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UNIVERSITY OF MISSOURI-ST. LOUIS
Department of Biology
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Evolution and biogeography of fire-eye antbirds (genus *Pyriglena*): Insights from molecules and songs

A Dissertation Submitted to The Graduate School of the University of Missouri-St Louis in Partial Fulfillment of the Requirements for the Degree Doctor of Philosophy in Biology

by

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GENERAL ABSTRACT

The importance of climatic and geologic factors as drivers of population differentiation and speciation in the Neotropical region has long been appreciated. However, many questions remain regarding their roles underlying the processes and patterns of diversification. Studies conducted in distinct regions containing a suite of geological and ecological conditions constitute ideal scenarios to assess the role of Pleistocene climatic changes, rivers, and mountain building as historical diversification mechanisms. In chapters 1 and 2, I used an integrative approach combining molecular phylogenetics, phylogeography and population genetics to elucidate the importance of climatic and geological factors as engines of diversification. I focused on the South American fire-eye antbirds (genus *Pyriglena*), which occur in forested areas in southeastern Amazonian basin, Pantanal basin, Andes and Atlantic Coast.

My study suggests that fire-eyes represent a young and rapid diversification in South America. It is conceivable that the origin of the major clades in this group trace back to the formation of the modern course of large rivers in the Amazonian basin and Atlantic Forest, with subsequent diversification fostered by more recent Pleistocene climatic oscillations creating opportunities for range expansion and geographic isolation in the Andes, Pantanal and Atlantic Forest. However, the role of large rivers as historical barriers to dispersal was apparently stronger in the Amazon basin relative to western South America (western lowlands and Andes) and Atlantic Forest. On the other hand, climatic oscillations seemed less important in creating opportunities for geographic differentiation within the Amazon comparative to the other regions. Fire-eyes seemed to have a complex history of diversification, involving large-scale geological and climatic processes acting over regional and continental scales during the last ~ 2.5 Mya.

In chapter 3, I examined in detail how songs varied across the range of Atlantic Forest fire-eye antbirds, and I tested several different hypotheses of the origin of song divergence in an attempt to explain their current vocal variation. Genetic differentiation and introgressive hybridization seemed to explain the overall song variation and geographic structuring in fire-eyes better than alternative factors such as body size, bill morphology and ecology.

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CHAPTER 1

Gene trees and historical demography of fire-eye antbirds (*Pyriglena*) of the Amazon basin: a test of the river and refuge models of biological diversification

1. Introduction

The role of historical factors in establishing patterns of geographic variation in diversity has been of interest for some time (Ricklefs & Schluter 1993; Rosenzweig 1995; Ricklefs 2006a). The latitudinal diversity gradient is the most striking example and various mechanisms have been proposed to explain the peak of species diversity in tropical regions (reviewed in Mittelbach *et al*. 2007), including regional differences in net diversification rates (Ricklefs 2006b; Weir & Schluter 2007). The tropics are considered to have higher diversification rates compared to temperate regions apparently because of higher rates of speciation in tropical regions (Mittelbach *et al*. 2007), although exceptions to this pattern have been reported (Weir & Schluter 2007). Unclear, however, are the relative roles of ecological, geological and climatic processes that might have elevated origination rates in tropical species lineages.

In the tropics, biological diversity is high in the Amazonian lowlands (Myers *et al*. 2000) and several hypotheses have been put forward to account for the origin (historical and biogeographical processes) and maintenance (ecological processes) of this diversity (Colinvaux 1996; Colinvaux *et al*. 1996; Haffer 1997a; Bates 2001). These ecological and historical hypotheses include the river (Wallace 1852; Sick 1967), refugium (Haffer 1969), gradient (Endler 1977; 1982), disturbance-vicariance (Colinvaux 1996; Colinvaux *et al*. 1996), and the palaeogeography hypothesis (mountain uplift and marine transgressions; Chapman 1917; Bates 2001). The relative importance of these hypotheses in explaining diversification of Amazonian organisms is contentious; the main criticisms stem from the fact that the same biogeographic patterns can be equally well explained by more than one hypothesis (Endler 1977; 1982; Tuomisto & Ruokolainen 1997) and because few contrasting predictions are available for, or can be derived from, the alternative hypotheses of speciation (Patton & Silva 1998; Moritz *et al*. 2000). One way to overcome this difficulty is through the application of integrative approaches that combine gene genealogies with analytical methods in the areas of population genetics and coalescent theory, which has allowed the falsification of certain hypotheses for some Amazonian lineages (*e.g.* Silva & Patton 1998; Lessa *et al*. 2003; Aleixo 2004; Cheviron *et al*. 2005).

Geographic mechanisms of speciation in Amazonia were first proposed in the 19th century (Wallace 1852) based on the observation that ranges of several closely related primates are separated by major Amazonian rivers (hereafter the river hypothesis). Modern versions of this hypothesis hold that ancestral populations were continuous across Amazonia and were spatially subdivided by the formation of large Amazonian rivers, with subsequent reduction or interruption of gene flow, leading, occasionally, to subspecific or specific differentiation of populations on opposite river banks (Capparella 1988; 1991; Haffer 1992; 1997). Several phylogeographic studies carried out in the Amazon basin have provided mixed support for this hypothesis. For example, studies carried out in western Amazonia have rejected the role of rivers as a significant barrier to gene flow among vertebrate populations (Patton *et al*. 1994; Lougheed *et al*. 1999; Aleixo 2004; Funk *et al*. 2007). In contrast, other studies have shown a south-north axis of genetic differentiation congruent with the position of rivers in this same region (Cohn-

Haft 2000; Aleixo 2004; Armenta *et al*. 2005; Cheviron *et al*. 2005) and a west-east phylogeographic break abutting large southeastern Amazonian rivers (Aleixo 2004), implying that rivers may be effective barriers to gene flow.

Geographic differences in the “river effect” were related to distinct geological histories and to topographic constraints in different parts of the Amazon Basin. For example, it has been postulated that extensive meandering of Amazonian tributaries that originate in the Andean slopes of western Amazonia could result in less of an isolation effect, compared to the rather straight eastern Amazonian rivers that flow down relatively steep slopes of the geologically stable Guianan and Brazilian shields (Raasanen *et al*. 1987; 1995; Webb 1995; Gascon *et al*. 2000; Haffer & Prance 2001; Aleixo 2004). Although it is clear that Amazonian geological history differs regionally, many questions remain regarding its reconstruction (Rosseti *et al*. 2005; Aleixo & Rossetti 2007; Rosseti & Toledo 2007). A recent geomorphological model (Rossetti & Valeriano 2007) proposed, for example, that the Tocantins River, one of the largest rivers in southeastern Amazonia, has had a recent dynamic geological history. This evidence suggests that a generalization on the role of rivers as drivers of vertebrate diversification in the Amazonian basin is still premature and requires additional consideration.

An alternative to the river hypothesis is the Pleistocene refuge hypothesis (Haffer 1969). It suggests that populations with continuous distributions became subdivided following forest contraction into multiple refugia during glacial maxima, leading to lineage splitting and species formation. Subsequently, these populations would have experienced range expansions during the warmer and more humid interglacial periods (Haffer 1969; 1997; Haffer & Prance 2001). Many aspects of the refuge model have been challenged in the Amazon region; some of the criticisms derive from studies that show forests may not have been as fragmented as hypothesized (Bush 1994; Colinvaux 1996; Mayle *et al*. 2004), that populations did not undergo suggested demographic changes (Lessa *et al*. 2003; Aleixo 2004), that estimates of the time since divergence among sister taxa predate the Pleistocene period (*e.g*. Hackett 1993; Hackett & Rosenberg 1990; Mustrangi & Patton 1997), and that a high degree of phylogeographic structure exhibited by some organisms over a relatively small geographic area (Patton *et al*. 1994) is inconsistent with an expected population expansion from a reduced forest refuge area (Lessa *et al*. 2003). At the same time, the influence of Pleistocene climatic changes as a diversification force were suggested to have been more pervasive on Neotropical mountain areas than in the lowlands (Weir 2006), but whether this a consistent pattern or merely an artifact of taxonomic biases has yet to be clarified (Bates & Demos 2001; see also Tobias *et al.* 2008). Despite the weak support for the refuge model, it is surprising, given its importance for the development of Amazonian biogeography, that only a few studies have explicitly tested the predictions of this hypothesis in a spatial-temporal framework (but see Lessa *et al*. 2003; Aleixo 2004).

In this paper, we used mtDNA sequence data to examine the genetic structure within White-backed Fire-eye (*Pyriglena leuconota*; hereafter referred to simply as fire-eyes) populations along the Tocantins River valley and to address two main questions concerning the evolutionary history of these populations. First, can either the river or refuge model explain the patterns of genetic variation observed in these populations? Second, do the patterns of genetic variation and the gene genealogies of these populations reflect the recently detected shift in the course of the Tocantins River?

1.1 *Study System: study area and geological and palaeoenvironmental history*

The Tocantins River is a long watercourse that spans a straight-line distance of over 2,000 km from the central high Brazilian plateaus near the city of Brasilia north to its junction with the Atlantic Ocean, near the city of Belém. Several smaller rivers and streams that constitute the headwaters are either bordered by gallery forests situated within the cerrado phytogeographic domain or are bordered by dry forests; lowland humid Amazonian forests are present along most of its middle and lower course. The area encompassing the Tocantins River valley is a good system for testing the river and refuge models of diversification because: (i) the Tocantins River is one of largest rivers in the Amazon basin; (ii) this river has been considered an important barrier for the dispersal of several bird species (Haffer 1992); (iii) forest refuges are hypothesized to have persisted on opposite banks of the Tocantins River during the last glacial maximum (Haffer 1969; Figure 1); (iv) this river delimits Amazonian lowland areas of endemism to the west (Belém) and to the east (Xingu) (Silva *et al*. 2005); and (v) the region in which the Tocantins River is located constitutes one of the few areas in the Amazonian basin that has been well documented from geological and sedimentological perspectives (Rossetti & Valeriano 2007). Therefore, the Tocantins provides a unique spatial and temporal framework to test the role of Amazonian river dynamics on genetic structure of vertebrate populations. In addition, the study area is characterized by a complex geological and climatic history. Geomorphological data demonstrate that the Tocantins River shifted its course to the northeast in response to tectonic reactivation, probably during the Pleistocene-Holocene (Rossetti & Valeriano 2007). From a palaeoenvironmental perspective, data indicate forest-savanna shifts during the last 30,000 years as a consequence of cycles of humid-dry periods in a central area of the Tocantins-Xingu interfluvium (Siffedine *et al.* 2001).

1.2 Predictions of Alternative Diversification Hypotheses

River hypothesis - Studies designed to test the river hypothesis have been criticized because of their failure to determine whether rivers had a primary role in the diversification of organisms or if they acted only as points of secondary contact (Patton & Silva 1998). The geographic distribution of *Pyriglena* fire-eye antbirds, with populations occurring in southeastern Amazon basin as well as in forests flanking the Amazon basin (Pantanal, Andes and Atlantic Forest), allows one to make this distinction. Fire-eyes constitute an ideal model to test the river hypothesis for two reasons. First, they have a widespread distribution in *terra-firme* forests in southeastern Amazonia. Second, a phylogenetic analysis based on a comprehensive geographic sampling of all populations of the entire genus revealed that fire-eye populations separated by the Tocantins River form a well-supported clade that is sister to a clade composed of the northeastern Atlantic Forest populations, whereas the population occurring in the Xingu-Tapajós interfluvium (*P. l. similis*) is sister to all western South America *Pyriglena* populations (Maldonado-Coelho *et al. in prep.*). Thus, we can rule out the possibility that fire-eyes studied here diversified in interfluvia other than the Tocantins. Accordingly, we can test three predictions of the river hypothesis (Haffer 1997a; Patton and Silva 1998; Cheviron *et al*. 2005; Funk *et al*. 2007) for the Amazonian populations of *Pyriglena* (Table 1): (1) haplotypes in opposite river banks will be reciprocally monophyletic, (2) genetic differentiation will be larger across the river rather than within the same interfluvium, taking into account the effect of geographic distance, and (3) if the Tocantins river had a primary role in subdividing fire-eye populations, the populations geographically close or bordering the river should not show evidence of demographic expansion.

Refuge hypothesis - This study covers populations inside one proposed large refuge area east of the Tocantins River (the Belém refuge; Figure 1 populations 10-13) and populations in areas outside both this refuge and a narrow and small unnamed refuge area along the eastern bank of the middle Xingu River (Figure 1; populations 1-9 and 14-18;see Figure 5 of Haffer 1969 for refuge locations), which was not sampled in the present study. If the current population genetic structure of fire-eyes was affected by fragmentation of forests into Pleistocene refuges as proposed by Haffer (1969), we would predict that (Table 1): (1) populations occurring in the Belém refuge area should possess higher levels of genetic diversity compared to populations in non-refuge areas (Figure 1). This prediction derives from theoretical (Nichols & Hewitt 1994; Ibrahim *et al*. 1996) and empirical (reviewed in Hewitt 2004a,b) studies, which show that episodes of range expansion have dramatic effects on the spatial pattern of genetic diversity; (2) divergence time congruent with the duration of the last glacial period (*i.e.* not older than 80,000 yrs. bp; Hooghiemstra *et al.* 2000) and shallow levels of genetic divergence representing isolation of populations in two refuge areas during the last glaciation (Hewitt 2004a); and (3) a signature of recent demographic expansion with a time corresponding to the period of forest expansion after the last glacial maximum (*i.e*. during the last 20,000 years; Hewitt 1996; Haffer 1997a). Importantly, if the magnitude of population bottlenecks were proportional to the size of the two postulated refuges, the population size increment should be larger on the western river bank (Figure 1).

Here, the criterion adopted to reject a hypothesis is whether there is a correspondence between biogeographical events and population processes. In other words, we will consider the hypothesized geological (river course shift) and climatic (forest fragmentation and expansion) processes to be consistent with the historical demographic processes (population subdivision and changes in population size) if they have overlapping confidence intervals.

