 3	OVC	OVC south	[cytb] (RC)
	5-Felixlândia, MG. 18° 44'W, 44° 48'S (Cerrado)	B1625 ^B		Not considered	OVC	OVC south	[cytb] (RC)
	6-Bocaiúva, MG. 17° 5'S, 43° 48'W (Cerrado)	B1790 ^B , B2215 ^B		Not considered	CAF	OVC south	[cytb] (RC)
	7-Simonésia, MG. 20° 7'S, 42° 00'W (Atlantic Forest)	B431 ^B		NP Caparaó, Espírito Santo (ES). 20° 26'S, 41° 24'W. n = 3	CAF	CAF	[cytb] (RC)
	8-Araponga, MG. 20° 39'S, 42° 32'W (Atlantic Forest)	B930 ^B		NP Caparaó, n = 3	CAF	CAF	[cytb] (RC)
	9-NP Itatiaia, Rio de Janeiro. 22° 25'S, 44° 36'W.	LGEMA P1326 ^B	MZUSP, photo	NP Itatiaia, n= 10	CAF	CAF	[cytb] (RC)

	(Atlantic Forest)						
	10- Três Lagoas, Mato Grosso do Sul. 20° 46'S, 51° 43'W (Cerrado)	LGEMA11444 ^M	MZUSP	Três Lagoas, n=6	OVC	OVC south	[cytb] (RC)
	11- Morro do Diabo State Park, São Paulo, (SP). 22° 30'S, 52° 18'W (Atlantic Forest)	All LGEMA: P1496 ^B , P1901 ^B , P1912 ^B , P1948 ^B , P1952 ^B	Photo	Morro do Diabo State Park, n=2	SAF	CAF	[cytb] (RC)
	12- Barreiro Rico, SP. 22° 38'S, 48° 13'W (Atlantic Forest)	P1696 ^M	XXX	Victoria, SP. 22° 46'S, 48° 2'W. n =3	CAF	CAF	[cytb] (RC)
	13- Morro Grande State Park, SP. 23° 42'S, 46° 59'W (Atlantic Forest)	All LGEMA: P1033 ^B , P2066 ^B , P2480 ^B , P2520 ^B , P2544 ^B , P2553 ^B , P2668 ^B , P2699 ^B ,		Eng. Ferraz, SP, 23° 58'S, 46° 36'W, n = 8	SAF	CAF	[cytb] (RC)
	14- Burí, SP. 23° 39'S, 48° 32'W (Atlantic Forest)	LGEMAP862 ^M	MZUSP	Burí, n = 1	CAF	CAF	[cytb] (RC)
	15- Pinhalão, Paraná, (PR). 23° 46'S, 50° 3'W (Atlantic Forest)	LGEMAP885 ^M	MZUSP	Pinhalão, n = 1	SAF	SAF	[cytb] (RC)
	16-Wenceslau Braz, PR. 22° 5'S, 48° 47'W (Atlantic Forest)	LGEMAP957 ^M	MZUSP	Wenceslau Braz, n = 1	SAF	SAF	[cytb] (RC)
	17-Ortigueira, PR. 24° 12'S, 50° 55'W (Atlantic Forest)	LGEMA11429 ^M	MZUSP	Ortigueira, n = 1	SAF	SAF	[cytb] (RC)
	18- Rancho Queimado, Santa Catarina (SC). 27° 40'S, 49° 1'W (Atlantic Forest)	LGEMAP1750 ^B		Hansa, SC, 26° 26'S, 49° 14'W, n = 3	SAF	SAF	[cytb] (RC)
	19- Arroio do Padre, Pelotas, Rio Grande do Sul (RS). 31° 31'S, 52° 23'W (Atlantic Forest)	LGEMAP1736 ^B LGEMAP1738 ^B	photo	Sinimbu, RS, 29° 32'S, 52° 32'W, n = 2	SAF	SAF	[cytb] (RC)
	20- San Antonio, Concepción, Paraguay. 23° 33'S, 56° 56'W. (Atlantic forest and Cerrado)			Not considered			AY442990 (Irestedt et al., 2004)
<i>D. picumnus</i>	Amazonas, Br	All LGEMA*: P355					[cytb]

		(=LSUB-35687) ^M , P360 (=LSUB-35704) ^M , P368 (=LSUB-35727) ^M , P369 (=LSUB-35728) ^M LGEMA9791 ^M	
<i>D. certhia</i>	Amazonas, Br		[cytb]
<i>D. sanctithome</i>			[EF212895] (Weir and Schluter, 2007)
<i>Xiphocolaptes</i>			[AY089798] (Aleixo, 2002)
<i>promeropirhynchus</i>			

TISSUE, tissue type. B: blood. M: muscle. *1 Whenever plumage data was extrapolated from the nearest location, this locality is given. Tissue collections: LGEMA—Laboratório de Genética e Evolução Molecular de Aves, Universidade de São Paulo, São Paulo. B—Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Belo Horizonte. LSUB—Louisiana State University, Museum of Natural Science, Baton Rouge. *2 Samples deposited in two collections.

Table 2: Divergence time (years, above diagonal) and migration (females per generation, below diagonal) between pairs of lineages of *D. platyrostris* obtained by the isolation migration methods (Hey and Nielsen, 2004) based on the control region of the mtDNA.

