Molecular Phylogenetics and Evolution 49 (2008) 760–773

Contents lists available at ScienceDirect

Molecular Phylogenetics and Evolution

journal homepage: www.elsevier.com/locate/ympev

Nuclear and mitochondrial phylogeography of the Atlantic forest endemic *Xiphorhynchus fuscus* (Aves: Dendrocolaptidae): Biogeography and systematics implications

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ARTICLE INFO

Article history:
Received 30 January 2008
Revised 5 July 2008
Accepted 19 September 2008
Available online 30 September 2008

Keywords:
Atlantic Forest
Caatinga
Phylogeography
Nuclear markers
Woodcreepers
Areas of endemism
Xiphorhynchus

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.

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, 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) (Veloso, 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 (Veloso, 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;

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doi:10.1016/j.ympev.2008.09.013

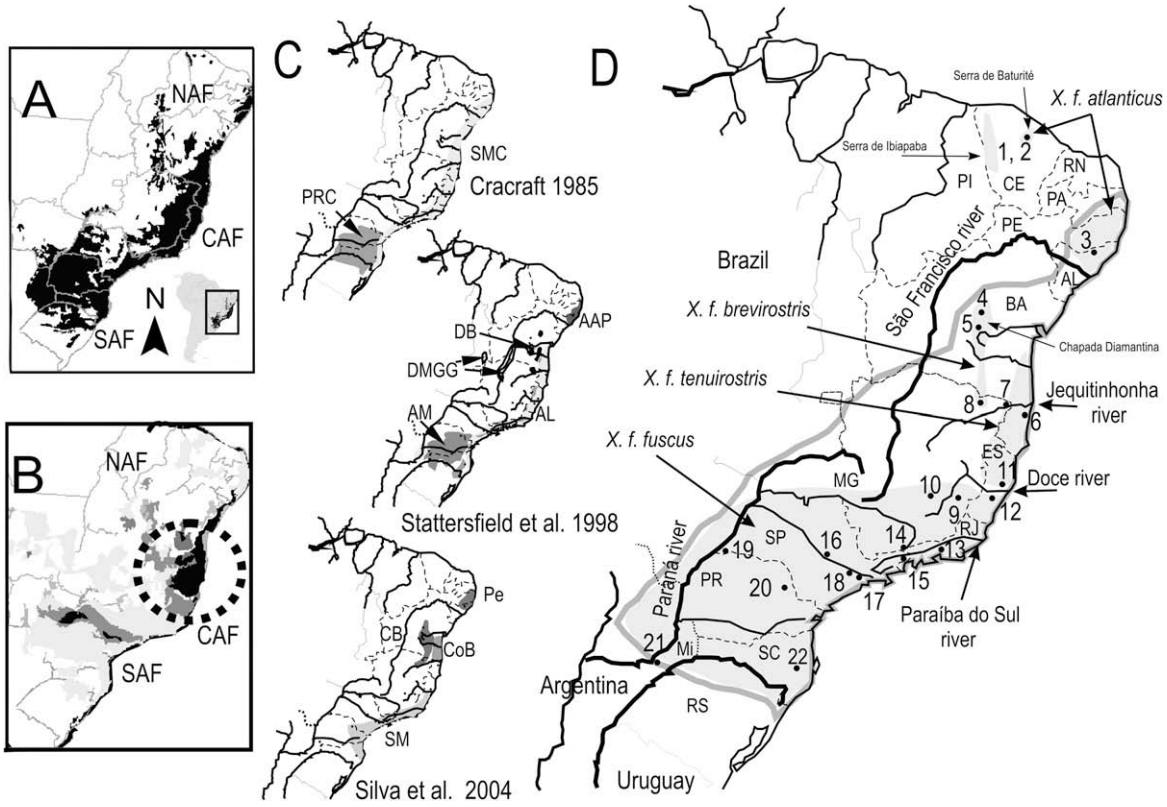

Fig. 1. Distribution of the Atlantic forest (AF), endemic bird areas, sampling locations and distribution of *Xiphorhynchus fuscus* and its subspecies. (A) Distribution of AF biome. NAF, northern AF; CAF, central AF; SAF, southern AF. (B) Historically stable areas for the AF during the last 21,000 years. Modified from Carnaval and Moritz (2008). The darker the area the higher the forest stability; i.e., black areas present 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 (Ridgely and Tudor, 1996; Marantz et al., 2003; Cabanne unpublished). Collection localities indicated by numbers (see Appendix B for details). States, PI, Piaui; 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).