2. Materials and methods

2.1 Geographical Sampling and Laboratory Molecular Procedures

We sampled one outgroup taxon (*P. leuconota pernambucensis*) and a total of 32 individuals from the eastern bank and 52 individuals from the western bank of the Tocantins River. From one to 16 individuals were sampled per site (for details on sampling locations, population sample size and voucher information see Figure 1, Table 3 and Appendix A).

Mitochondrial DNA from tissue and dry skin samples of specimens collected during the last 25 years was extracted using a Qiagen tissue extraction kit (QIAGEN, Inc.). Contamination risk of the museum samples was taken into account by extracting the DNA in a different room reserved for handling only bird skin samples and by always performing PCRs with negative controls. For all the tissue and some museum samples, we sequenced all or most of the NADH dehydrogenase subunit 2 (ND2; 1041 bp) in two fragments by using the primer pairs L5219/H5766 and L5758/H6313 (Johnson & Sorenson 1998). The ND2 gene from degraded skin samples was amplified in four fragments using the primer pairs described below, or by a nested PCR process. DNA amplification via nested PCR was performed by first sequencing the whole ND2 using the primers L5219 and H6313 followed by a second PCR using the product of the first PCR as a template. In this second PCR, we used primer pairs that included some internal primers, designed specifically for this project, to amplify shorter fragments: L5219 and HND2P1A (5’-GGTGGGTGAGTTGGGTAATG-3’) or HND2P1B (5’GCACCTTGGAGAACTTCTGG-3’); H5766 and LND2P2A (CATCGAGGCCACAACAAAAT) or LND2P2B (5’-AAAATCTCACCACCCACGAG-3’); L5758 and HND2P3 (5’-GGCAATGATTGTTGCTGTTG-3’); H6313 and LND2P4 (5’CTCCATTAACGGGCTTTCTG-3’).

PCR profiles included an initial 2 min denaturation cycle at 95°C, followed denaturation at 94°C for 45s, annealing varying from 46 to 52°C for 45 s, with a final extension of 1 min at 72°C. This was followed by 39 cycles of denaturation at 94°C for 45 s, annealing varying from 46 to 52°C for 45 s and extension at 72°C for 1 min, finishing with an additional extension at 72°C for 10 minutes. When multiple DNA bands were obtained, products were electrophoresed in low-melting point agarose gels stained with ethidium bromide, excised from the gels, and purified using QiaQuick PCR Kit (Qiagen, Inc.). Clean products were used as templates for sequencing both light and heavy strands. DNA sequencing was carried out with BigDye v 3.0 Dye Terminator Cycle Sequencing Kit (Applied Biosystems, Inc.), and the same primers used for amplification. Cycle sequencing reactions were purified with an ethanol-sodium acetate solution and run on an ABI 3100 automated sequencer. Sequences were assembled and edited using the program SeqMan (DNAstar) and aligned by eye. The following measures were taken to ensure that the amplified DNA fragments did not include pseudogenes of nuclear origin: 1) inspection for deletions, insertions and stop codons that would result in a nonfunctional protein, 2) confirming a high transition rate at third codon positions and 3) confirming a high transition to transversion substitution ratio characteristic of mitochondrial DNA (Arctander 1995; Sorenson & Quinn 1998).

2.2 Phylogenetic Analyses

Phylogenetic relationships among unique haplotypes based on the ND2 mtDNA gene were assessed with maximum parsimony (MP) and maximum likelihood (ML) using PAUP v4.0 (Swofford 2002) and Bayesian inference using MrBayes v3.1.2 (Huelsenbeck & Ronquist 2001; Ronquist & Huelsenbeck 2003). Maximum parsimony analyses were performed using a heuristic search, 100 random addition replicates and tree-bisection-reconnection (TBR) branch-swapping algorithm. Nodal support was assessed using non-parametric bootstrap (Felsenstein 1985) with 1,000 replicates. The best-fit model of nucleotide substitution for ML and Bayesian analyses was selected using Modeltest v3.7 (Posada & Crandall 1998) and MrModeltest v2.22 (Nylander 2004), respectively, based on the Akaike Information Criteria (AIC). The K81uf + G + I (Kimura model with gamma distributed rate variation among sites and a proportion of invariant sites) model was selected for the ND2 gene data. Bayesian analyses were performed with four heated and one cold Markov chains for 6,000,000 generations, with the first 100,000 generations discarded as a burn-in.

2.3 Population Genetic Analyses

2.3.1 Haplotype network, AMOVA and patterns of genetic diversity

Given the problems associated with reconstructing relationships among recently diverged haplotypes (Posada & Crandall 2001), we also inferred the relationships among haplotypes through networks using the statistical parsimony program TCS v.1.2.1 (Clement *et al*. 2000). Ambiguous connections among haplotypes in the network were solved following coalescent theory predictions as outlined in Crandall and Templeton (1993).

We tested for the effect of the Tocantins River on the structure of genetic variation of fire-eyes populations that contained more than one individual using an Analysis of Molecular Variance (AMOVA; Excoffier *et al*. 1992) as implemented in Arlequin (version 3.1, Schneider *et al*. 2000). Apportionment of the genetic variation was broken down among three hierarchical levels: among populations from opposite river banks, among populations on the same river bank and among individuals within populations. Significance tests were based on 10,000 random permutations of the data set.

Haplotypic (*h*) and nucletotide (π) diversity were calculated for population samples that contained more than one individual as described in Nei and Kumar (2000), using Arlequin (Schneider *et al*. 2000). Uncorrected pairwise distances among haplotypes are presented as mean ± SD.

2.3.2 Historical Demography

Because simulation studies have shown that the F*s* (Fu 1997) and R_2 (Ramos-Onsins & Rozas 2002) tests are statistically more powerful than other tests (Ramos-Onsins & Rozas 2002), we use these tests to assess whether the populations would fit a population-stationary or a population-expansion scenario using DnaSp (Rozas *et al*. 2003). Estimated values for these tests were compared to an empirical distribution based on 10,000 coalescent simulations assuming an infinite-sites model and a large population size. Significant *P* values (<0.05) were taken as evidence for departure from a model of constant population size in favor of an alternative scenario of demographic expansion. The generalized non-linear least-squares approach of Schneider and Excoffier (1999) implemented in Arlequin, was used to estimate the parameters of past demographic expansion from the mismatch distribution (Rogers & Harpending 1992) for those populations that showed significant departures from the null hypothesis of constant size. The estimated parameters were: Θ_0, the theta value before population expansion (where $\Theta_0 = 2N_e u$, where N_e is the female effective population size and u is the mutation rate); Θ_1, the theta value after population expansion and τ (where $\tau = 2ut$, where t is the time since the expansion in generation units). Confidence intervals (95%) were estimated for the parameters Θ_0, Θ_1, and τ through a parametric bootstrap with 10,000 re-samplings.

The estimated values of the tau (τ) parameter were converted into years-before-present by solving $t = \tau/2u$, where u is the mutation rate per sequence per generation (Rogers & Harpending 1992; Rogers 1995). Here, we used two mutation rates: the widely assumed mutation rate of 2.1 % per site per million years (reviewed in Weir & Schluter 2008) and the mutation rate estimate of 4.0% per site per million years of mitochondrial ND2 from the Galapagos mockingbirds (Arbogast *et al*. 2006). We assumed a generation time of 2.33 years, determined as $T = \alpha + [s/(1 - s)]$, where α refers to the age at which first breeding of females occurs and s is the expected adult survival rate (Sæther *et al*. 2005). One year was used for α as suggested for fire-eyes by Willis (1981) and 0.57 was used for *s* based on the average annual survival rate across ten typical antbird species in lowland Ecuador (Blake & Loiselle 2008).

Although Fu's F*s* and R_2 statistics provide evidence of historical changes in population size, these methods only provide point estimates that average out the whole history of the population and, therefore, do not allow inferences of population size fluctuations through time. Thus, we constructed Bayesian Skyline plots in BEAST v1.4.6 (Drummond & Rambaut 2007) to estimate historical changes in population size over time. This method uses Markov Chain Monte Carlo (MCMC) sampling techniques to estimate the posterior distribution of effective population size given a set of aligned DNA sequences and a model of molecular evolution, taking into consideration uncertainty in the genealogical process (Drummond *et al.* 2005). We used the best-fit model of molecular evolution selected by Modeltest. This analysis was run for 2×10^8 generations with model parameters and genealogies sampled every 1,000 generations under a strict molecular clock, of which the first 10% were discarded as a burn-in. Skyline plots were constructed using Tracer v1.4 (Rambaut & Drummond 2007).

2.3.3 Divergence Time and Migration Rates

We estimated divergence time between populations of fire-eyes west and east of the Tocantins River using two different approaches. First, we estimated time-to-most-recent-common-ancestor (TMRCA) using a Bayesian approach incorporated in the program BEAST. This analysis samples the TMRCA values from the posterior density distribution generated by MCMC simulations. Since substitution rate variation among lineages can be substantial, independent of the divergence time frame under consideration (Arbogast *et* *al*. 2002), we first estimated divergence times using a relaxed clock. This method infers the date of origin for the lineages without relying on a molecular clock and considers uncertainty in branch length and tree topology (Drummond *et al*. 2006). Our preliminary runs estimated that the coefficient of variation of the mean branch rate variation was larger than one, indicating substantial rate variation among branches (Ho *et al*. 2005; Drummond *et al*. 2006; 2007). For comparative purposes, however, we estimated divergence times between fire-eye populations of western and eastern riverbanks assuming both a relaxed and strict molecular clock. We ran the MCMC under the K81uf + G + I model of nucleotide substitution and assuming a constant population size. Overestimation of divergence can potentially occur when interspecific mutation rates are used to estimate intraspecific divergence events (Ho 2007); thus we employed here both the interspecific rate of 2.1% (1.0 x 10^{-8} substitutions per site per lineage per year) and the intraspecific rate of 4.0 % (2.0 x 10^{-8} s/s/y) divergence per MYA to estimate time of divergence. A total of four analyses were performed and each analysis consisted of one model type (clock constrained or unconstrained) and one mutation rate (two types). For each analysis, the three independent runs of 10,000,000 steps were combined to obtain an estimate of the posterior distribution. For each model type and mutation rate analysis, three independent runs were performed and combined, using 10,000,000 generations, discarding the first 1,000,000 as burn-in, and sampling parameter values every 1,000 generations. Populations on opposite river banks were constrained as monophyletic in this analysis (see Shimodaira-Hasegawa test below). In each independent run, we inspected for convergence of the chain to the stationary distribution using the program Tracer.

Second, we used the Bayesian coalescent method developed by Hey and Nielsen (2007) implemented in the program IMa to simultaneously estimate population divergence time (t = Tu; where T is time in units of years and u is the mutation rate per gene) and migration rate (M = 2Nm; where m is the migration rate per gene copy). IMa is able to distinguish between the retention of ancestral polymorphism and recent gene flow assuming no further population subdivision within the diverging groups of populations. We ran multiple initial runs assuming different priors to assess whether convergence in the modes of posterior distribution was being reached. Three final runs with identical conditions and different random seeds were performed using prior distributions empirically obtained from the initial runs and always choosing upper bounds values that were not included in the flat tail of the initial distributions (Won & Hey 2005). We ran each run for 2×10^7 generations with a burn-in of 200,000 steps, always checking during and at the end of each run the autocorrelation values, absence of trends in trendline plots and that effective sample sizes (ESS) among parameter values are at least 50 throughout the run (Hey & Nielsen 2004; Hey 2007). The peaks of the posterior distributions were taken as the estimates of the parameter values. Here, we estimated divergence time using the divergence rates of 2.1% and 4% per million years.

2.4 Partial Mantel Test

If the Tocantins River were a long-term historical barrier to gene flow, genetic distances between populations on opposite river banks would be greater than genetic distances between populations on the same side of the river. However, ongoing geographically-structured gene flow, as predicted by an isolation-by-distance model (IBD; Hutchison &

Templeton 1999), could also account for genetic variation among fire-eye populations and would potentially confound evaluation of this historical model. Here, we use partial Mantel tests (Legendre & Legendre 1998; Manly 2007), to decompose the relative contributions of the long-term historical effect of the Tocantins River versus IBD in explaining the genetic structure of fire-eye populations. In addition, we also evaluated the interaction between the historical and IBD processes (Telles & Diniz-Filho 2005). These analyses were performed considering the historical and IBD processes as predictors of genetic distance both separately, in simple Mantel tests, and combined in a multiple Mantel regression design, using the three following matrices: 1) a matrix of pairwise corrected genetic distances, 2) a matrix of pairwise geographic distances as a surrogate for IBD and 3) a pairwise binary matrix coding the position of populations relative to the river as reflecting the historical hypothesis (populations located on the same river side as 0 and populations on different river sides as 1). Geographic distances were measured as straight-line distances between populations. Geographic distances were log-transformed *prior* to the analyses because the relationship between genetic distance and geographic distance was not linear.

We estimated genetic distances among populations correcting for within-population sequence divergence using the standard function pAB(corrected) = pAB - 0.5(pA + pB), where pAB is the mean sequence divergence between populations A and B and pA and pB are the mean sequence divergences within populations A and B (Avise & Walker 1998). We used Arlequin to estimate the corrected pairwise genetic distances. Significance values of the partial Mantel correlations were obtained by 10,000 permutations, using the software FSTAT (Goudet 1995).

3. Results

3.1 DNA Sequence Variation

Ingroup mtDNA base frequencies were similar to those found in other bird species, with an overall deficit of guanines (Johnson & Sorenson 1998): A = 0.318, C = 0.309; G = 0.091; T = 0.282. A total of 17 and 14 unique haplotypes were recovered from the western and eastern sides of the Tocantins River, respectively; none of the haplotypes were shared between eastern and western banks of the river. A total of sixteen (1.5%) characters were parsimony informative.