	Lineage OVC (n = 14)	Lineage CAF (n = 21)	Lineage SAF (n = 8)
Lineage OVC		23154 (7763-47253)*	23594 (6142-50628)*
Lineage CAF	2.42 (0.33-16.92)		2*2
Lineage SAF	1.81 (0.07-12.83)	1.97 (0.08-18.79)	

* Approximated 90% confiability interval.

*2 Not convergence of the Markov chain obtained.

Table 3. Historical demography analysis of *D. platyrostris* lineages based on the control region of the mtDNA. 95% confiability intervals are shown between parentheses.

	Lineage OVC (n = 14)	Lineage CAF (n = 21)	Lineage SAF (n = 8)
R ₂	0.174 (P=0.79)	0.093 (P=0.12)	0.1382 (P=0.022)
Population parameter (Θ)	0.0156 (0.0051-0.065)	0.0221 (0.009-0.058)	0.00534 (0.001-0.017)
Exponential growth parameter (g)	245.4 (-207.0 – 949.3)	124.5 (-117.2 – 496.7)	494.6 (-577.4 – 5188.1)

OVC, open vegetation corridor. CAF, central Atlantic Forest. SAF, southern Atlantic Forest.

Figures

Figure 1: Distributions of the Atlantic Forest, of the open vegetation corridor (Caatinga, Cerrado and Chaco), of sampling localities for the plumage and genetic data of *Dendrocolaptes platyrostris*. See Table 1 for details of genetic sampling localities. Distribution of biomes followed Olson et al.(2001) and of *D. platyrostris*, Ridgely and Tudor (1996).

Figure 2: Working hypotheses and their predictions for humid forest organisms. (A) Expected genealogical pattern if the open vegetation corridor (OVC) is not biogeographically independent from the Atlantic forest. Specifically, forest and OVC contiguous populations belong the same major lineage, and phylogeographic latitudinal structures within the forest domains are also observed in the OVC. (B) Alternative hypothesis to A, both domains as independent biogeographic regions and corresponding populations are reciprocally monophyletic. (C) Expected pattern of plumage versus genetic divergences if selection (but not population history – drift-) is responsible for plumage variation. For similar levels of genetic divergence between localities, it is expected to find a larger plumage divergence in inter domain comparisons than in within domain comparisons (adapted from, Moritz et al., 2000; Smith et al., 2005b). (D) Expected pattern of plumage and genetic divergences if plumage variation accompanies population structure (evaluated by neutral markers) and not domain of occurrence.

Figure 3. Geographic distribution of plumage variation in *D. platyrostris*. Circles represent plumage units of analysis (population samples) and different levels of gray represent character scores. See materials and methods section for a detailed description of characters' states. Pop I: population I. Pop II: population II.

Figure 4: (A) Bayesian phylogenetic tree based on the cytochrome *b* of *Dendrocolaptes platyrostris* and closely related species. Model: TN 93 + G ($\alpha = 0.133$), purines Tr/Tv: 10.294, pyrimidines Tr/Tv ratio: 20.541. Estimated base frequencies: A = 0.29138, C = 0.31412, G = 0.12605, T = 0.26844. Values at nodes: ML bootstrap (% of 500 replicates) / posterior probability. Divergence time obtained using corrected distances and a calibration of 4.5% of divergence / MYr (see Material and Methods). (B) Median joining network based on the control region of the mtDNA of *D. platyrostris*. Numbers indicate sampling localities of Table 1 and Figure 1 (underlined numbers represent localities at the OVC-AF ecotone). See column AMOVA II of Table 1 for the assignment of samples to geographic regions. (C) Distribution of genetic samples (black triangles) and their relationship with plumage stability regions (Populations I and II).

Figure 5: Graphical representation of the pattern of genetic distances between individuals of *D. platyrostris* across the study area, following Miller (2006). (A) Raw results as obtained from the AIS 1.0. software and the control region of the mtDNA. Conditions of the analysis were: # coordinates bins (X and Y)= 100; distance weight value= 5. The darker the higher the was the genetic distance. (B) Interpretation of results of the analysis with AIS and distribution of CR lineages. Circles and gray levels represent sampling localities of the genetic study and lineages, respectively. (C) mtDNA lineages of *D. platyrostris* (see Fig. 4B for details). OVC: open vegetation corridor. CAF: central Atlantic forest. SAF: southern Atlantic forest.

Figure 6: Plumage divergence versus genetic distances within and between domains. TN 93: Tamura and Nei (1993). OVC: open vegetation corridor. AF: Atlantic Forest.

Figure 7: Genetic versus geographic distances pattern between localities within each domain. R^2 obtained by a non-parametric procedure (Mantel test). TN 93: Tamura and Nei (1993). OVC: open vegetation corridor. AF: Atlantic Forest.