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 darwinii* 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 endemicity (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.

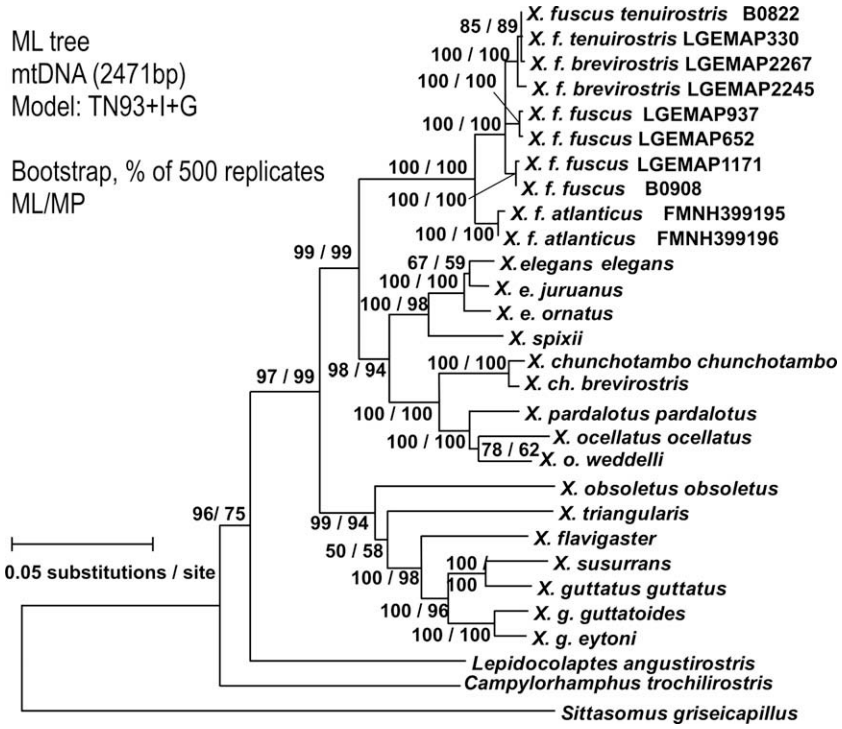

Fig. 2. Maximum likelihood (ML) tree based on 2471 bp of the cyt*b*, ND2 and ND3 (mtDNA, dataset 1). Model: Tamura and Nei (1993) + *I* (*I* = 0.6036) + *G* (α = 2.1493). Estimated base frequencies: *A* = 0.32, *C* = 0.33, *G* = 0.11, *T* = 0.24. Bootstrap values (percentage of 500 replicates) at the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

Xiphorhynchus 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, unpublished; 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, unpublished; 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.