3.2 Phylogenetic Analyses, Haplotype Network and Genetic Structure in Fire-eyes

The ML analyses for the ND2 data set produced a moderately supported clade composed uniquely of haplotypes from the western bank of the Tocantins River, while the relationships among haplotypes recovered from the eastern river bank could not be resolved (Figure 2). The MP tree (not shown) was similar to the tree generated by the ML analysis. The relationships between all the eastern and western haplotypes could not be resolved by the Bayesian analysis (not shown). To test if the reciprocal monophyly scenario predicted by the river hypothesis could be rejected, a Shimodaira-Hasegawa (SH) test (Shimodaira & Hasegawa 1999) with full optimization and 10,000 bootstrap replicates was performed in PAUP between the recovered ML tree ($-\ln L = 1790.6$) and the most-likely tree from a search in which haplotypes in either river bank were constrained as monophyletic ($-\ln L = 1794.0$). The SH test indicated that the reciprocal monophyly scenario cannot be rejected ($P = 0.323$).

Results from the statistical parsimony network show that haplotypes from opposite river banks separate in two distinct groups (Figure 3). Haplotypes from these two groups were separated by two mutational steps, and many haplotypes found on the same river bank were recovered in multiple populations, indicating a lack of strong within-basin phylogeographical structure (Figures 1 and 3). The mean pairwise uncorrected sequence divergence among unique haplotypes recovered on the western side of the river was 0.0033 ± 0.0014 (range 0.00096-0.0064) and similar to the value estimated for the eastern side (0.0031 ± 0.0013; range 0.00096-0.0058). Mean pairwise uncorrected distances were, however, larger when unique haplotypes from opposite river banks were compared (0.0076 ± 0.0016; range 0.0039-0.0110). AMOVA showed that most of the variation (71%) in mtDNA observed in populations of fire-eyes along the Tocantins River valley was partitioned among eastern and western riverbanks (Table 2). The indices of haplotype diversity were generally low and uniform for fire-eye populations sampled throughout the Tocantins River valley. Indices of nucleotide diversity also had low values but varied considerably geographically (Table 3).

3.3 Historical Demographic Analyses

The Ramos-Onsins & Rozas' (2002) R_2 test did not detect evidence of historical changes in population size for fire-eyes throughout the Tocantins River valley, although a signature of historical population expansion was detected by Fu's (1997) test in two western river bank localities (localities 5 and 7; Table 3, Figure 1). For these localities, the generalized non-linear least-squares approach of Schneider & Excoffier (1999) yielded distinct estimates of mean dates of population expansion time (τ) for the two mutation rates (Table 4). These point estimates correspond to historical demographic expansion in years before present of about 12,804 and 26,950 for locality 5, and 16,752 and 35,260 for locality 7 (Table 4). The effective sample sizes (ESS) for each of the two Bayesian Skyline Plots were larger than 200, suggesting that the MCMC mixed properly and that the number of generations was sufficient to infer historical size changes of fire-eye populations on both river sides. Although the credibility intervals of the Bayesian estimates were wide, the analyses showed trends that indicate either growth or stable population sizes. However, these trends were not consistent with the expectation of a large population increase in the western river bank after the onset of humid periods as would be predicted by the refuge hypothesis (Table 1, Figure 4).

3.4 Divergence time and Migration Rates

Coalescent simulations implemented in the program BEAST to estimate the TMRCA under relaxed and strict clock models resulted in proper mixing of the MCMC chains, as determined by the program Tracer. Assuming a strict molecular clock, the initial divergence of fire-eyes across the Tocantins River seems to have occurred during the Pleistocene (0.01-1.8 mya). TMRCA estimates were 0.476 million years ago (mya) (95% HPD: 0.298-0.834) and 0.272 mya (95% HPD: 0.148-0.416), assuming mutation rates of 2.1% and 4% per million years, respectively. Estimated values for the relaxed clock were substantially larger, and placed the initial divergence across the Tocantins River in the late Pliocene and early Pleistocene, as divergence time and associated credibility interval values were 2.58 mya (95% HPD: 0.266-7.044) and 1.16 mya (95% HPD: 0.146-3.154), corresponding to divergence rates of 2.1% and 4%, respectively.

Posterior distribution for population divergence time (Figure 5a) and migration (Figure 5b) across the Tocantins River were estimated from IMa. The posterior distribution of population divergence time *t* peaked at 1.86 (95% HPD: 0.89-8.94), resulting in divergence estimates across the river of 0.179 mya (95% HPD: 0.085-0.859 mya) and 0.083 mya (95% HPD: 0.040-0.399 mya), assuming the divergence rates of 2.1% and 4% per million years, respectively. These estimates suggest that population divergence across the river occurred during the late-Pleistocene. A migration rate of zero into both east and west sides of the river cannot be rejected, indicating that no or negligible gene flow occurs across the river. Indeed, likelihood ratio tests applied to evaluate the fit of nested models within the full IMa model could not reject models assuming no gene flow across the river (results not shown).

3.5 Partial Mantel Test

The results of the partial Mantel test show that the Tocantins River has been an effective historical barrier to gene flow for fire-eye antbirds. We found that 89.3% of the variation in corrected pairwise genetic distances can be explained by the combined effects of long-term historical isolation on opposite river banks and isolation-by-distance (IBD). However, after separating the effects of these two processes, 71.9% of genetic variation among fire-eye populations was due to the historical (river) effect, independent of IBD, while only 0.02% of the variation can be explained by IBD alone, independent of long-term historical processes. There was no correlation between corrected genetic distances and geographic distances (Figure 6), as would be predicted by the IBD model. Rather, for similar geographical distances among populations, opposite river bank comparisons always had a higher corrected genetic distance than comparisons from the same river bank.

4. Discussion

Evolutionary biologists have long been interested in unraveling the historical and ecological factors underlying the elevated biological richness of the Neotropical region, factors that are central to explaining patterns of species diversity at a regional and global scale. This issue is especially relevant in the Amazon basin, where species diversity is high but where few adequate tests of hypotheses have been put forward to explain this diversity. In this study, we tested predictions of the river and refuge hypotheses, two of the main evolutionary processes that have been proposed as engines of diversification for Amazonian vertebrate fauna. Despite the controversy surrounding these hypotheses, the population genetics framework adopted here allow us to draw with confidence some conclusions and reject predictions of these hypotheses.

Testing the Predictions of the River Hypothesis

We proposed to test three key specific predictions of the river hypothesis. Our data partially supported predictions 1 and 3 and fully support prediction 2 (Table 1). Prediction 1 is partially supported since the recovered ML tree is not statistically incongruent with the presence of two sister clades across the river. However, paraphyly of eastern river bank haplotypes relative to western haplotypes cannot be discounted. In fact, population splitting across the river seems to be a recent event and thus we are unable at the moment to provide a complete distinction of competing phylogenetic and historical hypothesis (*e.g.* Neigel & Avise 1986; Patton & Smith 1994, Patton & da Silva 2005). Thus, a paraphyletic scenario could be reconciled with either an over water dispersal into the western Tocantins river bank or by an eastward Pleistocene shift in the river course (as recently proposed by Rossetti & Valeriano 2007). Over water dispersal is, however, less likely since some populations in the western river bank show no signature of demographic expansion (see below).

Prediction 2 is supported by the AMOVA, the migration estimates in IMa and the presence of eastern and western river bank haplotype groups in the statistical parsimony network, indicating that the Tocantins River was an important historical factor shaping the phylogeographical structure in fire-eye populations. This prediction is also supported by the results of the partial Mantel test, implying that clinal patterns of gene flow among populations cannot explain the observed genetic variation. All results of R_2 and some of the Fu's F_s did not show any signature of historical demographic change (prediction 3), as would be expected if the river was merely a secondary barrier. However, the Fu's F_s test for one locality geographically close to the river (locality 5) did not reject a scenario of recent population expansion and, therefore, is inconsistent with the river hypothesis.

The view that large rivers act as effective barriers to dispersal of vertebrates inhabiting opposite river banks in the Amazonian lowlands was suggested long ago (Wallace 1852). For many parts of Amazonia, however, the influence of rivers on gene flow and speciation of many taxa has been challenged (*e.g*. Sick 1967; Haffer 1992; Patton & Smith 1998). In fact, results from previous studies indicate that no generalization about the role of rivers as primary drivers of diversification for the vertebrate fauna in the whole Amazon basin is tenable. In western Amazon, for example, several phylogeographic studies have shown that some rivers are significant barriers to gene flow for terrestrial vertebrates (Cohn-Haft 2000; Aleixo 2004; Armenta *et al*. 2005; Cheviron *et al*. 2005; Funk *et al*. 2007) although exceptions to this pattern have been found (Patton *et al*. 1994; Patton and Silva 1998; Lougheed *et al*. 1999; Aleixo 2004). In southeastern Amazonia, major lineage splits of a passerine bird coincide with both Tapajós and Xingu rivers, but not with the Tocantins River (Aleixo 2004), and a study in the headwater of the Tapajós River found a significant river effect for some but not all forest bird species considered (Bates *et al*. 2004). Genetic divergence across the Tocantins River was detected for one monkey species (Vallinoto *et al*. 2006), but a primary role of this river for population differentiation cannot be claimed, given the gene phylogeny. Although we cannot determine whether the Tocantins River imposed itself on an ancestral fire-eye geographic range (concordant with a reciprocal monophyletic scenario) or if an episode of across-river transfer of fire-eyes individuals took place at some point in time (concordant with a paraphyletic scenario), our study suggests that this river had an important historical role on fire-eye population differentiation.

Testing the Predictions of the Refuge Hypothesis

We tested three predictions derived from the refuge hypothesis. We found no support for predictions 1 and only partial support for predictions 2 and 3 (Table 1). Prediction 1 was not supported since variation in genetic diversity was not geographically structured as predicted by this hypothesis. However, this prediction relies on the assumption that range expansion was rapid with frequent long distance dispersal events, a process that would involve bottleneck events and lead to a reduction of genetic diversity (Ibrahim *et al*. 1996; Hewitt 1996). Modes and rates of dispersal for fire-eyes are unknown and a further evaluation of this assumption should wait for additional data.

Shallow levels of genetic divergence were found and some population divergence times and IMa estimates between the two main haplotype groups recovered in the haplotype network are consistent with the temporal scenario posited by the refuge model (prediction 2). Assuming that glacial periods lasted approximately 60,000 years (Hooghiemstra *et al*. 2000), divergence estimates based on the assumed mutation rate of 4% (Arbogast *et al*. 2006) could be reconciled with the timing predictions of the refuge hypothesis.

In spite of some support for the refuge hypothesis, the pattern of historical demographic stability of sampled populations in the western bank of the river (localities 1, 2 and 4) as given by the Fu's F_s test, the lack of any signal of historical demographic change detected by the Ramos-Onsins & Rozas' R_2 test and the absence of a population expansion of the magnitude of the refuge area hypothesized for the western river side, refute the refuge hypothesis (prediction 3). However, confidence intervals of Schneider & Excoffier's (1999) method supports partially prediction 3, as the timing to expansion of some populations outside refuge areas on the western river bank were congruent with the onset of warmer periods following the last glacial maximum.

Studies based on fossil pollen records (Colinvaux *et al*. 2000; Bush & Oliveira 2006) and molecular data (Lessa *et al*. 2003; Aleixo 2004) have challenged the view that the Amazonian biota evolved in response to isolation in forest refuges during the late Pleistocene. Recent opinion holds that most of central Amazonia remained forested during the last glacial maximum (reviewed in Mayle *et al*. 2004). In the Tocantins River valley, Haffer (1969) suggested that forest populations contracted into a large refuge area east of the river and into a small refuge area along the eastern bank of the middle Xingu

River as a result of the expansion of dry climates during the last glacial maximum (Figure 1). Although some of the methods employed here cannot reject the hypothesis of population expansion, our analyses do reject the key prediction of population decline derived from Haffer's hypothesis. This decline in population size would be expected mainly on the eastern bank, given the hypothesized small forest refuge area. Thus, the null hypothesis that populations have been stable or even growing cannot be rejected in favor of the hypothesis that they have experienced drastic reduction in their effective population size, as would have been expected if range contraction into forest refuges occurred.

Estimated Divergence Times

Population divergence times based on the IMa estimate assuming mutation rates of 2.1%, and 4% per million years indicate divergence across the Tocantins River of 0.179 mya and 0.083 mya (*i.e.* not earlier than the late Pleistocene). For the TMRCA, estimates varied from as young as 0.272 mya to as old as 2.58 mya from BEAST. Our estimates, however, are not compelling since these coalescent methods rendered divergence time estimates with substantial uncertainty. Thus, it would be difficult to specify any particular event as being the underlying cause of fire-eye population differentiation. For example, it is possible to reconcile the temporal pattern of divergence documented here for fire-eyes across the Tocantins River with a geological scenario recently proposed for the formation of the modern Tocantins River, whereby it shifted its course eastward during the Pleistocene or Holocene (Rosseti & Valeriano 2007). However, confidence intervals of the BEAST relaxed clock estimate assuming a mutation rate of 2.1% per mya cannot discard the possibility that differentiation of fire-eye populations initiated during or since the formation of an ancient Plio-Pleistocene Tocantins River course west of its modern course (Rosseti & Valeriano 2007).

Part of the uncertainty of our divergence time estimates likely reflects the limitations inherent to inferring population history from recent biogeographic events and from a single locus. This may be the case in our study, as the splitting pattern between western and eastern populations is very shallow and TMRCA estimates invariably surpass population divergence time (T_{pop}) estimates. Increasing the number of loci in the analysis might allow an assessment of how much of the total gene divergence between populations actually results from divergence since population separation (Edwards & Beerli 2000; Arbogast *et al*. 2002; Knowles and Maddison 2002, Carstens & Knowles 2007), which may decrease uncertainty in the estimates and allow the rejection of alternative biogeographic scenarios with more confidence. However, nuclear loci show larger effective population size and deeper coalescent times (Palumbi *et al*. 2001) and, thus, represent a major difficulty for inferring shallow population history with confidence.

