Phylogeography of Xiphorhynchus fuscus (Passeriformes, Dendrocolaptidae): vicariance and recent demographic expansion in southern Atlantic forest

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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 analyse 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 analysis of molecular variance and the demographic history with multiple regression analysis, coalescence simulations, and demographic tests. Xiphorhynchus fuscus presented a significant population genetic structure ($\Phi_{st}$ = 0.57). Three mitochondrial lineages were described, one associated with Xiphorhynchus fuscus tenuirostris and the others with Xiphorhynchus fuscus fuscus. The data did not support the primary influence of geographical barriers or rivers in the intraspecific diversification of X. fuscus in the southern Atlantic forest. Instead, the data supported the influence of isolation by geographical distance, recent vicariance events, and demographic expansions apparently related to Pleistocene and Holocene forest dynamics. © 2007 The Linnean Society of London, Biological Journal of the Linnean Society, 2007, 91, 73–84.


The Atlantic forest is distributed along eastern Brazil, eastern Paraguay, and north-eastern Argentina (Gusmão Câmara, 2003). Its unique biota is probably the result of a complex evolutionary history; however, few studies have attempted to clarify the biogeographical processes that shaped it (Mustrangi & 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 (Moritz et al., 2000), the evolution in palaeorefuges and the influence of geographical 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’Saber, 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 be in contact. This hypothesis requires the expansion and contrac-
tion of open areas and the formation of forest refuges. Palinological (Ledru et al., 1998; Behling & Negrelle, 2001; Behling, 2002) and other types of studies (Brown & Ab'Saber, 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 phylogeographical study of the gecko Gymnodactylus darwinii (Gekkonidae, Squamata), proposed that rivers play an important role in the diversification of Atlantic forest biota. Other authors proposed that the tectonic activity associated with the formation of geographical landmarks could be relevant to biodiversity modelling, as suggested by Silva & Straube (1996) who observed that the geographical range of some passerines was limited by a graben, the valley of the Paraíba do Sul river (VPSR). Those tectonic episodes could 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 & Fulfaró, 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), eastern Paraguay and north-eastern Argentina (Marantz et al., 2003). There are four subspecies defined by slight variations in colour and morphological measurements (Marantz et al., 2003). Xiphorhynchus fuscus tenuirostris and Xiphorhynchus fuscus 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

Figure 1. Study area, sampling localities, and distribution of Xiphorhynchus 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.
Table 1. Localities, sample sizes (N), haplotypes, and nucleotide diversity (π) in percentage