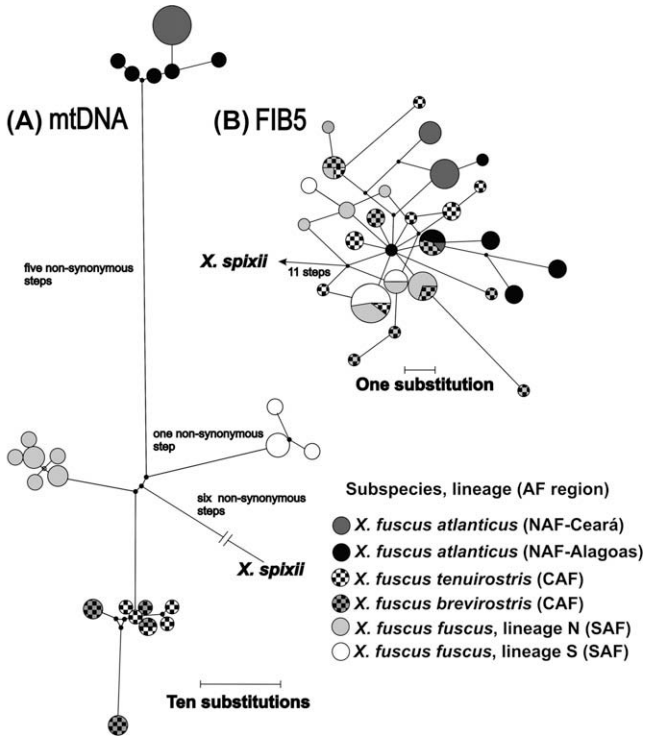

Fig. 3. Median joining networks based on (A) mitochondrial DNA of *X. fuscus* (2499 bp of concatenated cyt*b*, ND2 and ND3, dataset 2, *n* = 34 sequences) and on (B) FIB5 of *X. fuscus* (547 bp, dataset 2, *n* = 68 sequences). Number of aminoacidic changes are shown. See Table 1 for summary statistics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

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 trochilirosstris* and, to root the phylogeny, *Sittasomus griseicapillus*. The sequences for this dataset were: ~1000 bp of cytochrome *b* (cyt*b*), ~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 six samples of 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 Appendices 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 cyt*b*—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 Mc Donald 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 *Xiphorhynchus*. 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 for protein-coding sequences of the avian mtDNA and resolved numerically the Eq. (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