CAPÍTULO 5

PHYLOGEOGRAPHY OF *SCHIFFORNIS VIRESCENS* (AVES: TYTYRIDAE): LACK OF A STRONG MATRILINEAL PHYLOGEOGRAPHIC STRUCTURE IN AN ATLANTIC FOREST ENDEMISM

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Abstract

We used mitochondrial DNA to study the population genetic structure of the Atlantic Forest (AF) passerine *Schiffornis virescens* (Tyrtyridae). We expected to find similar results to those obtained in other studies of AF organisms that described a common strong phylogeographic pattern. As the sister species of *S. virescens*, *S. turdina*, presents a considerable intra specific genetic structure, we first used cytochrome b DNA sequences to address the monophyly of *S. virescens*. Subsequently, we studied intraspecific relationships by using sequences of the control region of the mtDNA (476 bp and 57 individuals). The analysis indicated that *S. virescens* is monophyletic and presents a weak population genetic structure (mean $\Phi_{st} = 0.32$). Contrarily to what was expected according to results obtained with other AF organisms, and also with other Neotropical birds, *S. virescens* did not present a strong phylogeographic structure (no intraspecific clades were supported). Comparison of nucleotide diversity with other passerines from the same biome suggested that the mtDNA genetic diversity of *S. virescens* is low. A possible origin for this pattern is the combined effects of gene flow and historical demography within the AF (a bottleneck followed by a range or demographic expansion). Other ecologically similar species of the same biome are more diversified, a contrast interesting for future biogeographical issues

Recent palinological studies (i.e., Behling, 2002; Behling and Negrelle, 2001; Ledru et al., 2005) and models of forest distribution (Carnaval and Moritz, 2008) suggest that the southern Atlantic Forest (AF) was strongly affected by the advance of grasslands during the late Quaternary, in contrast to central and northern regions of the biome. During the late Quaternary, the southern grassland-forest ecotone seems to have shifted 750 km northwards from its current location, which was possibly established at the late Holocene (Behling, 2002). Under this scenario, it is expected that the majority of the forest dependent organisms were similarly affected by the retraction and fragmentation of forests, and common phylogeographic patterns may have evolved.

In the present work we used mitochondrial DNA (mtDNA) to study the population genetic structure of the passerine *Schiffornis virescens* (Tytridae, Remsen et al., 2009). *S. virescens* is endemic to the AF (Fig. 1A) and only occurs with low to moderate density in well preserved forests; therefore it is a good model to address issues related to the AF history. We expected to find similar results to those obtained by other studies of AF organisms that described a common phylogeographic pattern (i. e., Chapter 4 of this Thesis, Batalha, 2008; Cabanne et al., 2008; Cabanne et al., 2007; D'Horta, 2009; Grazziotin et al., 2006; Mustrangi and Patton, 1997; Pessoa et al., 2006). Specifically, the common pattern consists in the existence of two main mtDNA clades southwards the Doce river, in central Minas Gerais, that get into contact along with no evident gene flow barrier in central-southern São Paulo (Fig. 1A). Additionally, some species present a third lineage northward from the Doce river. Another characteristic of the common pattern is that the corresponding southern clades present evidences of population expansion. We believe that those common phylogeographic patterns are the product of alterations in the forest cover that occurred during the late Quaternary. However, phylogeographic studies of AF organism still are few and a synthetic robust hypothesis about the evolution of AF organisms is not possible yet.

The objective of this study was to describe the mtDNA genetic variation of *S. virescens* to address the following questions. 1) Does *S. virescens* present the phylogeographic structure shared by other AF organisms? 2) How did the species' phylogeographic pattern originate? and 3) What are the implications of results for understanding the evolution of AF organisms?

As Nyari (2007) showed that the sister species of *S. virescens*, *S. turdina*, presents a considerable intra specific genetic structure, we first used cytochrome b DNA sequences to address the monophyly of

S. virescens. Subsequently, we studied evolutionary relationships among *S. virescens* populations by using sequences of the hypervariable control region of the mtDNA.

Material and Methods

Genetic datasets

For the phylogenetic analysis we used 840 bp of the cytochrome b (*cytb*) from 28 *S. virescens*, 12 *S. turdina*, one *S. major* and one *Laniisoma hypopyrra* as the outgroup. Procedures for DNA purification and for obtaining *cytb* sequences followed Bruford (1992) and Cabanne et al. (2008), respectively. For the population genetic study we used 476 bp from the first domain of the mtDNA control region (CR) from 57 *S. virescens* collected in 19 localities (Fig. 1A). We selected CR for the population analyses because it is more variable than the *cytb*. Amplification (~1000 bp) and sequencing of the CR were performed using specifically designed primers: forward LSvCB GATCTATCCCCAATAAACTTGGAGG and reverse HSvCR GATTACGAACTCGAAGTTGGACGG. PCR annealing temperature for amplifying CR were 60° C for *S. virescens* and at 53° for *S. turdina*. A detailed list of the localities, samples, sequences and GenBank accession numbers is provided in Table 1

Genetic data exploration

We used maximum likelihood and neighbor joining analyses in PHYML 2.4.4 (Guindon and Gascuel, 2003) and MEGA 4 (Tamura et al., 2007), respectively, to obtain phylogenetic trees for the *cytb* sequences from the genus *Schiffornis*. Molecular evolution models were selected in MODELTEST 3.7 (Posada and Crandall, 1998). Visualization of relationships among CR haplotypes was done by constructing a median joining network in NETWORK 4.1.0.8 (<http://www.fluxus-engineering.com>). Nucleotide diversity values were based on non corrected distances. We compared *cytb* nucleotide diversity of *S. virescens* with nucleotide diversity values of similar markers (*cytb* or other coding mtDNA sequences) obtained in other AF passerines. As genetic diversity values may depend on population size and mutation rate, we only used large samples that represented most of each species range (Table 2).