<table>
<thead>
<tr>
<th>Locality</th>
<th>N</th>
<th>Haplotypes</th>
<th>π% (SD%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Minas Gerais (MG), Jequitinhonha, left bank of the Jequitinhonha river, 16°20'0S, 41°00'W</td>
<td>5a</td>
<td>T2, T4, T6, T7</td>
<td>0.5186 (0.2198)</td>
</tr>
<tr>
<td>2. MG, Salto da Divisa, left bank of the Jequitinhonha river, 16°05'0S, 40°02'W</td>
<td>6a</td>
<td>T1, T5, T7</td>
<td>0.4606 (0.1785)</td>
</tr>
<tr>
<td>3. Bahia, Porto Seguro, 17°22'0S, 40°17'W</td>
<td>1b</td>
<td>T3</td>
<td></td>
</tr>
<tr>
<td>4. MG, Nova Lima, 19°59'3S, 43°49'W</td>
<td>1a</td>
<td>N8</td>
<td></td>
</tr>
<tr>
<td>5. MG, Marliéria, 19°43'5S, 42°44'W</td>
<td>1a</td>
<td>N9</td>
<td></td>
</tr>
<tr>
<td>6. MG, Caratinga, 20°50'5S, 42°05'W</td>
<td>1a</td>
<td>N12</td>
<td></td>
</tr>
<tr>
<td>7. MG, Simonésia, 20°07'5S, 42°00'W</td>
<td>1a</td>
<td>N13</td>
<td></td>
</tr>
<tr>
<td>8. MG, Araponga, 20°40'5S, 42°31'W</td>
<td>3a</td>
<td>N2, N10, N11</td>
<td>0.4710 (0.2374)</td>
</tr>
<tr>
<td>9. Rio de Janeiro, Itatiaia National Park, 22°25'S, 44°36'W</td>
<td>10</td>
<td>N2, N6, N14, N15</td>
<td>0.2274 (0.1293)</td>
</tr>
<tr>
<td>10. São Paulo (SP), Bananal State Park, 22°41'S, 44°19'W</td>
<td>2a</td>
<td>N3, N6</td>
<td>0.7109 (0.3368)</td>
</tr>
<tr>
<td>11. SP, Picinguaba, 22°31'S, 44°50'W</td>
<td>4a</td>
<td>N2</td>
<td>0.0</td>
</tr>
<tr>
<td>12. SP, Caragatuatabu, 23°37'S, 42°26'W</td>
<td>2</td>
<td>N2, N14</td>
<td>0.1760 (0.1771)</td>
</tr>
<tr>
<td>13. SP, Morro do Diabo State Park, 22°30'S, 52°18'W</td>
<td>1a</td>
<td>S1</td>
<td></td>
</tr>
<tr>
<td>14. Paraná (PR), Ortigueira, 24°12'S, 50°55'W</td>
<td>1a</td>
<td>S8</td>
<td></td>
</tr>
<tr>
<td>15. PR, Wenceslau Braz, 22°51'S, 49°47'W</td>
<td>7a</td>
<td>S1, S8</td>
<td>0.1677 (0.1237)</td>
</tr>
<tr>
<td>16. SP, Barreiro Rico, 22°38'S, 48°13'W</td>
<td>3a</td>
<td>N2, S8</td>
<td>0.9479 (0.3195)</td>
</tr>
<tr>
<td>17. SP, Itaberá State Park, 23°51'S, 49°08'W</td>
<td>7</td>
<td>S4, S6, S10</td>
<td>0.2014 (0.1154)</td>
</tr>
<tr>
<td>18. SP, Buri State Park, 23°39'S, 48°32'W</td>
<td>9a</td>
<td>S4, S7, S8, S9</td>
<td>0.1760 (0.1098)</td>
</tr>
<tr>
<td>19. SP, Caboclos, 24°28'S, 48°35'W</td>
<td>8</td>
<td>S4, S8</td>
<td>0.0943 (0.0949)</td>
</tr>
<tr>
<td>20. SP, São Roque, 23°34'S, 47°09'W</td>
<td>9</td>
<td>N1, N2, S4</td>
<td>0.9199 (0.2815)</td>
</tr>
<tr>
<td>21. SP, Morro Grande State Park, 23°42'S, 46°59'W</td>
<td>14</td>
<td>N1, N2, S1, S6, S7</td>
<td>0.8830 (0.2589)</td>
</tr>
<tr>
<td>22. SP, Juquitiba, 23°53'S, 47°00'W</td>
<td>7a</td>
<td>N1, N4, S4, S6, S7</td>
<td>1.0172 (0.3925)</td>
</tr>
<tr>
<td>23. SP, Serra do Mar State Park, Station Curucutú, 23°58'S, 46°44'W</td>
<td>4a</td>
<td>N2, N4, N5, N7</td>
<td>0.4424 (0.1858)</td>
</tr>
<tr>
<td>24. Argentina, Misiones (MI), Campo San Juan State Park, 27°22'S, 55°39'W</td>
<td>3</td>
<td>S1, S2</td>
<td>0.1173 (0.1065)</td>
</tr>
<tr>
<td>25. MI, Yaboti Biosphere Reserve, 26°48'S, 53°55'W</td>
<td>3</td>
<td>S1, S3</td>
<td>0.1173 (0.1181)</td>
</tr>
<tr>
<td>26. Santa Catarina, Botuverá, 27°13'S, 49°03'W</td>
<td>1a</td>
<td>S1</td>
<td></td>
</tr>
</tbody>
</table>

*Tissue samples deposited at the Universidade Federal de Minas Gerais; all the other samples are deposited at the Universidade de São Paulo.

Samples with museum voucher or specimen field (f) numbers: *Museu Paraense Emílio Goeldi fAA568; *Museu de Zoologia da Universidade de São Paulo (MZUSP) f76, f91; *MZUSP fITA257, fITA302, f ITA283; *MZUSP f31, f59, f60, *MZUSP 76134, MZUSP 75588; *MZUSP fITA141, fITA171, fITA182, fITA152, fITA172, fITA157, 75565; *MZUSP f9; *MZUSP 75032.

Numbers in superscript indicate the number of individuals with the corresponding haplotype.

Bahia (Xiphorhynchus fuscus brevirostris) and the north-eastern part of the country (Xiphorhynchus fuscus atlanticus). The distribution limits of these subspecies are not well known. Given its geographical 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 analyse the mitochondrial DNA variation of populations of X. 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.