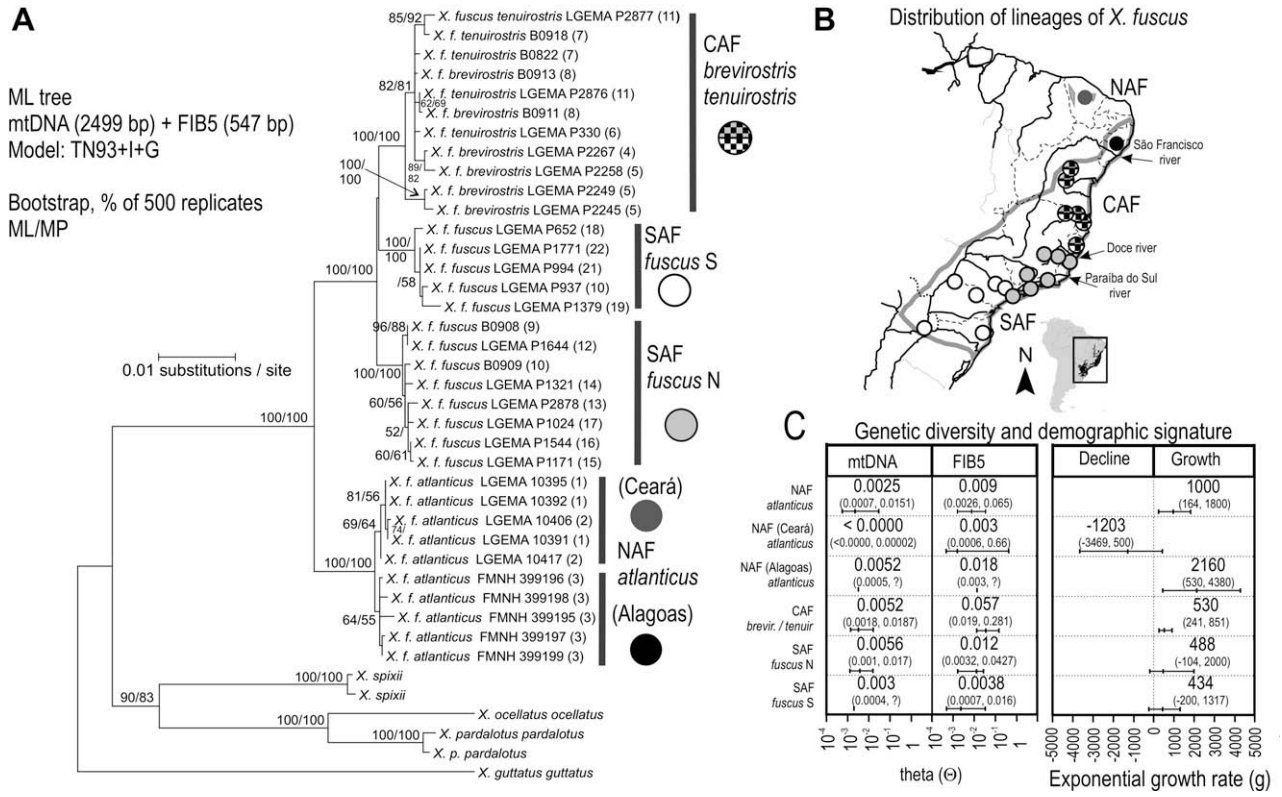

Fig. 4. Phylogenetic relationships, genetic diversity and demography of intraspecific lineages of *X. fuscus*. (A) Maximum likelihood (ML) tree based on a total evidence analysis of mtDNA and FIB5 (dataset 2, total alignment of 3046 bp, cyt*b*, ND2, ND3 and FIB5). Model of evolution: Tamura and Nei (1993) + *I* (*I* = 0.501) + *G* (α = 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*.

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 forest (dataset 1) and 4–4.6% 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 analyses 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

Table 1
Summary statistics of the mtDNA (concatenated cyt*b*, ND2 and ND3) and FIB5 sequences of the complete dataset and intraspecific lineages of *X. fuscus*

	X. fuscus, complete dataset 2		Lineages (geographic region)							
			atlanticus (NAF)		*brevirostris/tenuirostris* (CAF)		*fuscus* N (SAF)		*fuscus* 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
π%	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 sequences; *K*, number of haplotypes; π%, 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.

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 1 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 Φst 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 $M = (1 - \Phi\text{st})/2\Phi\text{st}$ (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 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 $r << 0.0001$).

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 *Xiphorhynchus* 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 0.4 to 0.8 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 and 0.15 MYr and suggested that migration

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

Hypothesis	$-\ln L$ constrained tree	delta $-\ln L$	P
X. fuscus 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

$-\ln L$ of the unconstrained tree is 7648.95470.
* (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 cyt*b*, ND2 and ND3) above the diagonal and standard errors below the diagonal

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

Geographic regions indicated between parentheses.
NAF, northern Atlantic Forest; CAF, central Atlantic forest; SAF, southern Atlantic forest.

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* × *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.

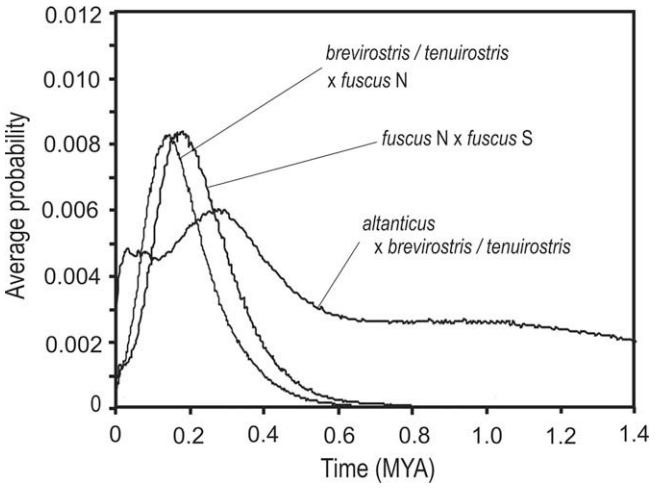

Fig. 5. Distributions of migration events for each pairwise comparison between geographically contiguous lineages of *X. fuscus* obtained from the IM program (Won and Hey, 2005), averaged across markers.