Evidence for the River-Refuge Model of Diversification?

Although we have shown that it is unlikely that fire-eye populations have undergone a population decline through range contractions into refuge areas, as envisioned by Haffer (1969), regional and local fluctuations in population size might still have been of historical importance in southeastern Amazonia, likely involving populations geographically close to the southern edges of the forest. Our analysis suggests that population bottlenecks of the magnitude predicted by the refuge model do not seem to have occurred over the fire-eye history, but is not entirely clear that population expansion did not occur. This lack of clarity is a result of conflicts among different historical demographic estimators and wide confidence intervals in coalescent inferences. If population expansion is to be confirmed, it could imply that regional range expansion could have occurred recently to limited extent, most likely in the southern edge of the forest. Thus, support for an additional model of diversification, not necessarily mutually exclusive of the refuge and river models, would be provided.

Both the river and refuge models propose that genetic divergence can originate by geographical isolation due to geological (rivers) or climatic-vegetational (refuges) processes. However, the possibility that geological features and climatic oscillation can simultaneously promote diversification in Amazonia remains untested. An alternative model that includes both types of processes and that could potentially lead to diversification is the river-refuge hypothesis (Ayres & Clutton-Brock 1992; Haffer 1992; 1997). This hypothesis proposes that cladogenesis can occur by a combination of geographic isolation on opposite sides of the lower courses of Amazonian rivers and by forest retraction northwards, from the headwater regions toward the lower courses of the rivers during Pleistocene glacial periods (Haffer 1992; 1997). This scenario would lead to an additional prediction of recent population expansion southward following the onset of forest re-expansion since the last glacial period (last 20,000 years; Haffer 1997a). However, this model would be validated only if the retraction of populations on northern fronts during the glacial maxima was temporally congruent with the onset of the isolation effect due to the river, an arguable prediction since glacial periods are characterized by lower water levels on the Amazon River basin (Irion *et al*. 1995; 1997; Vital & Stattegger 2000), a period in which the isolation effects of large Amazonian rivers on *terra firme* forest biota are presumably smaller.

Given this scenario, can our data support the river-refuge model? For populations closer to the southern edge of Amazonia (localities 5 and 7), the Fu's F_s test detected a signature of demographic expansion indicating that fire-eye populations may have expanded into these areas following the end of the last glacial period. Point estimates of dates of population expansion for the two localities sampled in our study fall in the late Pleistocene and Holocene periods, independent of the mutation rate employed. The detected signal of demographic expansion in the western Tocantins River localities may suggest that small magnitude episodes of forest regression and expansion during the late Pleistocene and Holocene could have impacted populations of fire-eyes, at least at a regional scale. It is noteworthy that our data could provide some support for the model of regional forest replacement by open vegetation at the Carajás site (locality 5, Figure 1; Ledru *et al*. 2001; Siffedine *et al.* 2001; but see Bush & Oliveira 2006). Despite the wide confidence intervals of some estimates of the time to the population expansion, they coincide with the proposed time of forest expansion at this locality (last 30,000 yrs; Ledru *et al*. 2001; Siffedine *et al.* 2001) and with another area from the southern border of Amazonia (last 3,000 yrs; Mayle *et al*. 2000; Burbridge *et al*. 2004).

Although a full test of the river-refuge model will require a more complete geographic sampling in the eastern river bank, the presumable isolation effect of the Tocantins River, initiating before or roughly at the same time as population expansion events (Table 4), and the signature of demographic stability and expansion recovered for some populations in the lower (localities 1, 2 and 4) and medium (localities 5 and 7) course of the western river bank may allow us to reconcile our data with the predictions of this model. Future phylogeographic sampling and palynological studies along the edges and central parts of the Amazonian forest are crucial not only for testing the predictions of the river-refuge model as proposed here, but also for an accurate definition of headwaters regions of Amazonian rivers, a challenging task given the influence of both contemporary and historical factors (Bates *et al.* 2004).

Conclusions

The pattern of mtDNA variation observed for fire-eye populations provides little support for the view that populations were isolated in glacial forest refuges as proposed by Haffer (1969). Episodes of population demographic expansion are not entirely ruled out; therefore there may be effects of demographic processes induced by climatic changes; however, our data provide stronger support for the predictions of the river hypothesis. These results add to mounting evidence that, regardless of its popularity as a main process driving speciation in the Amazonian biota, climatic oscillations throughout the Pleistocene period do not seem to have played a substantial role in the diversification of many taxa in this region. Instead, our results agree with previous studies that have shown that physiographic events (Patton & da Silva 2005; Aleixo & Rossetti 2007) may have had a primary role in vicariance events.

Future studies should be designed to test whether climatically driven range contractions could have occurred along the southern edge of the forest, hence promoting an intensification of the isolating effect of large Amazonian Rivers. It has long been shown that Amazonian rivers can be significant ecological barriers to animal dispersal in the Amazon basin (Wallace 1852; Sick 1967; Haffer 1992; 1997a,b), yet a primary role of rivers in phyletic splitting of lineages has been contentious; our study adds another piece of information to this discussion by showing that, although a recent event (*i.e.* late Pleistocene), the Tocantins River has likely been an important historical barrier for fire-eye antbirds. It is also noteworthy that the ML and MP trees might suggest that the postulated river channel shift could have promoted across-river transfer of individuals, as would have been expected from meandering river episodes (Salo *et al*. 1986; Patton *et al*. 1994; Peres *et al.* 1997; Colwell 2000). Finally, the difficulty in obtaining precise and accurate dating for recent biogeographic events in Amazonian lowlands represents a major obstacle to implementing robust tests of predictions about the timing of speciation derived from alternative diversification scenarios.

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Table 1 Predictions and evidence for the river and refuge hypotheses of diversification for fire-eye (*Pyriglena leuconota*) in the Tocantins River Valley. See text for details

Hypothesis	Predictions	Prediction supported?
River	(1) haplotypes on opposite river banks will be reciprocally monophyletic; (2) genetic differentiation will be greater across the river rather than within individual interfluvia, after controlling for the effect of geographic distance; (3) populations geographically close to the river should not show evidence of demographic expansion.	(1) partial support. The reciprocal monophyly scenario cannot be rejected in a maximum-likelihood framework; (2) yes. Several tests indicated higher genetic divergence among populations across the river than within the same interfluvium; (3) partial support. No evidence of demographic expansion for most populations geographically close or bordering the river.
Refuge	(1) lower genetic variability in non-refuge areas; (2) timing of population divergence and shallow levels of genetic divergence representing isolation in refuge areas; (3) recent demographic expansion from refuge areas following the onset of humid periods. The magnitude of range expansion will be significantly larger in the western river bank.	(1) no. Variation in genetic diversity was not geographically structured; (2) yes. Shallow levels of divergence found and some population splitting and TMRCA estimates consistent with temporal prediction; (3) partial support. The Fu's F_s test cannot reject population growth for two populations, but Rozas' R_2 and the Bayesian analyses show no signature of a large population expansion in the western river bank.

Table 2 AMOVA analysis for fire-eye (*Pyriglena leuconota*) populations grouped into western vs. eastern banks of the Tocantins River

Source of variation	d.f.	Percent of variation
Among population from opposite river banks	1	70.96**
Among populations from the same river bank	16	3.96*
Within populations	65	25.08**
Total	82	

*$P < 0.01$; **$P < 0.001$.

Table 3 Sample size, number of haplotypes, haplotype diversity (*h*), nucleotide diversity (π), and historical demographic analyses (Fu's F_s and Ramos-Onsins and Rozas' R_2 tests) for the 18 populations of fire-eye (*Pyriglena leuconota*) on opposite banks of the Tocantins River

No.		Sample size	Number of haplotypes	Haplotype diversity	Nucleotide diversity ($x10^{-3}$)	Fu's F_s	R_2
	Western Bank	52	17	0.83 ± 0.04	7.0 ± 4.1	-12.07***	0.11**
1	Senador José Porfírio	5	3	0.70 ± 0.21	4.2 ± 3.7	-0.19	0.29
2	Altamira region	7	2	0.48 ± 0.17	1.3 ± 1.4	0.59	0.27
3	Caraipé Valley	3	2	0.67 ± 0.31	4.7 ± 4.8	-	-
4	Region of Jacunda	7	5	0.90 ± 0.10	6.1 ± 4.6	-1.89	0.23
5	Serra dos Carajás	16	8	0.83 ± 0.07	5.0 ± 3.5	-4.00**	0.16
6	Ourilândia do Norte	2	2	1.0 ± 0.5	11.8 ± 13.1	-	-
7	Santana do Araguaia	12	6	0.85 ± 0.07	5.4 ± 3.9	-2.09*	0.18
	Eastern bank	33	14	0.88 ± 0.04	5.3 ± 3.2	-11.16***	0.12
8	Santa Bárbara	3	3	1.0 ± 0.27	3.9 ± 4.0	-	-
9	Peixe-Boi	1	1	-	-	-	-
10	Moju	1	1	-	-	-	
11	Tailândia	5	3	1.0 ± 0.27	10.0 ± 8.8	-0.08	0.20
12	Paragominas	2	2	1.0 ± 0.5	3.4 ± 2.7	-	-
13	Canoal	1	1	-	-	-	-
14	Açailândia	9	4	0.75 ± 0.11	5.9 ± 7.2	-0.72	0.21
15	Amarante	2	2	1.0 ± 0.5	2.8 ± 3.9	-	-
16	Grajaú	6	2	0.33 ± 0.21	0.9 ± 1.2	-0.00	0.31
17	Porto Franco	1	1	-	-	-	-
18	Feira Nova	2	2	1.0 ± 0.50	8.8 ± 10.1	-	-

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. The Fu's test was considered as significant at the 5% level if its *P* value was below 0.02 (Fu 1997).

Table 4 Population estimates of Θ_0, Θ_1, τ and estimated time since the population expansion in y.b.p for fire-eye (*Pyriglena leuconota*) populations, assuming three mitochondrial mutation rates (2.1% and 4.0%). Western and eastern bank samples of the Tocantins River were pooled to estimate global population expansion in either river side. Sampling localities numbered as in Figure 1

Sampling locality	Θ_0	Θ_1	T	Time of expansion	
				2.1%	4.0%
Western bank	0.00 (0.00-0.028)	99,999 (9.859-99,999)	0.834 (0.447-1.410)	18,096 (9,698-30,594)	8,596 (4,608-14,536)
Eastern bank	0.454 (7.708-99,999)	787.500 (7.708-99,999)	1.836 (1.277-3.389)	39,838 (27,708-73,538)	18,938 (13,164-34,938)
Serra dos Carajás (Locality 5)	0.000 (0.0-0.014)	9,999 (1.899-9,999)	1.242 (0.604-2.295)	26,950 (13,106-49,798)	12,804 (6,226-23,658)
Santana do Araguaia (Locality 7)	0.000 (0.0-0.369)	9,999 (2.608-9,999)	1.625 (0.547-2.932)	35,260 (11,868-63,620)	16,752 (5,638-30,226)

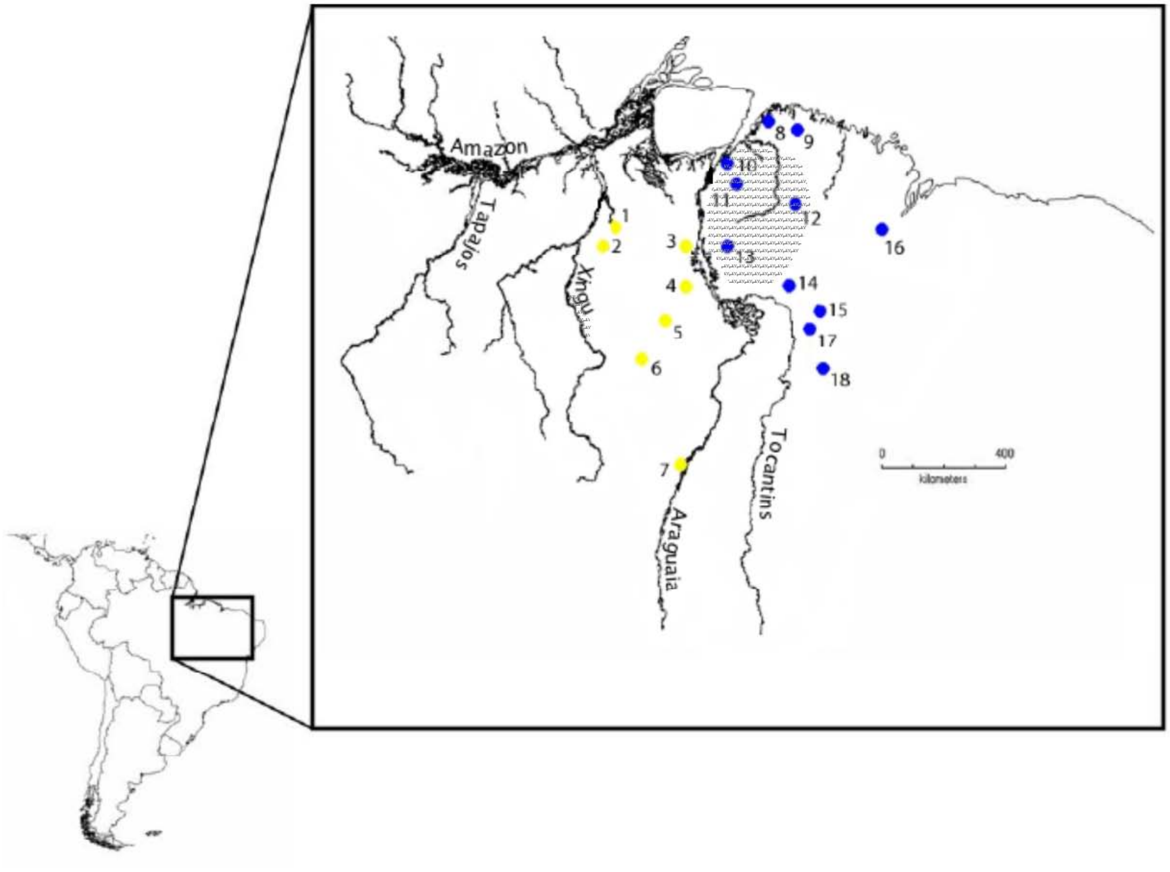

Fig. 1 Sampling localities of fire-eye (*Pyriglena leuconota*) antbird populations along the Tocantins River Valley, southeastern Amazon, Brazil. Gray shaded areas on the western and eastern river banks indicate respectively the location of an unnamed and the Belém glacial forest refuges, following Haffer (1969).