**MATERIAL 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 a.s.l.

Blood was collected (approximately 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. Each bird was captured with mist nets, photographed and marked with an aluminium 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 nuclear mtDNA inserts, we amplified an approximately 1600-bp fragment with the primer pair LXfB2 (5′-TCAATTCCAAACAACTAGGAGG-3′, present study)/HPRO (5′-GCTTGGAGTTGGAGATATAAGG-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, 1X of Taq buffer (Pharmacia Biotech), 200 µM of each dNTP, 1 µM of each primer, and 0.1 U of Taq polymerase (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 the primer pair HXfRC1 5′-GGGGAAAATAAACGTTTATTA-3′, HXfRC2 5′-CAAGATGGACATGTTCGAC-3′, pre

Analytical Methods
The complete control region (1275 bp) and flanking genes (tRNAThr 70 bp, tRNAPro 70 bp) from one X. f. fuscus sample (tissue IBUSP P652) were sequenced. The analysis of 1048 bp of the control region (positions 57–1104) from 20 individuals revealed that the most variable portion encompasses 575 bp (positions 57–631); thus, this segment

Sequences were aligned with the program Clustal X (Thompson et al., 1997). The likelihood ratio test as implemented in the software Modeltest, version 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 (α = 0.6467); Hasegawa et al., 1985]. We reconstructed the phylogenies by Neighbour-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 Xiphorhynchus pardalotus (tissue IBUSP P264) was used to root the resulting trees. The support of the nodes was evaluated by nonparametric 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 available in the software we used. The haplotype network was constructed in accordance with Templeton, Crandall & Sing (1992) with the program TCS, version 1.13 (Clement, Posada & Crandall, 2000). The net divergence between sets of sequences was obtained in MEGA, version 3.0 (Kumar, Tamura & Nei, 2004) with the formula \( \delta = \delta_y - 0.5(\delta_x + \delta_y) \), where \( \delta_x \) is the mean divergence between all the sequences, and \( \delta_x \) and \( \delta_y \) 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 per nucleotide, as 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 1 year (Klicka & Zink, 1999).

To test whether the X. f. fuscus 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 invariant until total coalescence. Four arbitrary \( N_e \) were tested: 100 000, 50 000, 25 000, 12 000, and 6000. The
discordance between these gene trees and the alternative model of two isolated populations (one in the north and the other in the south in the Valley of the Paraiba do Sul river, VPSR; Fig. 1) was measured using statistic s (Slatkin & Maddison, 1989). The discordance between the reconstructed gene tree (NJ tree of X.f.fuscus sequences) and the two populations’ model was also evaluated with the statistic s and this value was compared with 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 (α = 0.05), the null model is rejected. We used the nucleotide diversity (π, Nei & Kumar, 2000) calculated in Arlequin, version 2.0 (Schneider, Roessli & Excoffier, 2000) to approximate theta (Θ) and to calculate the empirical N_e according to the relationship π = Θ = 2N_eµ (Watterson, 1975; Nei & Kumar, 2000).

We estimated the nucleotide diversity for each locality where two or more individuals were available 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, version 2.0. We performed global (all the localities) and regional analyses (subgroups of localities). The ΦST was obtained using corrected distances and its statistical significance was estimated by a nonparametric 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 geographical distance among the pairs of localities and their geographical location) on the average corrected genetic distances among individuals from pairs of locations. Genetic distances were estimated with the program MEGA, version 3.0. The matrix of geographical location was constructed as a dummy variable (Quinn & Keough, 2002) that indicated whether each pair of localities were within the same main geographical region (north-western Minas Gerais plus southern Bahia, south-eastern 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 distances reflect simple isolation by distance, geographical distance would be the best predictor. If vicariance were the most important process, the geographical 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, version 2.9.3.2 (Goudet, 2002), using 10 000 permutations to obtain the probability values.

Demographic expansion was inferred by calculating F_3 (Fu, 1997) and R_2 (Ramos-Onsins & Rozas, 2002) using DnaSP, version 4 (Rozas & Sánchez-Del Barrio, 2003). Significantly negative F_3 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, version 2.0. The expansion time was estimated according to t = τ/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 revealed evidence of being of mitochondrial origin: (1) the empirical base composition of the sequences used in the phylogeographical study (A, 27.7%; C, 25%; G, 16.3%; and T, 31%) is compatible with the expected one for avian mitochondrial DNA (Baker & Marshall, 1997); (2) 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^Phe at the 3’ end (70 bp), and this gene arrangement is the expected one for Suboscines (Mindell, Sorenson & Dimcheff, 1998); (3) the inferred secondary structure and the presence of expected anticodons (UGU and UGG) suggested that these tRNA genes are functional (data not shown); and (4) the amplicon analysed 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, Figs 2, 3), with 31 polymorphic sites (5.39% of all positions), 30 transitions and two transversions, and no indels.