The neutrality test of McDonald and Kreitman (1991) (MK test) was performed for *S. virescens* sequences in DNASP 4.0 (Rozas et al., 2003) using *S. turdina* as outgroup. For studying neutrality at the control region, we performed a MK like test following Eyre-Walker (2006). Specifically, this test uses a

procedure similar to that used by the conventional MK test, but considered third codon positions of the *cytb* as silent positions and control region as the putative selected region. See Eyre-Walker (2006) for details.

Population genetic analysis

Analyses of molecular variance (AMOVA), done with CR sequences in ARLEQUIN 3.1 (Excoffier et al., 2006) and based on uncorrected distances, tested the existence of phylogeographic gaps (gene flow barriers) at the Doce river and at southern-central São Paulo (Fig. 1A). Specifically, we evaluated Φ_{CT} values, which represent the proportion of the total molecular diversity variance that is explained by genetic differences among regions. These regions were limited by the phylogeographic gaps tested (Fig. 1A and Table 3). $\Phi_{CT} = V_a / V_t$, where V_a is the molecular variance among groups and V_t is the total molecular variance (Excoffier et al., 1992). Gene flow estimation was obtained using the formula $M = (1 - \Phi_{ST}) / 2\Phi_{ST}$ (Hedrick, 2000). $\Phi_{ST} = V_a + V_b / V_t$, where V_b is the molecular variance among populations within groups (Excoffier et al., 1992). Significance and confidence intervals of fixation indices were based on 20,000 bootstrap permutations.

Divergence time and historical demography

To obtain a rate of change and divergence dates for the *cytb* we used the method of Ho et al. (2005) as implemented in Cabanne et al. (2008). Ho et al. (2005) analyzed several calibrations of mtDNA molecular clock rates and developed a mathematical approach to directly relate DNA sequence distances to divergence dates (in million years, MYr). We used the model for protein-coding sequences of the avian mtDNA and resolved numerically the equation [7] from Ho et al. (2005). The *cytb* calibration that resulted after applying this method was 2.35% of divergence per MYr, which is in accordance with other calibrations obtained for bird *cytb* sequences (i.e., Lovette, 2004).

To estimate the parameter Θ we used the maximum likelihood and coalescent method implemented in LAMARC 2.1.2b (Kuhner, 2006). We used a relative effective size of one. We also used LAMARC and CR sequences to evaluate the demographic signature by estimating the exponential population growth rate g ($\Theta_t = \Theta_{now} e^{-gt}$, where Θ_{now} is the current Θ and Θ_t is the value of the parameter t time ago). Positive g -values indicate population growth and negative values, population decline. Final

runs used the F84 model of sequence evolution with empirical base frequencies and transition/transversion ratios, and 10 short chains (500 sampled trees every 50 iterations and burn-in of 1000 trees) followed by two to ten long chains (20,000 sampled trees every 20 iterations and burn-in of 5000 trees).

We also evaluated the historical demography of the species using CR sequences and the neutrality tests of Tajima (1989) and of Fu (1997) in DNASP 4.0 (Rozas et al., 2003). If the marker is selectively neutral (not positively selected), significant negative values of statistics of both tests suggest demographic expansion. Significance was determined based on 10,000 coalescent simulations under a model of 2000 bp (program default), population stability using empirical sample sizes and estimates of theta (Θ).

Results

Genetic data, neutrality and variation

The MK test did not reject neutrality for the *cytb* sequences used for the monophy analysis (G-test $P = 0.269$). For the CR-alignment, the MK like test rejected neutrality at the 5% level of significance (G-test $P = 0.043$) and indicated a slight excess of CR polymorphism (results not shown). We interpreted this result as indicative of the presence of nearly neutral mutations at the CR, a result expected for mtDNA (Nachman, 1998). See Nachman (1998) for further details.

We evaluated the relative diversity of *cytb* and CR. We estimated Θ for CR ($\Theta_{CR} = 0.01218$, 95 % c.i.: 0.0067-0.0219) and for *cytb* ($\Theta_{cytb} = 0.00491$, 95 % c.i.: 0.0023-0.0096) and obtained the ratio $\Theta_{CR}/\Theta_{cytb} = 2.48$. This ratio suggests that the mutation rate at the CR is ~2.5 times higher than the *cytb*'s rate. A similar result was obtained when nucleotide diversities were compared ($\pi_{CR} = 0.0041$, SD = 0.0013; $\pi_{cytb} = 0.00187$, SD = 0.00062; $\pi_{CR}/\pi_{cytb} = 2.19$). These results indicated that the CR was a suitable marker for the population genetic analyses.

We compared the *cytb* genetic diversity of *S. virescens* with other available genetic data of AF passerines (Table 2). Nucleotide diversity values of *cytb* from other passerines ranged from 0.002 to 0.015 (average $\pi = 0.07$, $n = 7$), suggesting that the mtDNA genetic diversity of *S. virescens* is low ($\pi_{cytb} = 0.00187$).