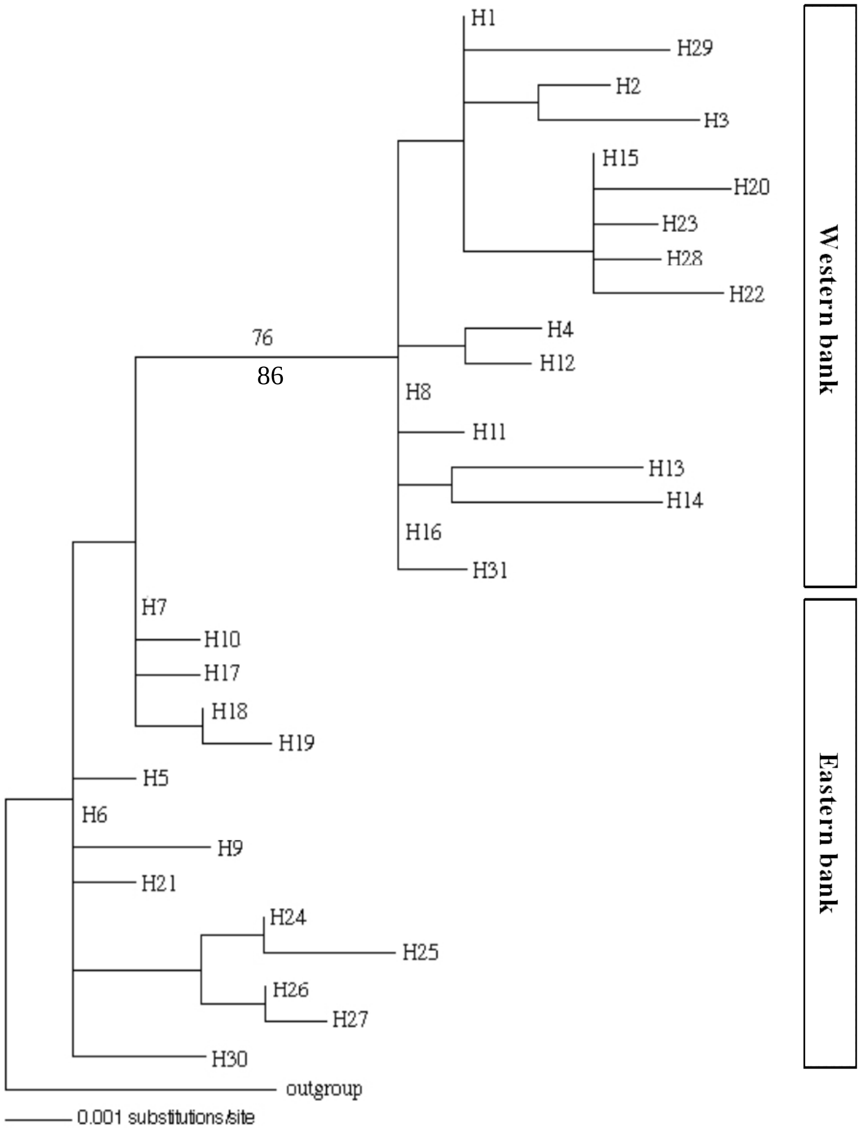

Fig. 2 Phylogram of unique haplotypes using the ML tree search. Numbers and letters in the terminals represent the haplotype number. The geographic origin (western or eastern river bank) of haplotypes is indicated by vertical bars. The fifty-percent majority-rule consensus tree obtained using the equally weighted MP search in PAUP produced a similar tree topology (not shown). The numbers above and below the western clade are the ML and MP bootstrap values, respectively.

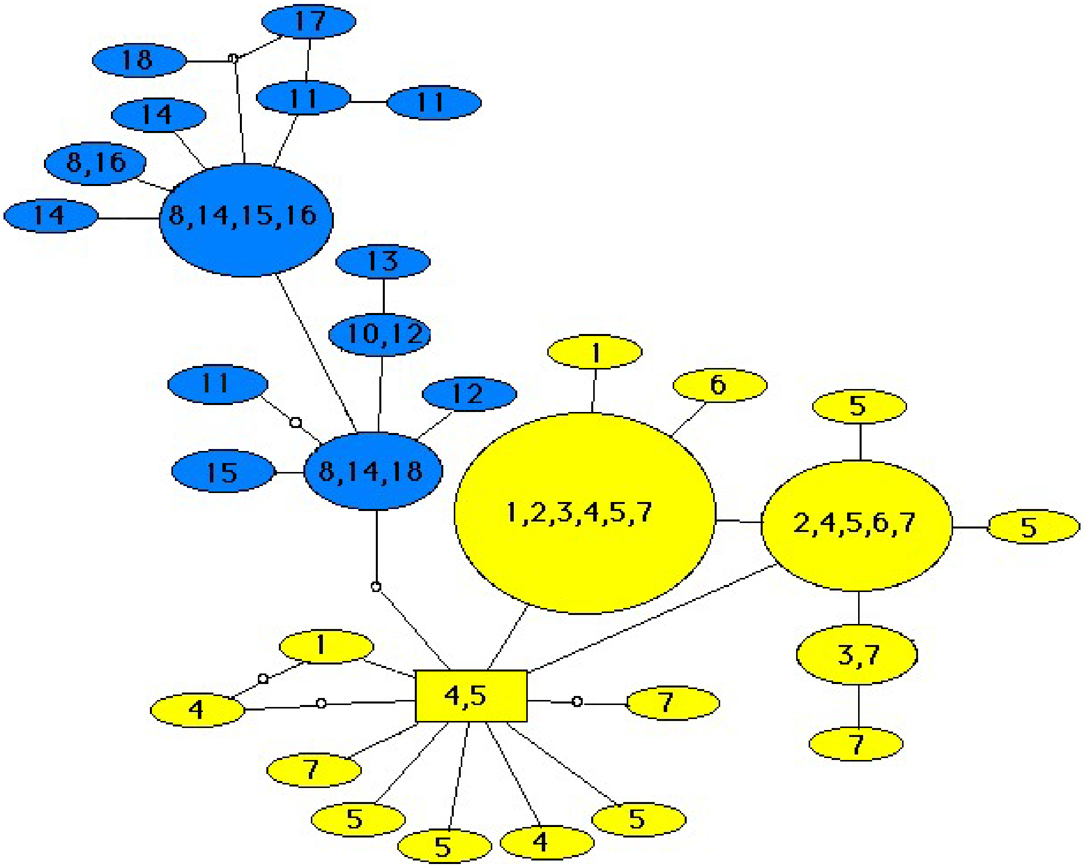

Fig. 3 Statistical parsimony network among haplotypes of Amazonian fire-eye (*Pyriglena leuconota*) populations based on ND2 sequences. Each circle represents a different haplotype with size proportional to its relative frequency. Yellow and blue circles represent haplotypes recovered in the western and eastern banks of the Tocantins River, respectively. The numbers correspond to sampling localities labeled in Figure 1.

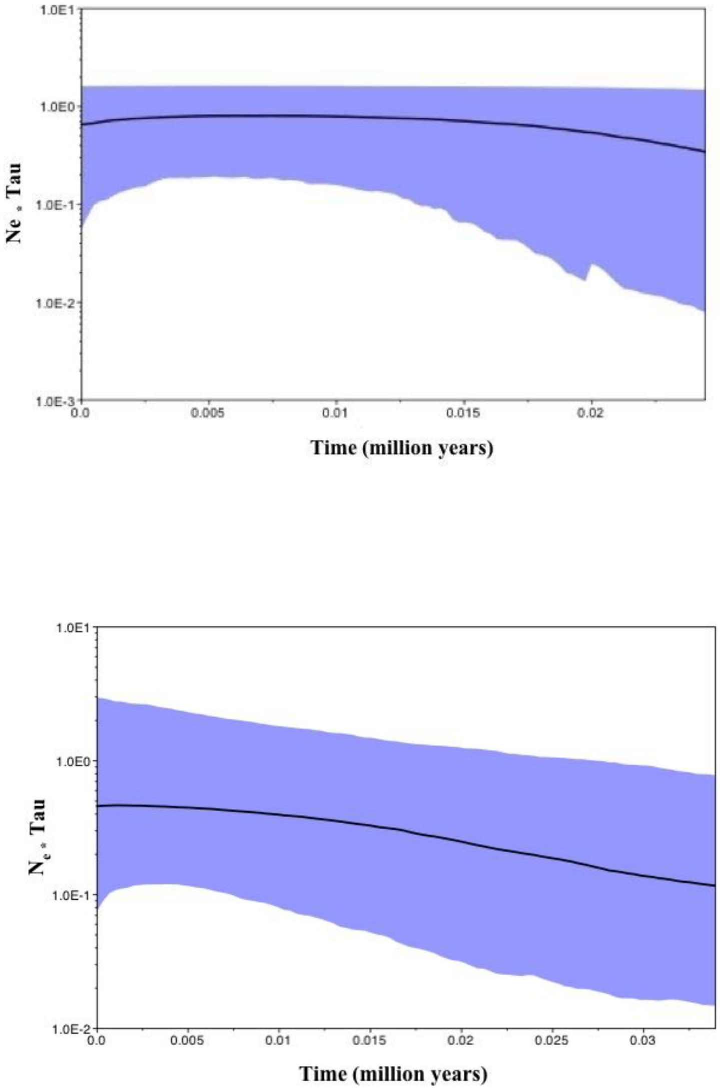

Fig. 4 Bayesian Skyline Plots depicting the demographic history of fire-eye (*Pyriglena leuconota*) populations in western (top figure) and eastern (bottom figure) banks of the Tocantins River, with time axis scaled to the mutation rate of 2.1% per MYA. The solid line represents the median value for the log of the effective population size and the gray area represents the upper and lower 95% credible intervals. Time zero is the present, with values indicating time increasing towards the past.

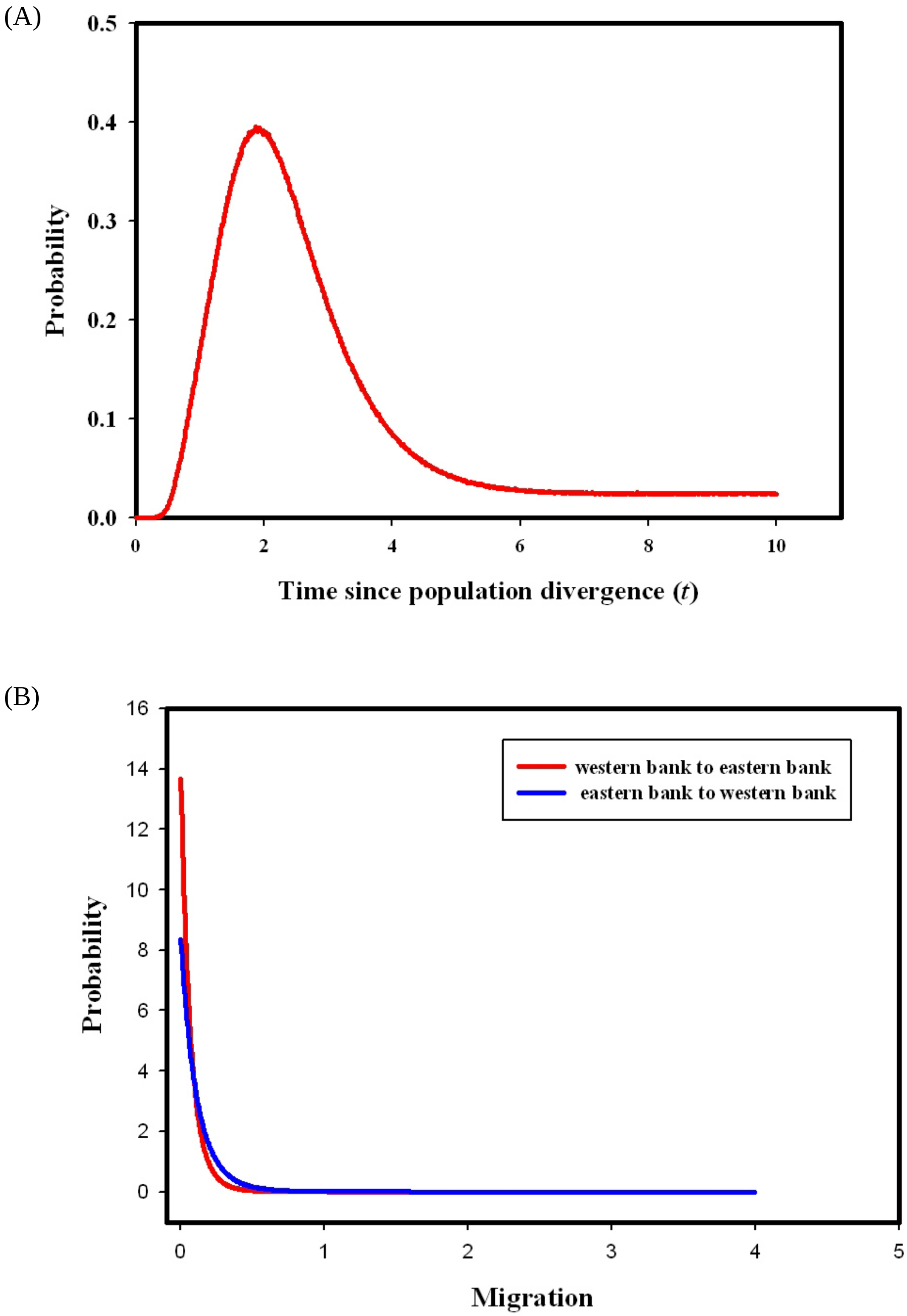

Fig. 5 IMa posterior distributions of population divergence time (A) and migration (B) between fire-eye (*Pyriglena leuconota*) populations on opposite banks of the Tocantins River.