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 val-

Table 4
Population divergence times and migration between populations of *X. fuscus* based on the isolation–migration model of Hey and Nielsen (2004) and the model of LAMARC 2.0 (Kuhner, 2006)

Estimation	Divergence with gene flow, IM (90% c.i.)			Migration per marker, LAMARC (95% c.i.)		
	θ	t (MYr)	M	M_{FIB5}	M_{mtDNA}	$M_{mtDNA\text{-}4Ne}$
Inter lineages (AF region)						
1 [*atlanticus*] × [*brevirostris*/ *tenuirostris*] (NAF × 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 [*atlanticus*] × [*fuscus* N] (NAF × 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 [*atlanticus*] × [*fuscus* S] (NAF × 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 [*brevirostris/tenuirostris*] × [*fuscus* N] (CAF × 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 [*brevirostris/tenuirostris*] × [*fuscus* S] (CAF × 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 [*fuscus* N] × [*fuscus* 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 *atlanticus* [CE] × [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 *brevirosris/tenuirostris* [CH] × [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)

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.
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. $M_{mtDNA\text{-}4Ne}$, 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.

ues 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* (Figs. 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 *Xiphorhynchus* 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 and 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, the 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 highest 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 personal communication), 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 personal communication), 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. Event though populations are currently isolated, the migration-time graph showed recent gene flow for the pair *atlanticus–tenuirostris*/*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 (Omland et al., 2006). These results support the model of Fig. 1B, 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 he 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 km^2). 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, unpublished; 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 (Figs. 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 (se 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 are 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.

Acknowledgments

We thank A. Martensen, A. Uezu, J.P. Metzger, L.F. Silveira, M. Francisco, M. Maldonado-Coelho, A. Aleixo, M. Marini and the Field Museum of Natural History (J. Bates and D. Willard) for providing us with some tissue samples. We also thank IBAMA, Inst. Florestal (SP, Brazil), Inst. Estadual de Florestas (MG, Brazil), Min. de Ecología de Misiones (Argentina), and G. Patané for permits to collect samples. We are also grateful to AQUASIS (W. Silva, C. Albano and A. Campos), J. Albuquerque, R. Pessoa, I. Roesler, S. Seipke, F. Nodari, T. Matsumoto and E. Krauczuk for assistance during field work. D. Meyer, R. Pessoa, M. Kuhner, L. Smith and E. Rynes provided useful comments. We also thank the Associate Editor R. Tashian and two anonymous reviewers who helped to improve the article. This study was funded by FAPESP, FAPEMIG, CNPq, CAPES, PELD/MCT/CNPq and WWF-US. Part of this work was carried out by using the resources of the Computational Biology Service Unit from Cornell University which is partially funded by Microsoft Corporation.

Appendix A

GenBank accession numbers of sequences other than those of *Xiphorhynchus fuscus* used for phylogenetic analyses (see Appendix B for *X. fuscus* sequences)

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

Underlined accession numbers indicate sequences obtained from GenBank. All samples are muscle.
Cyt*b*, 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 accession numbers

Locality	Sub-species	*N*	Cracraft (1985)*	Stattersfield et al. (1998)*	Silva et al. (2004)*	Sample ID[TISSUE]	cyt*b*	ND2	ND3	FIB5
1 Mulungú, Serra de Baturité, Ceará (CE), 4°17′45′S, 38°55′W, 724 m	*atlanticus*	3	—	—	—	LGEMA10391[B]	EU073025	EU073030	EU073035	EU073040 EU073041
			—	—	—	LGEMA10392[B]	EU073026	EU073031	EU073036	EU073042
			—	—	—	LGEMA10395[B]	EU073027	EU073032	EU073037	EU073043

Appendix B (*continued*)