Cytb sequences showed that the *S. virescens* lineage is monophyletic and diverged from its sister species, *S. turdina*, ~4.2 MYr ago (Fig. 2A). Unexpectedly, no particular intraspecific structure was

suggested by the *cytb* sequences for *S. virescens*. The CR data presented 17 haplotypes and the network evidenced the inexistence of a strong phylogeographic structure in *S. virescens* (Fig. 2B).

The AMOVA analyses indicated that most of the variation in CR sequences was organized among and within populations, not among regions (i.e., all Φ_{ct} values are non significant, $P > 0.05$) (Table 3). Therefore, none of the specifically tested genetic gaps was supported. The global Φ_{st} value was 0.3184 (line 4 in Table 3) and corresponded to ~ 1.1 (95% c. i.: 0.56-6.64) females migrant per generation among populations, indicating a weak population genetic structure.

The historical demography analysis with LAMARC suggested that *S. virescens* expanded recently ($g = 932.3$, 95% c.i.: 367.28 - 1966.14) and neutrality tests of Fu and Tajima corroborated the result of LAMARC (Fu's $F = -7.5$, $P = 0.001$; Tajima's $D = -1.47$, $P = 0.038$).

Discussion

The analysis of mtDNA sequences indicated that *S. virescens* is monophyletic and presents a weak population genetic structure. Comparison of nucleotide diversity with other passerines from the same biome suggested that the mtDNA genetic diversity of *S. virescens* is low. However, this last result should be interpreted with caution because the comparison was based on a small sample of other AF birds (Table 2). These results are in opposition to what was observed in the sister species of *S. virescens*, *S. turdina*, which presented high levels of genetic diversity and a strong phylogeographic structure (Nyari, 2007). Within the lineage *S. virescens* / *S. turdina*, the most ancient split separated birds from the AF (*S. virescens*) and birds from the Amazon and Central America forests (*S. turdina*). Interestingly, and as already observed in other bird groups (i. e., Cabanne et al., 2008; Ribas et al., 2005), the radiation of the AF clade, compared to its Amazonian sister clade, showed shorter branches and lower genetic distances among terminals (Fig. 2A). This pattern suggests lower diversification rates in the AF (Ricklefs, 2007), aspect that would be interesting to address in future studies.

Contrarily to what was expected according to results obtained with other AF organisms (i.e., Batalha, 2008; Cabanne et al., 2008; D'Horta, 2009; Grazziotin et al., 2006; Mustrangi and Patton, 1997; Pessoa, 2007; Pessoa et al., 2006), and also with other Neotropical birds (i.e., Aleixo, 2004; Bates, 2002; Cheviron et al., 2005), *S. virescens* did not present a strong phylogeographic structure. Three hypotheses can be proposed to explain the origin of the genetic pattern of *S. virescens*: 1) recent dispersion of the species,

or of its ancestral population, to the AF and lack of time for the evolution of a significant intraspecific structure, 2) existence of high gene flow rates across all the species' range, and 3) a bottleneck and recent expansion that resulted in the current species' range.

The option of recent dispersion to the AF and lack of time for the evolution of a significant intraspecific structure in *S. virescens* is not well supported. Even though our estimative of the divergence time between *S. virescens* and its sister species may not be accurate (~ 4.2 MYr), the origin of the lineage *S. virescens* is not comparatively more recent than those from other AF organisms that present a strong population structure. Therefore, it seems that enough time passed to allow the evolution of a significant structure. This becomes clear when analyzing other similar cases of species that are as old as *S. virescens* but present intraspecific structure. For example, D'Horta (2009) and Cabanne et al. (2008) studied two AF passerines (*Sclerurus scansor* and *Xiphorhynchus fuscus*, respectively) that diverged from their sister clades ~ 1.1 and ~ 3 MYr ago, respectively, and present a very strong intraspecific genetic structure (i.e., maximum divergences between intraspecific lineages were about 0.6 to 0.8 MYr). On the other hand, *S. virescens* diverged from its sister species around 4.2 MYr ago but did not present a strong phylogeographic structure. In spite of that, if *cyt b* nucleotide diversity (average divergence between sequences) is used as a proxy to evaluate intraspecific divergence, and by using the calibration method of Ho et al (2005) (see Methods), an intraspecific divergence of only about 40,000 years is estimated for *S. virescens*.

Alternatively, it seems that the phylogeographic pattern of *S. virescens* could be explained by the combined effects of gene flow and historical demography within the AF. Gene flow rate among populations is moderated (point estimation ~ 1.1 females / generation), but enough to avoid allele fixation and strong divergence (Wang, 2004), and a recent population expansion was indicated by the Fu's and Tajima's tests and by the coalescence analysis in LAMARC. Furthermore, *S. virescens* apparently has low levels of mtDNA genetic diversity than other AF birds. Therefore, the demographic expansion may have occurred after a strong population bottleneck that reduced genetic diversity. It is likely that the bottleneck contributed to the extinction of intraspecific lineages, hence to determine the observed lack of a strong phylogeographic pattern in *S. virescens*.