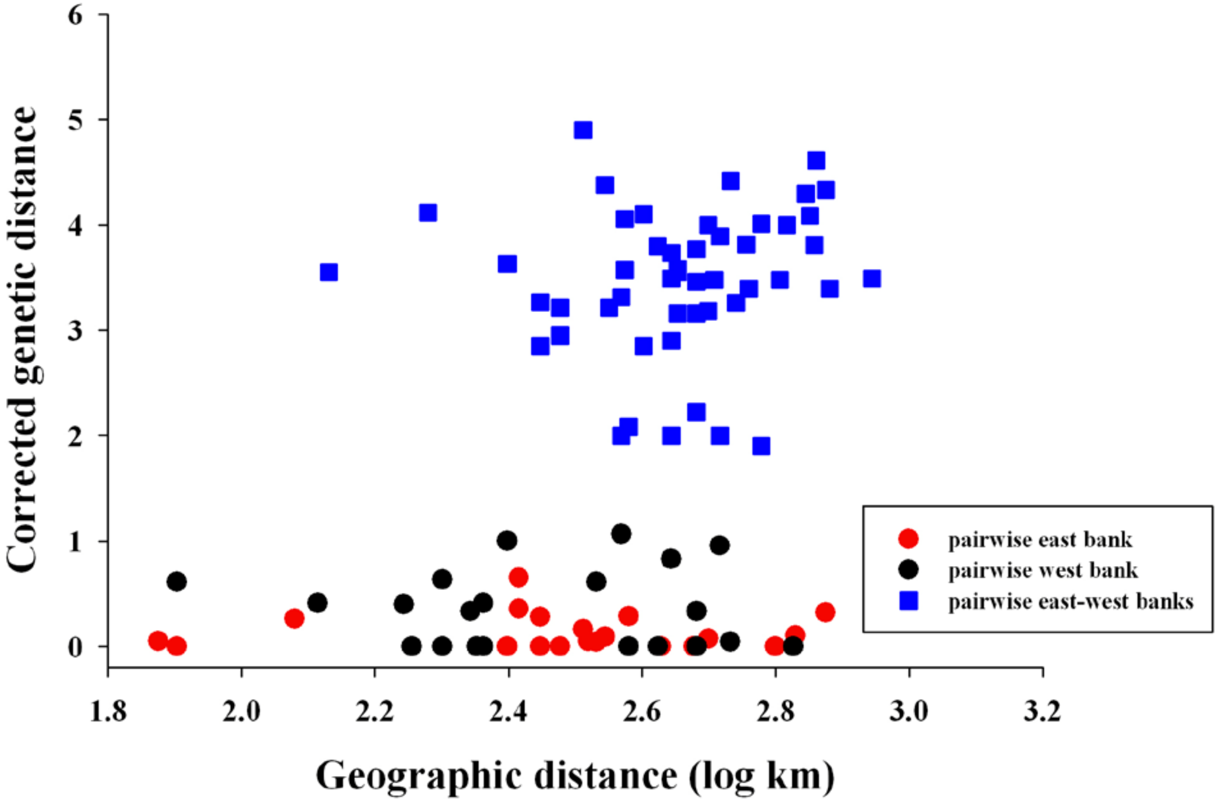

Fig. 6 Correlation of corrected genetic distances and geographical distances between fire-eye (*Pyriglena leuconota*) populations sampled in the same (dots) and on opposite (squares) banks of the Tocantins River.

Appendix A

Collection locality, sample size, tissue or dry skin source, voucher number and GenBank accession number for specimens of White-back fire-eye (*Pyriglena leuconota*) sequenced in this study.

Locality	Sample size	Tissue/Skin source	Catalogue number	Gen Bank accession nos.
1. Pará:Senador José Porfírio, Eastern Bank of Xingu River; 3º 331’47.2”S-51º 43’58.6”W	5	MPEG	UHE353, UHE356, 55715, 55716, 55717.	
2. Pará: Altamira, east Bank of Xingu River -	7	USNM		
3. Pará: Rio Tocantins, west bank Tocantins River, 40 km north of Tucurui dawn.	5	MPEG	35701, 36248, 36246, 36247,	
4. Pará:West bank of Tocantins River, Jacunda	8	MPEG	35925, 35926, 35927, 35929, 36094, 35928, 35930, 35931	
5. Pará:Paraupebas, Serra dos Carajás; 5º48'S, 50º30'W	17	MPEG, FMNH	1565, 37251, 38038, 38174, 38180, 38181, 38182, 38183, 36776, 38184, 38177, 38179, 38178, 36175, 53799, 53800, 53801, 39147	
6. Pará: Ourilândia do Norte	2	MPEG	DPN093, DPN143	
7. Pará: Santana do Araguaia, Fazenda Fartura	13	MPEG	48920, 48766, 48754, 48920, 48754, 48755, 48756, 48757, 48762, 48763, 48767, 48920	
8. Pará:Santa Bárbara	3	MPEG	55900, 58649, 59091	
9. Pará:Peixe-Boi, Fazenda Monte Verde	1	MPEG	50988	
10. Pará:Moju, Fazenda Latomix	1	MPEG	51978	
11. Pará:Tailândia; 2º 31´48º 47'			P2586, P2870, P2872, P2873, P2875	
12. Pará: Paragominas, Fazenda Rio Capim	2	MPEG	54994, 68978	
13. Pará:Canoal	1			
14. Maranhão:Rio Itinga, Açailândia, BR 010 Km 21, Fazenda Cobrás	10	MPEG	38487, 38488, 38491, 38492, 38493, 38494, 38495, 38497, 38489, 38490	
15. Maranhão:Amarante	2	MPEG	37865, 40859	
16. Maranhão:Grajaú	9	MPEG	37709, 37710, 37711, 37712, 37713, 37714, 37715, 37716, 37717	

17. Maranhão:Porto Franco	1	MZUSP	76549
18.Maranhão:Riachão, Povoado Feira Nova, Fazenda do Arroz	2	MPEG	42322, 42323

CHAPTER 2

Phylogeny and phylogeography of a circum-Amazonian avian complex: the role of geological and climatic factors in the diversification process of fire-eye antbirds (*Pyriglena*)

1. Introduction

The importance of climate and landform as drivers of population differentiation and speciation has long been appreciated (Chapman 1917; Mayr 1942). In the Neotropical region, although both factors are thought to create barriers to gene flow and potentially lead to speciation, many questions remain regarding their influence on diversification. For example, previous studies on Neotropical organisms suggest that the effects of Pleistocene glaciation events on population differentiation differ with respect to latitude, habitat and topography and, therefore, their relative importance in the process of speciation varies regionally (e.g. Wüster et al. 2005; Noonan and Gaucher 2006; Weir 2006; Carnaval and Bates 2007). Similarly, the impacts of mountain building and the formation of river barriers on genetic divergence and speciation may vary geographically and over time (e.g. Aleixo and Rosseti 2007; Brumfield and Edwards 2007). Whereas several recent studies have tried to elucidate the importance of climatic and geological factors as engines of diversification in a limited geographic extent, few have made an attempt to clarify their relative roles on a continental scale.

One major geological factor that profoundly influenced the ecology and evolution of South American plants and animals was the rise of the Andes (Haffer 1974; Hooghiemstra and van der Hammen 1998). This prolonged event created high-elevation forest and non-forest habitats that were colonized by taxa from different regions (e.g. Fjeldså1985; Bates and Zink 1994; Hackett 1995; Peréz-Emán 2005), after which subsequent *in situ* diversification likely occurred. The complex topography (e.g. deep inter-Andean valleys), coupled with glacial and interglacial shifts in vegetation belts, might have created many opportunities for colonization and allopatric differentiation for lowland and foothill forest organisms that became isolated on either slope of the mountains (Patterson et al. 1992; Brumfield and Caparella 1996; Ribas et al. 2005; Brumfield et al. 2008) or on different Andean ridges (Graves 1988; Patton and Smith 1992; Arctander and Fjeldså 1994). Although studies focusing exclusively on lowland organisms distributed on either side of the Andes or on organisms occurring along Andean foothills may provide insights into the role of some historical processes, studies of organisms composing both types of distribution may constitute opportunities to assess a more complete suite of diversification mechanisms acting within this region.

A second major geological factor in South America has been the formation of major river systems that can be effective dispersal barriers even for birds. The palaeo-drainage of South American rivers started to be formed in the late Oligocene, but it was not until late Miocene and Pliocene that the present-day pattern of most river systems began to be established (Martin et al. 1993; Potter 1997; Lundberg et al. 1998, Ribeiro 2006). The role of rivers in delimiting the spatial distribution of Neotropical terrestrial vertebrates has been contemplated since earliest naturalists started to explore the region (Wallace 1852). Subsequent studies based on distributions of organisms in different parts of South America indeed suggested that some rivers might serve as dispersal barriers (e.g. Hershkovitz 1990; Haffer 1992; Vielliard 1996; Hayes and Sewlal 2004). Similarly, some molecular studies of Neotropical organisms have provided evidence that rivers restrict gene flow effectively (Capparela 1988; 1991; Aleixo 2004; Bates et al. 2004; Armenta et al. 2005; Pellegrino et al.

2005) although other studies find weak or no effects (Patton et al. 1994; Lougheed et al. 1999; Aleixo 2004; Bates et al. 2004; Funk et al. 2007; Cabanne et al. 2008). This implies that research in distinct biogeographic regions containing a suite of geological and ecological conditions will be required to provide a complete picture of the relative role of rivers as a historical diversification mechanism in the Neotropical region.

Pleistocene glacial events during the last 2.4 My are thought to have influenced the genetic structure and geographic distribution of populations in temperate regions (reviewed in Hewitt 2000; 2004), but their influence on the spatial distribution and geographic differentiation of populations in the Neotropics remains less well understood (but see Noonan and Gaucher 2006; Carnaval and Bates 2007; Rull 2006, 2008). Previous studies suggest that the influence of Pleistocene refuges on the genetic structure and range dynamics of forest organisms may have been more pervasive in highlands (e.g. Andean forests; Weir 2006; Ribas et al. 2007, Koscinski et al. 2008) and at higher latitudes (e.g. southern Brazilian Atlantic Forest; Behling 1995, 2002; Cabanne et al. 2007; Martins et al. 2007) than in the Amazonian lowlands (e.g. Lessa et al. 2003, Aleixo 2004). Thus, organisms that occur in different biogeographic regions, that possess broad latitudinal geographic distributions, and that are present in both lowlands and highlands are best suited to elucidate the role of Pleistocene climatic changes on processes of population colonization and differentiation.

Given their broad geographic distribution, with populations occurring in forested areas in southeastern Amazonian basin, Pantanal floodplain basin, Andes and the Pacific and Atlantic Coasts, the South American fire-eyes, Thamnophilid antbirds of the genus *Pyriglena* (hereafter referred to as fire-eyes) provide a model system to investigate the influence of geological and climatic processes on patterns of diversity. The genus as a whole, and populations within species, extend across rivers, glacial refuges, and mountains, providing a link between genetic diversification and historical processes. The geographic extent of our sampling (see below) allows us to distinguish whether potential barriers to dispersal (i.e. rivers, mountains, and dry habitats) were primary sites of diversification or secondary meeting points for populations that diversified elsewhere (Patton and da Silva 1998, 2005). In addition, because dispersal capabilities presumably do not vary across the geographic range of *Pyriglena,* among-region differences in phylogeographic patterns can be assigned with confidence to historical processes as opposed to evolved differences in life-history traits. Finally the geographical distribution of fire-eyes conforms to a partial circum-Amazonian pattern (*sensu* Remsen et al. 1991) that is shared by a number of South American avian and plant groups (Remsen et al. 1991, Prado and Gibbs 1993, Bates 1997, Pennington et al. 2000, Lovette 2004), suggesting that the processes underlying the evolutionary patterns in this group may have been shared among other circum-Amazonian taxa.

In this study, we provide a densely sampled survey spanning the geographic distribution of South American fire-eyes and place the history of these populations in the context of geology and past climate change. Specifically, we ask whether the spatial and temporal patterns of diversification within and among distinct biogeographic regions can be explained by geological factors, Pleistocene climate change, or by an interplay between both. To address this question, we first test whether potential barriers to dispersal, such as the uplift of the Andes, formation of modern river systems in Amazon, Pantanal and Atlantic Forest, and the presence of dry inter-Andean valleys, correlate with genetic differentiation of fire-eye populations. Second, we investigate whether climatic fluctuations and refuges proposed by palynology (e.g. Behling 1997, 2002; Behling and Negrelle 2001), geomorphic features (e.g.

Auler et al. 2004; Wang et al. 2004) and historical climatic modeling (Carnaval and Moritz 2008) can explain patterns of genetic differentiation among and within each biogeographic region.