GENEALOGY, DIVERGENCE, AND GEOGRAPHICAL 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 north-eastern Minas Gerais and southern Bahia. Haplotypes of the X.f.fuscus northern lineage only occurred in south-eastern Minas Gerais, southern Rio de Janeiro and north-eastern São Paulo, and two transversions, and no indels.

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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 NJ and 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 ± SD Tamura & Nei (1993) distance between X. pardalotus and X. fuscus was 12.366 ± 1.481%. The distance between X. f. fuscus and X. f. tenuirostris was 2.368 ± 0.561%, and between X. fuscus lineages N and S was 1.493 ± 0.400%. The net divergence between the two X. f. fuscus lineages was 1.228 ± 0.432%, which corresponds to 73 500 ± 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 ± 32 000 years) was based on the mean distance between groups (2.368 ± 0.561%).

**COALESCENCE SIMULATIONS**

Sequences within X. f. fuscus grouped in two main lineages that were strongly associated with specific geographical regions, suggesting that each lineage possibly evolved in allopatry. 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 whether 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 Ne used (P < 0.01); thus, the null model of a single population was rejected. Also, the empirical N, was 52 000 and is compatible with the range of N, (6000–100 000) used in the simulations.
PHYLOGEOGRAPHY OF *X. FUSCUS*

**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 geographical regions where the main lineages occur (north-eastern Minas Gerais plus southern Bahia, south-eastern Minas Gerais plus southern Rio de Janeiro, and south of the VPSR). A regional AMOVA without the samples from the *X. f. tenuirostris* lineage (north-eastern Minas Gerais and southern Bahia) was performed subdividing each main region in two subregions. The subregions of the first region are Minas Gerais and Rio de Janeiro, whereas the subregions 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 subregions within regions, and that 56.23% ($P < 0.01$) was allocated within subregions. The $\Phi_{ST}$ 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 geographical distance among pairs of localities (partial regression coefficient = 0.585, $P < 0.01$) and the geographical location (partial regression coefficient = 0.251, $P < 0.01$).

**Historical demography**

The $F_s$ value for *X. f. tenuirostris* is nonsignificant, 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. Because the $R_2$ test is more suitable for the analysis of small samples than the $F_s$ test (Ramos-Onsins & Rozas, 2002), we analysed *X. f. tenuirostris* ($N = 12$) using the $R_2$ test. The result was nonsignificant ($R^2 = 0.1800$, $P = 0.6960$), indicating that this taxa presented past demographic stability. Based on the estimations of $\tau$, the demographic expansions of the northern and southern lineages of *X. f. fuscus* started approximately 57,000 and 19,000 years ago, respectively.

**Discussion**

**Phylogeographical structure of *X. Fuscus* in the south-eastern 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, Figure 3. Neighbour-joining (NJ) tree based on 575 bp of the control region of *Xiphorhynchus fuscus*. The numbers at nodes show NJ and maximum likelihood bootstrap values above 50%, respectively. *Xiphorhynchus pardalotus* was used to root the tree. Haplotype identification: T1 to T7, *Xiphorhynchus fuscus tenuirostris* lineage; N1 to N15, *X. f. fuscus* northern lineage; S1 to S10, *X. f. fuscus* southern lineage.
Three main mitochondrial lineages were revealed in the *X. fuscus* populations studied. One lineage was associated with the subspecies *X. f. tenuirostris* and the others with the subspecies *X. f. fuscus* (Figs 1, 2, 3). Haplotypes of *X. f. fuscus* northern lineage were found from the Doce river basin in Minas Gerais to north-eastern 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 appear to have diverged in the late Pleistocene, around 130 000 years ago. The separation of the two *X. f. fuscus* clades was estimated to have occurred approximately 70 000 years ago. A contact zone between the two clades of *X. f. fuscus* was located to the south-west 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 suggests that there was a past range fragmentation somewhere between São Paulo and northern Paraná, and this needs to be tested in future studies with a more detailed sampling.