Even though *S. virescens* is a not new lineage, its intraspecific differentiation was low. Other ecologically similar species of the same biome are more diversified (i. e., *Conopophaga lineata*, *S. scansor* and *X. fuscus*), a contrast interesting for future biogeographical issues. It seems that the long term effective size

in *S. virescens* is smaller than those of other species, but considering that distribution ranges, ecological characteristics and abundances are not very different (Ridgely and Tudor, 1996; Stotz et al., 1996), causes for the observed differences are not evident. A study incorporating simulation of genealogies could contribute to test whether the observed differences among birds are caused by different histories, or are consequence of stochastic effects during the evolution of genealogies (Carstens and Richards, 2007; Knowles, 2004; Knowles and Carstens, 2007). More studies are necessary to describe common phylogeographic patterns and to make possible a synthetic model about the evolution of the Atlantic forest.

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Table 1: Collection localities of *S. virescens*, sample characteristics and GenBank accession numbers. Samples are deposited at the Laboratório de Genética e Evolução Molecular de Aves (LGEMA), Instituto de Biociências, Univ. de São Paulo, Brazil, and at the Departamento de Biologia Geral, Instituto de Ciências Biológicas, Univ. Federal de Minas Gerais, Brazil (B). All samples are from Brazil, except when indicated.

Taxa	Locality	Sample size	Tissue ID ^{TISSUE}	GenBank Access, <i>cytb</i>	GenBank Access, CR	Origin
<i>S. virescens</i>	1- Bonito, Bahia. 11° 56'S, 41° 15'W	1	LGEMA P2263 ^b		XXX	This study
	2-Ecological station Acauã, Leme do Prado, Minas Gerais (MG). 17° 4'S, 42° 40'W	1	B2284 ^b		XXX	
	3-Turmalina, MG. 17° 16'S, 42° 43'W	7	B1990 ^b , B1989 ^b , B2279 ^b , B2280 ^b , B2281 ^b , B2282 ^b , B2283 ^b		XXX	
	4-Jequitinhonha (Mata Escura), MG. 16° 20'S, 41° 00'W	1	B1091 ^b		XXX	
	5-José Gonçalves de Minas, MG. 15° 00'S, 40° 25'W	1	B2285 ^b		XXX	
	6-Nova Lima, MG, 19° 50'S, 43° 49'W	1	B1570 ^b		XXX	
	7-São Bartolomeu (Grande Mata), MG, 20° 17'S, 43° 35'W	1	B1022 ^b		XXX	
	8- Santa Teresa, Espírito Santo (ES). 19° 56'S, 40° 34'W	1	B1711 ^b		XXX	
	9- Morro do Diabo State Park, São Paulo (SP). 22° 30'S, 52° 18'W	9	All from LGEMA: P1965 ^b , P1867 ^b , P1957 ^b , P1960 ^b , P1959 ^b , P1973 ^b , P2158 ^b , P1494 ^b , P1388 ^b	XXX	XXX	
	10-Barreiro Rico, SP. 22° 38'S, 48° 13'W	2	LGEMA P1703 ^M , LGEMA P1686 ^M	XXX	XXX	
	11-Itatiaia Natl. Park, Rio de Janeiro. 22° 25'S, 44° 36'W.	5	Al from LGEMA: P1322 ^b , P1346 ^b , P1339 ^b , P1332 ^b , P1333 ^b	XXX	XXX	
	12-Piedade, SP.	1	LGEMA P681 ^M	XXX	XXX	
	13-Morro Grande State Park, SP. 23° 42'S, 46° 59'W	10	All from LGEMA: P519 ^b , P493 ^b , P542 ^b , P463 ^b , P435 ^b , P448 ^b , P461 ^b , P544 ^b , P618 ^b , P518 ^b	XXX	XXX	
	14-Juquitiba, SP, 23° 53'S, 47° 00'W	3	LGEMA P714 ^M , LGEMA P773 ^M , LGEMA P713 ^M	XXX	XXX	
	15-Pinhalão, Paraná (PR). 23° 46'S, 50° 3'W	1	LGEMA P869 ^M	XXX	XXX	
	16-Wenceslau Braz, PR. 22° 5'S, 48° 47'W	2	LGEMA P958 ^M , LGEMA P980 ^M	XXX	XXX	

	17-PETAR, Caboclos station, SP, 24° 28'S, 48° 35'W	3	LGEMA P1211 ^b , LGEMA P1220 ^b , LGEMA P1221 ^b	XXX	XXX	
	18-State Park Urugua-í, Misiones, Argentina, 25° 51'S, 53° 55'W	5	All from LGEMA: 10369 ^M , 10341 ^M , 10348 ^M , 10359 ^M , 10370 ^M	XXX	XXX	
	19-Rancho Queimado, Santa Catarina. 27° 40'S, 49° 1'W Paraguay.	2	LGEMA P1753 ^b , LGEMA P1756 ^b	XXX EF458569, EF458570	XXX	Nyari (2007)
<i>S. turdina</i>	Novo Airão, Amazonas. 1° 57'S 62° 21'W. See original paper for details		LGEMA 9705		XXX	This work Nyari (2007)
<i>S. major</i> <i>Laniisoma</i> <i>hypopyrra</i>				EF458548, EF458560, EF458563, EF458557, EF458536, EF458538, EF458539, EF458532, EF458541, EF458551, EF458554, EF458565, EF458571 EF458572		
TISSUE, tissue type. ^b : blood. ^M : muscle						

Table 2: Nucleotide diversity of mtDNA (*cytb* any other coding gene) from Atlantic Forest passerines.