The fire-eyes study system: synopsis of current taxonomy and distribution

The fire-eye genus *Pyriglena*, as currently recognized, includes three species: the Fringed-backed Fire-eye (*P. atra*), the White-backed Fire-eye (*P. leuconota*), and the White-shouldered Fire-eye (*P. leucoptera*) (Willis and Oniki 1982; Ridgely and Tudor 1994; Zimmer and Isler 2003); although different authors have considered *Pyriglena* to comprise one (Zimmer 1931) or four (Chapman 1923) species. Notably complicated is the taxonomic status of *P. atra*, which between the geographic ranges of *P. leuconota* and *P. leucoptera*. Willis and Oniki (1982) suggested that the relationship of *P. atra* is uncertain, given its intermediate vocal and plumage characters in relation to *P. leuconota* and *P. leucoptera*. Equally enigmatic is *P. leuconota,* which is currently considered to include 10 parapatrically or allopatrically distributed subspecies (Figures 1and 2). One subspecies (*P. l. pernambucensis*) is isolated in the coastal areas of northeastern Brazil, north of the São Francisco River. Three other subspecies occur in the Amazonian region south of the Amazon River and east of the Tapajós River. Six subspecies occur from western lowlands of Brazil across central Bolivia and north along the eastern slopes of the Andes to central Colombia. In western Andes, another isolated population occurs in the Tumbesian center of endemism in northwestern Peru and western Ecuador (Willis and Oniki 1982; Parker et al. 1995). Finally, the monotypic *P. leucoptera* occurs in parapatry with *P. atra* in the northern Brazilian Atlantic Forest (Figure 1B). The relationships, boundaries and monophyly of the species, subspecies and populations have never been rigorously examined. Several authors have questioned the interpretation of a single widely distributed species of *P. leuconota* (Ridgely and Tudor 1994; Parker et al. 1995; Zimmer and Isler 2003), whereas morphological intermediates between *P. leucoptera* and *P. atra* in eastern Brazil (Willis and Oniki 1982) raise the possibility of hybridization and introgression between these phenotypically distinct forms.

2. Methods

Geographic sampling and data collection

Most of samples used in our study were vouchered and were obtained during fieldwork throughout South America or were borrowed from museums (see Appendix 1). We lack vouchers of *P. atra*, an endangered taxon from coastal Brazil; only blood samples were collected. Since we were unable to obtain tissue samples from the Peruvian endemic *P. l. picea*, we extracted DNA from several old specimens housed at the AMNH. The DNA was too degraded for most of the *P. l. picea* specimens, and we could obtain sequences from only a single individual (Appendix 1).

We conducted our sequencing analyses using two strategies to perform historical demographic estimates and to establish evolutionary relationships among taxa and populations at distinct hierarchical levels. To explore phylogeographic and phylogenetic variation within and among biogeographic regions, we used complete mtDNA NADH dehydrogenase subunit II (ND2; 1041 bp) from 442 samples spanning the entire taxonomic and geographic distribution of the fire-eyes (Appendix 1). Although the analyses of the ND2 sequences revealed highly divergent, geographically structured mtDNA clades, relationships within and among regions could not be fully resolved using this data set (results not shown). We thus sequenced three other mitochondrial genes and one nuclear gene from a subset of 74 individuals to further explore phylogeographic structure and to resolve relationships. The second dataset included the mtDNA ND2, NADH dehydrogenase subunit III (ND3; 351 bp), cytochrome b (cyt *b*; 1045 bp), ATP-synthase 6 and 8 (ATPase; 776 bp) and the nuclear intron beta-fibrinogen 5 (BF5; 535 bp). To amplify and sequence samples yielding low quality DNA, we designed external primers for ATPase6 and ATPase8 and internal primers for ND2 that permitted us to sequence fragments of 300-400 bp (Table 1). For amplification and sequencing we used the following primers: ND2, L5219, H5766, L5758 and H6313 (Johnson and Sorenson 1998); ND3, primers L10755 and H11151 (Chesser 1999); cyt *b* L14990, H16065, cytb.intf and cytb.intr (Brumfield and Edwards 2007); BF5, FIB5L and FIB5H (Brumfield et al. 2007).

Total genomic DNA was extracted from frozen tissues, blood, and dry skin samples of specimens collected during the last 25 years using a Qiagen tissue extraction kit (QIAGEN, Inc.). Contamination risk of the museum samples was minimized by extracting the DNA in a different room reserved for handling only bird skin samples and by always performing PCRs with negative controls. PCR profiles were designed for each primer set and included in general an initial 2 min denaturation cycle at 95°C, followed denaturation at 94°C for 45s, annealing varying from 46 to 52°C for 45 s, with a final extension of 1 min at 72°C. This was followed by 39 cycles of denaturation at 94°C for 45 s, annealing varying from 46 to 52°C for 45 s and extension at 72°C for 1 min, finishing with an additional extension at 72°C for 10 minutes. DNA sequencing was carried out with BigDye v 3.0 Dye Terminator Cycle Sequencing Kit (Applied Biosystems, Inc.) and the same primers used for amplification. Cycle sequencing reactions were purified with an ethanol-sodium acetate solution and run on an ABI 3100 automated sequencer. Sequences of both BF5 and mtDNA genes were assembled and edited using the program SeqMan (DNAstar) and aligned by eye. The following measures were taken to ensure that amplified fragments from the mitochondria coding genes did not include pseudogenes of nuclear origin: 1) inspection for deletions, insertions and stop codons that would result in a nonfunctional protein, 2) confirming a high transition rate at third codon positions and 3) confirming a high transition to transversion substitution ratio characteristic of mitochondrial DNA (Arctander 1995; Sorenson and Quinn 1998).

Phylogenetic analyses

We first analyzed a terminal data set comprising the four mtDNA sequences (hereafter mtDNA data set; 3213 bp total) for one to twelve individuals per taxon. Second, we analyzed a subset of these individuals with the addition of the nuclear intron (hereafter full data set; 3748 bp total). In the intron data set, double peaks in the chromatograms were inferred as heterozygous sites and coded as polymorphic using the standard IUPAC degeneracy codes. No more than one heterozygous site was recovered in any BF5 individual. Phylogenetic relationships among individuals based on the mtDNA data set and the full data set were assessed with maximum parsimony (MP), maximum likelihood (ML) using PAUP v4.0 (Swofford 2002), and Bayesian inference in MrBayes v3.1.2 (Huelsenbeck and Ronquist 2001; Ronquist and Huelsenbeck 2003). All MP analyses were performed using a heuristic search, 100 random addition replicates and tree-bisection-reconnection (TBR) branch-swapping algorithm with all the characters equally weighted. Nodal support was assessed using non-parametric bootstrap (Felsenstein 1985) with 100 replicates. The best-fit model of nucleotide substitution for ML and Bayesian analyses was selected using Modeltest v3.7 (Posada and Crandall 1998) and MrModeltest v2.22 (Nylander 2004), respectively. MrModeltest was selected for the Bayesian analyses because it only considers models of nucleotide substitutions that were implemented in MrBayes v3.04b (Ronquist and Huelsenbeck 2003). We used the Akaike Information Criteria (AIC) for model selection because of its advantages over hierarchical likelihood-ratio tests (Posada and Buckley 2004). The Trn + I + G and K81 + I + G models of molecular evolution were selected for the mtDNA and full data sets, respectively, in Modeltest. MP and ML analyses were performed on the Beowulf Cluster at the University of Missouri-St Louis.

Data sets composed of multiple genes, as in this study, contain partitions (e.g. codon positions and nuclear and mitochondrial genes) that may evolve under different models of evolution. In these situations, simulations and empirical studies have shown that using a single model of evolution for the entire data set may not capture the complexity in nucleotide substitution for different codon positions and genes. This might potentially affect various aspects of the phylogenetic reconstruction, such as accuracy of posterior probability values (Huelsenbeck and Rannala 2004, Castoe et al. 2004, Brandley et al. 2005), tree topology (Nylander et al. 2004, Castoe and Parkinson 2006), branch lengths (Lemmon and Moriarty 2004) and resolution of deeper nodes (Castoe et al. 2004, Brandley et al. 2005). The issue of model complexity is more decisive with highly divergent sequences where homoplasy due to multiple substitutions becomes a serious issue (Huelsenbeck and Rannala 2004, Lemmon and Moriarty 2004). One way to overcome this problem is by employing independent models of nucleotide evolution for each partition in the data set (Nylander et al. 2004).

In this study, we used mixed-model Bayesian analyses in which the concatenated data sets were partitioned by codon position according to their adequate model of molecular evolution (except the intron gene that had a distinct model). Bayesian analyses were performed with flat Dirichlet distribution for base frequencies and estimation of nucleotide substitution and default values for all other parameter estimations. Codon partitions and parameters were allowed to vary independently by unlinking partitions. We used the Akaike Information Criteria (AIC) for model selection in MrModeltest and the HKY + I + G, HKY + I, GTR + I + G, and HKY + I models of molecular evolution were selected for the first, second and third mtDNA positions, respectively, and for BF5. We ran three independent analyses with different seed numbers. In each analysis, we implemented four heated and one cold Markov chains for 4,000,000 and 10,000,000 generations for the mtDNA and full data set, respectively. Stationarity of each individual analysis was confirmed in three ways. First, we visually inspected plots of likelihood scores against generation time. Second, we checked whether the average standard deviation of split frequencies was <0.005 and third, we assessed whether the three independent runs converged in similar tree topology and associated posterior probabilities. The first 500,000 generations were discarded as a burn-in for both data sets. Once we verified that the three independent runs had converged on similar posterior distributions, we presented our results based on a single run. Choice of outgroup taxa was based primarily on a recently published antbird phylogeny (Brumfield et al. 2007), with the inclusion of the Slender Antbird (*Rhopornis ardesiacus*), which has been suggested as closely related to *Pyriglena* based on behavior and voice (Willis and Oniki 1981, Ridgely and Tudor 1994, Zimmer and Isler 2003).

Assessing the conflict between traditional classification and mtDNA variation

Our phylogenetic analyses indicated that some relationships were contrary to those implied by traditional classifications. We thus assessed the robustness of conflicting placement of taxa recovered in our maximum-likelihood tree with those alternate topological placements of taxa implied by traditional classifications. To do this, we conducted a number of constrained ML searches in PAUP using the combined mitochondrial genes data set and contrasted their likelihood scores with the one obtained from of the unconstrained ML search employing the Shimodaira-Hasegawa tests (S-H, Shimodaira-Hasegawa 1999), with full optimization and 1,000 bootstrap replicates.

Mantel tests - Rivers as historical barriers

If South American rivers were a long-term historical barrier to gene flow, genetic distances between populations on opposite river banks would be greater than genetic distances between populations on the same side of the river. To test whether large rivers have had an important role in shaping the phylogeographic structure in fire-eyes, patterns of genetic variation between and within populations spanning major river systems across the South American continent were examined (Figure 1). When possible, partial Mantel tests with 10,000 random permutations were performed between a matrix of corrected pairwise genetic distances and a binary matrix (the river barrier) after controlling for geographic distance (see Chapter 1 for details).

Phylogeography and historical demography - Climatic changes and population history

We assessed the effects of Pleistocene climatic changes on timing of population differentiation, genetic diversity and population demography, contrasting our genetic data with previous knowledge of vegetation history of the different regions based on palynological, geomorphic and historical modeling approaches. In Atlantic Forest, an historical model for the distribution of moist forest during the last glacial maximum has been proposed (Carnaval and Moritz 2008). Specifically, these authors have hypothesized that most of the forest south of the Doce River had been replaced by savanna-like vegetation during the last glacial period, whereas two forest refuges persisted north of the river: i) a large area stretching from the southern bank of the Doce River northward to the southern border of the São Francisco River (hereafter "Bahia refuge") and ii) a smaller refugium north of the São Francisco River (hereafter "Pernambuco refuge"). If the current population genetic structure of fire-eyes was affected by fragmentation of forests into refuges during the last glacial maximum as proposed, we would predict that populations occurring in the areas where forest did not persist should present: i) a signature of recent population growth corresponding to the period of forest expansion after the last glacial maximum (i.e. during the last 20,000 years; Hewitt 1996; Haffer 1997a); ii) reduced genetic diversity relative to refuge areas. This prediction derives from theoretical (Nichols & Hewitt 1994; Ibrahim *et al*. 1996) and empirical (reviewed in Hewitt 2004a,b) studies, that show that episodes of range expansion into previously unoccupied areas have dramatic effects on the spatial pattern of genetic diversity; and iii) the set of populations in this area should not exhibit a regional pattern of isolation-by-distance because populations in recently colonized regions would not have existed long enough for the drift-mutation-gene flow equilibrium to have been achieved (reviewed in Hutchison and Templeton 1999). As such, measures of genetic distance between populations should not increase as a function of geographic distances separating the populations. One of the predictions of the refugium hypothesis is that we should recover shallow levels of genetic divergence representing isolation of fire-eye populations in the Bahia and Pernambuco refuges (Hewitt 2004); however, the test of this prediction is not tenable in our study because populations in the Pernambuco refuge are sister to eastern Amazonian populations (see below). We also ask whether Atlantic Forest fire-eyes (*P. leucoptera*) show evidence of range expansion towards the headwaters of the Paraguaçu River in response to Pleistocene climatic changes. *Pyriglena leucoptera* and *P. atra* occur parapatrically along the northern middle bank of this river (Figure 1B), where evidence of past gene flow and introgression has been recovered (see below).

Haplotypic (h) and nucletotide (π) diversity were calculated for population samples that contained more than one individual as described in Nei and Kumar (2000), using Arlequin (Schneider et al. 2000). We also inferred the relationships among haplotypes through networks using the statistical parsimony program TCS as outlined in Chapter 1.

In order to define geographic regions of large genetic changes, a spatial analysis of molecular variance (SAMOVA version 1.0; Dupanloup et al. 2002) was performed. This analysis was restricted to Atlantic Forest, where our sampling was most dense. SAMOVA defines *a posteriori* groups of populations that are geographically and genetically homogeneous and maximally differentiated from each other and, as a by-product, allows the identification of genetic barriers between these groups of populations. A simulated annealing procedure is used to maximize the proportion of total genetic variance due to differences between groups of geographically homogeneous populations. The analysis was based on pairwise differences for a varying number of groups (K). The final number of groups was based on the largest total genetic variance due to differences between groups of populations. For each grouping number, the simulated annealing process was repeated 100 times.