Locality	Sub-species	*N*	Cracraft (1985)*	Stattersfield et al. (1998)*	Silva et al. (2004)*	Sample ID[TISSUE]	cyt*b*	ND2	ND3	FIB5
2 Guaramiranga, Serra de Baturité, CE, 4°14′S, 38°56′W, 724 m	*atlanticus*	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	*atlanticus*	5	SMC	AAP	Pe	FMNH399195[M]	EF190601	EF190608	EF190645	EF190670 EF190683
						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	*brevirostris*	1	—	DB	CB	LGEMAP2267[M]	EF190597	EF190623	EF190660	EF190678
5 Lençois, BA. 12°25′S, 41°21′W, 520 m	*brevirostris*	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	*tenuirostris*	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	*tenuirostris*	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. *brevirostris*	2	SMC	DB	CB	B0913[B]	EF190581	EF190637	EF190643	EF190682 EF190694
						B0911[B]	EF190580	EF190636	EF190642	EF190678
9 Caratinga, MG. 20°50′S, 42°5′W, 55 m	*fuscus*	1	—	—	—	B0908[B]	EF190578	EF190634	EF190640	EF190674
10Nova Lima, MG. 19°59′S, 43°49′ W, 900 m	*fuscus*	1	—	—	—	B0909[B]	EF190579	EF190635	EF190641	EF190692
11Sooretama, Espírito Santo (ES). 19°3′S, 39°55′W, 50 m	*fuscus*	2	SMC	AL	CoB	LGEMAP2876[M]	EF190602	EF190629	EF190664	EF190695
						LGEMAP2877[M]	EF190603	EF190627	EF190665	EF190689 EF190691
12Santa Teresa, ES. 19°56′S, 40°34′W, 650 m	*fuscus*	1	SMC	AL	SM	LGEMAP1644[B]	EF190592	EF190618	EF190655	EF190676 EF190680
13Cantagalo, Rio de Janeiro (RJ). 22°S0′W, 42°33′W, 400 m	*fuscus*	1	SMC	AL	SM	LGEMAP2878[M]	EF190604	EF190628	EF190666	EF190689 EF190690
14Itatiaia, RJ. 22°24′S, 44°38′W, 800 m	*fuscus*	1	SMC	AM	SM	LGEMAP1321[B]	EF190589	EF190615	EF190652	EF190681
15Bananal, São Paulo (SP). 22°41′S, 44°19′W, 500 m	*fuscus*	1	SMC	AL	SM	LGEMAP1171[M]	EF190588	EF190614	EF190651	EF190692
16Barreiro Rico, SP. 22°38′S, 48°13′W, 550 m	*fuscus*	1	—	AL	SM	LGEMAP1544[M]	EF190591	EF190617	EF190654	EF190678
17São Roque, SP. 23°34′S, 47°09′W, 960 m	*fuscus*	1	SMC	AM	SM	LGEMAP1024[B]	EF190587	EF190613	EF190650	EF190692
18Morro Grande, SP. 23°42′S, 46°59′W, 800 m	*fuscus*	1	SMC	AM	SM	LGEMAP652[B]	EF190598	EF190624	EF190661	EF190692
19Morro do Diabo State Park, SP. 22°30′S, 52°18′W, 300 m	*fuscus*	1	—	—	—	LGEMAP1379[B]	EF190590	EF190616	EF190653	EF190688
10Wenceslau Braz, Paraná (PR). 22°51′S, 49°47′W, 800 m	*fuscus*	1	PRC	AM	SM	LGEMAP937[M]	EF190599	EF190625	EF190662	EF190681
21Campo San Juan, Misiones, Argentina. 27°22′S, 55°39′W, 150 m	*fuscus*	1	PRC	AM	—	LGEMAP994[B]	EF190600	EF190626	EF190663	EF190692
22Rancho Queimado, Santa Catarina, Br. 27°41′S, 49°2′W, 850 m	*fuscus*	1	PRC	AM	SM	LGEMAP1771[M]	EF190593	EF190619	EF190656	EF190692

FIB5 heterozygous individuals present two GenBank accesses, one for each chromosome.
Cyt*b*, 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.

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