Species	π - nucleotide diversity	Sample size	Marker	Reference
<i>Thamnophilus ambiguus</i>	0.005 (SD=0.002)	22	<i>cytb</i> (837 bp)	(Lacerda et al., 2007)
<i>Thamnophilus pelzengi</i>	0.003 (SD=0.002)	21	<i>cytb</i> (837 bp)	(Lacerda et al., 2007)
<i>Automolus leucophthalmus</i>	0.00242 (SD=?)	22	<i>cytb</i> (900 bp)	(D'Horta, 2009)
<i>Sclerurus scansor</i>	0.01042 (SD=?)	47	Concatenated <i>cytb</i> -ND2-ND3 (2400 bp)	(D'Horta, 2009)
<i>Xiphorhynchus fuscus</i>	0.0151 (SD=?)	34	Concatenated <i>cytb</i> -ND2-ND3 (2499 bp)	(Cabanne et al., 2008)
<i>Dendrocolaptes platyrostris</i>	0.00448 (SD=0.00026)	31	<i>cytb</i> (983 bp)	Chapter 4, this Dissertation
<i>Tachyphonus coronatus</i>	0.00512 (SD=0.00042)	50	<i>cytb</i> (990 bp)	Cabanne & Miyaki unpubl.

Table 3. Analyses of molecular variance (AMOVA) of 57 *S. virescens* based on 476 bp of the mtDNA control region. See Figure 1 for localities and gene flow barriers tested (CSSP - Central-southern São Paulo, and DR- Doce river, .

Tested barrier	Population structure*1 (numbers represent localities from Table 1)	Φ_{st} (95% c.i.)	Φ_{st} Significance	Φ_{ct} (95% c.i.)	Φ_{ct} Significance
CSSP	[(2-3), (4-5), (6-7), 10, 11, (12-13), 14] [9, (15-15), 17, 18, 19]	0.3348, (0.0911-0.4825)	$P = 0.0000$	0.0528 (0.0148-0.0896)	$P = 0.0733$
DR	[(2-3), (4-5)] [(6-7), 10, 11, (12-13), 14, 9, (15-15), 17, 18, 19]	0.3367 (0.0513- 0.5043)	$P = 0.0000$	0.0405 (-0.1253-0.2871)	$P = 0.2308$
CSSP and DR	[(2-3), (4-5)] [(6-7), 10, 11, (12-13), 14] [9, (15-15), 17, 18, 19]	0.3227 (0.0766-0.4720)	$P = 0.0000$	0.0220 (0.0627 - 0.1598)	$P = 0.1899$
No barrier	[(2-3), (4-5), (6-7), 10, 11, (12-13), 14, 9, (15-15), 17, 18, 19]	0.3185 (0.0701-0.4717,	$P = 0.0000$	-	-

*1- localities within parenthesis were pooled to form a new sample. Localities 1 and 8 were excluded from the analyses due to small sample sizes. Specific structures indicated by samples within [].

Figure 1: Distribution of the Atlantic Forest (AF) and of the sampling localities for the genetic study of *Schiffornis virescens*. See Table 1 for details of the localities. Distribution of the biome followed Olson et al.(2001) and of *S. virescens* Ridgely and Tudor (1996). Gene flow barriers observed in AF organisms: I) passerine *Dendrocolaptes platyrostris* (Chapter 4, this Thesis), bee *Melipona quadrifasciata* (Batalha, 2008) and viper *Bothrops jararaca* (Grazziotin et al., 2006), II) passerine *Xiphorhynchus fuscus* (Cabanne et al., 2008), III) opossum *Marmosops incanus* (Muistrangi and Patton, 1997), passerine *Sclerurus scansor* (D'Horta, 2009), passerine *Conopophaga lineata* (Pessoa et al., 2006), IV) passerine *Sclerurus scansor* (D'Horta, 2009) and passerine *Xiphorhynchus fuscus* (Cabanne et al., 2008).

Figure 2: (A) Maximum Likelihood (ML) tree based on 840 bp of the *cytb* of *Schiffornis virescens* and closely related species. Model: HKY + G ($\alpha = 0.9$), Tr/Tv ratio: 20.959. Estimated base frequencies: A = 0.2844, C = 0.3100, G = 0.1165, T = 0.2880. Bootstrap values (percentage of 500 replicates) at the nodes: neighbor joining / ML. Time divergence obtained using corrected distances and a calibration of 2.35% of divergence / MYr (see Material and Methods). Clades of *S. turdina* are identified according to Nyari (2007) (B) Median joining network based on 476 bp of the mtDNA control region of *S. virescens*.

CAPÍTULO 6

DISCUSSÃO FINAL

O objetivo geral desta Tese foi estudar a evolução de organismos da Floresta Atlântica (FA), utilizando aves florestais como modelos e sob uma perspectiva filogenética – filogeográfica. Foram estudadas as estruturas filogeográficas de três passeriformes: *Xiphorhynchus fuscus*, *Dendrocolaptes platyrostris* e *Schiffornis virescens*.