Even though studies on Atlantic forest organisms are scant, the biogeographical pattern observed in *X. fuscus* with three phylogeographical groups that appear to be limited by geographical landmarks or specific geographical 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 biogeographical divergence among north-eastern Minas Gerais, south-eastern Minas Gerais, and southwards regions (Mustrangi & Patton, 1997; Costa et al., 2000). In the gecko *G. darwinii* (sampled in the same geographical region studied here), Pellegrino et al. (2005) also found 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. darwinii* present similar phylogeographical patterns. 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 Mya) 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 the barriers generating the phylogeographical 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, which would favour secondary contact (Moritz et al., 2000). The samples that we had available did not permit these predictions to be tested in all the main river systems of the study area. For the area around the Paraíba do Sul river, the number of samples allowed us to start to explore this issue. Because the two *X. f. 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 appear to be a primary barrier for the gene flow of *X. fuscus*. Nevertheless, the limits of the distribution of the subspecies *X. f. tenuirostris* and *X. f. fuscus* appear 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.

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

<table>
<thead>
<tr>
<th>Clade</th>
<th>N</th>
<th>Fₛ⁺</th>
<th>Fₛ⁺ (P(Fₛ⁺))</th>
<th>Θ₀</th>
<th>Θ₁</th>
<th>Expansion dates</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Xiphorhynchus fuscus tenuirostris</em></td>
<td>12</td>
<td>−1.400</td>
<td>0.160</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td><em>Xiphorhynchus fuscus</em> N</td>
<td>46</td>
<td>−7.080</td>
<td>0.001</td>
<td>0.0010</td>
<td>7.0460</td>
<td>10 130–57 370</td>
</tr>
<tr>
<td><em>Xiphorhynchus fuscus</em> S</td>
<td>56</td>
<td>−3.789</td>
<td>0.015</td>
<td>0.0000</td>
<td>3415</td>
<td>4 690–19 460</td>
</tr>
</tbody>
</table>

N, sample size; P(Fₛ⁺), probabilities of the Fₛ⁺ statistic value being lower than the observed one based on 1000 coalescent simulations; Θ₀ and Θ₁, parameters of the model of exponential growth of Rogers & Harpending (1992); NA, not applicable. *Fₛ⁺* was calculated *sensu* (Fu, 1997).

ON THE ORIGIN OF THE PHYLOGEOGRAPHIC STRUCTURE OF X. FUSCUS

The partial regression analysis suggested that both isolation by geographical distance and the history of vicariance (geographical location) were important in shaping the population genetic structure of *X. fuscus*. The coalescence simulations supported the idea 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 latter divergence is focussed on below.

Evolution in isolation and secondary contact provides a possible explanation for the phylogeographical structure of *X. f. fuscus*. Two vicariant events could have separated the ancestral population: the geological 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 *Lepidocolaptes falcinellus*, Silva & Straube, 1996; *Helioletus contaminatus contaminatus* and *Helioletus contaminatus camargoi*; Silva & Stotz, 1992) and marsupials (i.e. *Marmosops paulensis* and *Marmosops 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) come into contact in the same geographical 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 for dispersion improved, they came into contact. The 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 from São Paulo (Albuquerque, 1996). The prediction regarding the age of the valley, however, is not accepted. According to Petri & Fulfaro (1983), the formation of the valley started in the Miocene-Pliocene (approximately 15 Mya) 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 do not support the formation of the current VPSR as the vicariant event that produced the two *X. f. fuscus* lineages.

Brown & Ab’Saber (1979) suggested that the forest cover along the VPSR was not discontinued in the last glacial period. Based on this hypothesis, Silva & Straube (1996) suggested that the putative forest fragmentation that resulted in the distribution pattern of *L. falcinellus* and *L. squamatus* could not be related to this historical climatic oscillation. Clapperton (1993), however, 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 the scenario of Clapperton (1993), the palaeorefuge 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. f. fuscus* lineages has a high proportion of derived haplotypes, especially from the northern lineage (i.e. haplotypes N1, N4, and N5; Table 1, 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 south-eastern 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 proposed that the modern forest cover of south and south-east 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 geographical 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 geographical barriers (VPSR) or rivers in the divergence between the two main mitochondrial lineages of X. f. fuscus of the south-eastern Atlantic forest. Instead, the data supported the influence of isolation by geographical distance, recent vicariance events, and demographic expansions in shaping the phylogeographical structure of X. fuscus. These vicariance and expansions events appear to be related to recent natural forest landscape dynamics. There are more than 1000 bird species in the Atlantic forest, many of them distributed along the same geographical range of X. fuscus and with comparable ecological requirements. It is plausible that the same forest dynamics that appear to have modelled the phylogeographical 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

The present study has revealed a strong phylogeographical structure in X. f. fuscus that defined two divergent populations in different geographical areas with dissimilar histories in the southern Atlantic forest: (1) south-eastern Minas Gerais plus southern Rio de Janeiro and (2) São Paulo plus southwards regions. As indicated by AMOVA, genetic differences between the two regions explained approximately 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 the 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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