Os padrões filogeográficos de *X. fuscus*, e de *D. platyrostris* no domínio central da FA mostraram semelhança com o padrão de outros passeriformes endêmicos e de ampla distribuição na FA, especificamente *Sclerurus scansor* (D’Horta, 2009) e *Conopophaga lineata* (Pessoa, 2007). Nessas quatro espécies existe uma quebra filogeográfica na região central de São Paulo. Além disso, em *S. scansor* e *X. fuscus* existe outra disjunção filogeográfica na região do baixo Rio Doce (Espírito Santo). Além das quebras filogeográficas, em todas estas espécies mencionadas foram encontrados sinais de expansão demográfica nas populações do extremo sul de distribuição. Estruturas filogeográficas semelhantes são observados também em outros grupos, como uma abelha (Batalha, 2008) e uma serpente (Grazziotin et al., 2006), o que sugere a existência de um padrão filogeográfico comum para alguns organismos da FA. Isto sugere um histórico compartilhado por grupos diversos.

No entanto, nem todos os organismos da FA apresentam o mencionado padrão comum. A terceira espécie aqui estudada, *S. virescens*, apresentou um padrão filogeográfico sem disjunções (ausência de estrutura no DNA mitocondrial). Isso também é encontrado em outros passeriformes, como *Automolus leucophthalmus* (D’Horta, 2009) e *Tachyphonus coronatus* (Cabanne e Miyaki não publicado). A origem deste padrão indiferenciado não é clara, mas uma explicação possível seria considerar a existência combinada de gargalos populacionais seguidos de expansões demográficas e alto fluxo gênico em toda a área de distribuição.

D. platyrostris é a única espécie estudada que habita grande parte das florestas de galeria e encraves florestais da diagonal aberta (Caatinga, Cerrado e nordeste do Chaco). Nessa região, foi encontrada uma linhagem isolada do domínio central da FA. Este resultado não era esperado, pois a continuidade entre as matas de galeria e o domínio central da FA deveria refletir se na ausência de divergência entre domínios.

Nossos resultados, assim como os obtidos por outros autores com outras aves (p. ex., D'Horta, 2009; Pessoa, 2007), sustentam um modelo para a FA no qual a distribuição temporal e espacial das florestas é muito dinâmica. O norte e o sul da FA seriam as regiões mais instáveis, devido à influência de mudanças climáticas locais e globais (p. ex., ciclos de glaciações e de períodos interglaciais). Esse cenário é congruente com dados de palinologia (p. ex., Behling, 2002) e os modelos obtidos com simulação de cobertura florestal (Carnaval e Moritz, 2008). Esta dinâmica florestal poderia ter contribuído para a evolução dos padrões filogeográficos observados, nos quais cada linhagem poderia ter evoluído em regiões com enclaves ou refúgios distintos.

Nosso trabalho, assim como o de D'Horta (2009), não sustentou a idéia de que o geotectonismo (p. ex., eventos neotectônicos no sudeste da FA) tenha sido importante na evolução dos organismos da FA. Isto se contrapõe ao estudo de Pessoa (2007). Além disso, nosso trabalho e o de D'Horta (2009) tampouco sustentaram uma ação primária dos rios da FA como barreira ao fluxo gênico. Porém, alguns rios teriam atuado como barreiras secundárias (p. ex., Rio Doce para *X. fuscus*).

Embora a dinâmica florestal associada a mudanças climáticas parece importante para a evolução da estrutura populacional de algumas aves da FA, ainda não está claro se a fragmentação natural de florestas foi / é importante para a especiação ou para gerar variação em caracteres não neutros (p. ex., plumagem, medidas morfológicas, etc). Por exemplo, se encontrou em *D. platyrostris* que a variação de coloração da plumagem não acompanha a história das populações, mas que deve estar relacionada a mudanças de habitat / bioma. Isto sugere que, pelo menos para a plumagem, a ação de fatores não históricos (possivelmente seleção) seria importante na evolução da morfologia externa da ave. Este resultado sustenta a hipótese de gradientes ambientais. Além disso, não ficou claro se as linhagens diferentes que ocorrem dentro do domínio central da FA (p. ex., linhagens CAF e SAF de *X. fuscus* e de *D. platyrostris*), e que não possuem características diagnósticas morfológicas evidentes (Capítulos 4 e 5, e Cabanne não publicado), possuem algum grau de independência evolutiva. Futuras pesquisas podem se concentrar nas regiões de contato entre essas linhagens e estudar a existência de introgressão diferencial entre marcadores mitocondriais e autossômicos, pois a existência deste tipo de introgressão indicaria certo grau de isolamento genético entre linhagens (Carling e Brumfield, 2008).

No contexto sistemático, nossos resultados sugerem que: 1) *X. fuscus atlanticus* seja uma espécie plena e 2) que as subespécies de *D. platyrostris platyrostris* e *D. platyrostris intermedius* não deveriam ser consideradas como espécies, pois não foram encontradas linhagens evolutivas independentes.

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