Tajima's D (1989), F*s* (Fu 1997), and R_2 (Ramos-Onsins and Rozas 2002) tests were used to assess whether the populations would fit a population-stationary or a population-expansion scenario using DnaSP (Rozas et al. 2003). Estimated values for these tests were compared to an empirical distribution based on 10,000 coalescent simulations assuming an infinite-sites model and a large population size. Significant P values (<0.05) were taken as evidence for departure from a model of constant population size in favor of an alternative scenario of demographic expansion. The generalized non-linear least-squares approach of Schneider and Excoffier (1990) implemented in Arlequin, was used to estimate the parameters of past demographic expansion from the mismatch distribution (Rogers and Harpending 1992) for those populations that showed significant departures from the null hypothesis of constant size. The estimated parameters were: Θ_0, the theta value before population expansion (where $\Theta_0 = 2N_e u$, where N_e is the female effective population size and u is the mutation rate); Θ_1, the theta value after population expansion and τ (where $\tau=2ut$, where t is the time since the expansion in generation units). Confidence intervals (95%) were estimated for the parameters Θ_0, Θ_1, and τ through a parametric bootstrap with 10,000 re-samplings.

The estimated values of the tau (τ) parameter were converted into years-before-present by solving $t = \tau/2u$, where u is the mutation rate per sequence per generation (Rogers and Harpending 1992; Rogers 1995). Here, we used two mutation rates: the widely assumed mtDNA mutation rate of 2.1 % per site per million years (reviewed in Weir and Schluter

2008) and the mutation rate estimate of 4.0% per site per million years of mitochondrial ND2 from the Galapagos mockingbirds (Arbogast et al. 2006). We assumed a generation time of 2.33 years, as determined in Chapter1.

We constructed Bayesian Skyline plots in BEAST v1.4.6 (Drummond and Rambaut 2007) to estimate historical changes in population size over time. This method uses Markov Chain Monte Carlo (MCMC) sampling techniques to estimate the posterior distribution of effective population size given a set of aligned DNA sequences and a model of molecular evolution, taking into consideration uncertainty in the genealogical process (Drummond et al. 2005). We used the best-fit model of molecular evolution selected by Modeltest. This analysis was run for 3×10^8 generations with model parameters and genealogies sampled every 1,000 generations under a strict molecular clock, of which the first 10% were discarded as a burn-in. Skyline plots were constructed using Tracer v1.4 (Rambaut and Drummond 2007).

Here, the criterion adopted to reject a hypothesis was a lack of correspondence between biogeographical events and population processes. In other words, we will consider the hypothesized geological (e.g. river course shift and mountain building) and climatic (forest fragmentation and expansion) processes to be consistent with the historical demographic processes (population subdivision and changes in population size) if they have overlapping confidence intervals.

Dating Historical Events

In order to validate the application of the 2.1% corrected sequence divergence per My for cyt *b* in birds (Weir and Schluter 2008) for our entire mtDNA data set, we compared pairwise model corrected genetic distances of the cyt *b* mtDNA gene alone with the genetic distances of the three remaining mtDNA genes, excluding the cyt *b*. In this comparison, we employed the GTR-I model for corrected distances (Weir and Schluter 2004), and the corrected genetic distances between cyt *b* only when the other mtDNA samples were strongly and significantly correlated ($r^2 = 0.95$; $P < 0.001$). In this case, we used the 2% rate of divergence to estimate divergence times.

We estimated the dates of origin of all fire-eye clades and sub-clades using a Bayesian statistical approach incorporated in the program BEAST. This analysis samples the time-to-most-recent-common-ancestor (TMRCA) values from the posterior density distribution generated by MCMC simulations. We estimated divergence times using an uncorrelated lognormal relaxed clock. This method infers the date of origin for the lineages without relying on a molecular clock and considers uncertainty in branch length and tree topology (Ho *et al*. 2005; Drummond *et al*. 2006; 2007). When estimating divergence times between species or populations, it is necessary to assume either a Yule tree prior or a coalescent tree prior, depending on whether coalescent events are completely relevant. Here, we estimated divergence times assuming a Yule tree prior for the well-supported clades and sub-clades identified in the phylogenetic analyses described above. We used a reduced data set, which included one individual from each phylogeographic lineage of fire-eyes. To incorporate evolutionary information specific to each codon position, we performed partitioned analyses in BEAST after having inferred the appropriate nucleotide substitution model for each codon position in MODELTEST. Two independent MCMC analyses were run for 60,000,000 generations, discarding the first 6,000,000 as burn-in, and sampling parameter values every 1,200 generations. The two independent runs were combined to obtain an estimate of the posterior distribution. In each independent run, we inspected for convergence of the chain to the stationary distribution using the program Tracer. This strategy ensured that the TMRCAs were well sampled (ESS values > 200).

3. Results

Phylogenetic relationships within fire-eyes

The Bayesian, ML and MP analyses based on the four mitochondrial genes and seven outgroup taxa recovered fire-eyes as a monophyletic group with high support (Figure 2). Within fire-eyes, all three phylogenetic methods identified the same three major, well-supported clades. The first clade was composed of the two Atlantic Forest species *P. leucoptera* and *P. atra*. Haplotypes recovered from these two species were not reciprocally monophyletic for any of the distinct analyses (Figures 2, 3 and 5). As revealed by the S-H test, enforcing reciprocal monophyly of *P. leucoptera* and *P. atra* haplotypes produced a significantly worse explanation of the data than produced by the optimal tree we recovered, in which those two species were not monophyletic (Table 2). Relationships and geographical variation in mtDNA within this clade are discussed in detail below, based on a more comprehensive geographical sampling of populations (see population history).

The second clade was formed by the populations of *P. leuconota* from northeastern Atlantic Forest and from southeastern Amazon east of the Xingu River (Figures 1A, 1B and 2). Samples from northeastern Atlantic Forest (subspecies *P. l. pernambucensis*), as well as from populations in opposite sides of the Tocantins River (subspecies *P. l. leuconota* and *P. l. interposita*), each form well-supported monophyletic groups in all three analyses. Samples from populations on opposite sides of the Tocantins River appeared as reciprocally monophyletic in all analyses, a result moderately supported by the Bayesian search (0.78 posterior probability), but less so by ML and MP (52% bootstrap for both). The third clade consists of the *P. leuconota* population occurring between the Xingu and Tapajós rivers (subspecies *P. l. similis*) and all other western South American fire-eye populations. Within this group, a well-supported basal division (1.00 Bayesian posterior probability and 100% ML and MP bootstrap) separates the Amazonian *P. l. similis* from all the western populations (Figure 2).

Further sub-clades can be described within this third clade, according to tree topology and geographical distribution of populations. All analyses recovered a well-supported (1.00 Bayesian posterior probability and 100% ML and MP bootstrap) separation between a clade formed by populations in the eastern foothills of the southern Andes and southwestern lowlands and a clade composed by populations in the central Andes (central Peru north) and the *trans*-Andean subspecies *P. l. pacifica*. Relationships within the first clade are discussed in detail below. Within the latter clade, all analyses revealed that defined subspecies do not correspond well with the recovered mtDNA clades. The subspecies *P. l. castanoptera* is paraphyletic with respect to both the southern subspecies *P. l. picea* and the *trans*-Andean *P. l. pacifica*, a result strongly supported in Bayesian (1.00 posterior probability), ML and MP (100% bootstrap for both) analyses. More specifically, the northern samples of *P. l. castanoptera* and *P. l. pacifica* are grouped together with high nodal support and a second well-supported clade was formed by a single northern sample of *P. l. castanoptera* and all southern *P. l. castanoptera* plus an individual of *P. l. picea*. A ML search in which the monophyly of *P. l. castanoptera* was enforced provided a significantly worse explanation of the mtDNA data, as shown by S-H tests (Table 2).

The phylogenetic analyses did not resolve the early branching events in fire-eyes with strong support. Although a sister relationship between populations south of the São Francisco River in Atlantic Forest (*P. atra* and *P. leucoptera*) and the populations of western South America (including the Amazonian *P. l. similis*) was recovered in Bayesian searches with relatively high posterior probability values (0.90) for the mtDNA data set, this relationship was not recovered in both ML and MP bootstrap analyses. Instead, ML and MP bootstrap consensus trees collapsed the three major clades in a basal polytomy. Constraining the topology by enforcing *P. leuconota* as monophyletic also could not be rejected under a maximum-likelihood framework (Table 2).

Analyses performed with the combined analyses of mitochondrial and nuclear sequences (Figure 3) resulted in similar tree topologies to those produced by the mitochondrial data set. The nuclear BF5 sequences did not provide further resolution in the basal relationships within the fire-eyes, as low nodal supports were recovered in both Bayesian (0.60 posterior probability), ML (75% bootstrap) and MP (63% bootstrap) analyses. Although the information content in the BF5 was limited, the Bayesian analysis resulting from the combined data set increased support for the sister relationship between Amazonian populations separated by the Tocantins River from 0.76 posterior probability for mtDNA only to 0.94 posterior probability for the combined data set.

Tempo of diversification of fire-eyes

The age estimate of node A, the TMRCA for the genus *Pyriglena*, suggests diversification began during the early Pleistocene, approximately 2.5 My (Figure 4 and Table 3). Because the basal relationships within fire-eyes could not be resolved unambiguously, we estimated two combinations of the TMRCA for the three major clades (Table 3). Forcing monophyly between the major clades 1 and 3 rendered a TMRCA estimate (2.20) slightly larger relative to the TMRCA estimate (2.12) of clades 2 and 3. Sub-clades in clade 2 are estimated to have begun diverging approximately 0.49 My (node B), with the separation of northeastern Atlantic Forest from southeastern Amazon. Shortly after this, a cladogenic event splitting populations across the Tocantins River is estimated to have occurred around 0.34 My (but see first Chapter 1 for alternative time estimates). The earlier branching event in clade 3, representing the separation between the Amazonian *P. l. similis* and all western South American populations, was approximately 1.66 My (node E). After this, the southern Andean-western lowlands sub-clade separated from the central Andean sub-clade at about 0.67 My (node F). Within the central Andean sub-clade, north-south divergence is estimated to have occurred around 1.06 My. Finally, divergence between the population west of the Andes and the eastern Andean populations appears to have begun approximately 0.37 My (node H).

Population history and mitochondrial variation in Atlantic Forest

We recovered 76 distinct mtDNA ND2 haplotypes from 266 individuals of *P. leucoptera* and *P. atra*. The haplotype network reveals three main features (Figure 5). First, there is an extensive haplotype sharing between *P. leucoptera* and *P. atra* along the parapatric zone (i.e. along the Paraguaçu River, Figure 1B). The lack of reciprocal monophyly between haplotypes recovered from the two species may suggest both gene flow and incomplete lineage sorting (see discussion). Second, clusters of haplotypes from different regions of Atlantic Forest are segregated in different parts of the network and connected by as few as one fixed substitutional change. Despite this shallow divergence, the network depicts a pattern of significant geographical association of haplotypes, with clusters of haplotypes spanning a south-north axis (blue and red colors in Figure 5). In the lower and left side of the network, the first haplotype cluster includes populations sampled mostly in the southernmost region of Atlantic Forest south of the hypothesized Bahia refuge, although the most common haplotype in this cluster also has been recovered in two individuals in the northern populations (localities 35 and 39, Figures 1 and 5). Other clusters of haplotypes include populations in the central and northern part of Atlantic Forest, respectively (Figure 5). The network shows that some localities in the central (e.g. localities 17 and 23) and northern parts (e.g. localities 33 and 34) of Atlantic Forest harbor haplotypes from different haplotype clusters. The third important feature is the connection between the group of haplotypes shared by *P. leucoptera* and *P. atra* to the southernmost group of haplotypes, rather than to geographically nearer haplotypes, revealing a biogeographical knot (Figure 5).

SAMOVA did not recover an unambiguous grouping number because the F_{CT} values increased as the number of groups (K) increased (Figure 6). For all values of $K \geq 4$, some groups defined by SAMOVA were made of a single population, implying that group structure could have disappeared. Although retaining the grouping pattern corresponding to $K = 4$ would be considered valid by some (e.g. Heuertz et al. 2004), we consider such a decision arbitrary. Our SAMOVA results failing to identify distinct groups may be related to i) the presence of isolation-by-distance along Atlantic Forest (see below), a fact that could lead to an incorrect identification of groups of populations in the absence of real genetic barriers and ii) the fact that only one locus was used, which could have added a large stochastic component due to the coalescent process. Under these conditions, SAMOVA can return unreliable results (Dupanloup et al. 2002). We thus will neither draw conclusions based on SAMOVA nor discuss its results further.

Mantel tests revealed that fire-eye populations south of the Bahia refuge exhibit no association between geographic distance and genetic distance ($r = 0.09$, $P > 0.05$; Figure 7a), whereas populations inside the Bahia refuge were consistent with the predictions of an isolation-by-distance model either including ($r = 0.84$, $P < 0.05$; Figure 7b) or excluding ($r = 0.84$; $p < 0.001$; Figure 7c) areas south of the parapatric zone of *P. leucoptera* and *P. atra*. The nucleotide diversity showed a trend of decreasing values southward and outside the Bahia refuge, whereas no prominent geographic variation was detected for haplotype diversity values (Figure 8).

Populations from the hypothesized Bahia refuge show no signature of demographic change as detected by the Ramos-Onsins and Rozas' (2002) R_2, Tajima's D (1989) and Fu's (1997) test. The only exception is the locality in the southernmost part of this refuge, which presented a signature of historical population expansion as given by the R_2 test (Table 4). For populations south of the Bahia refuge, we only detected historical changes in population size for the southernmost localities (sites 1, 5 and 7, Figure 1B). Although the credibility intervals of the Bayesian skyline estimates were wide, the analysis showed trends that indicate stable population sizes inside the Bahia refuge during the last glacial maximum (ca. 0.020 My) followed by a short period of population decline during the last 2-3 thousand years (Figure 9a). By contrast, population expansion in the area south of this refuge was inferred during the last 30 thousand years, which roughly coincide with the onset of humid periods following the last glacial maximum (Figure 9b).

Population history and mitochondrial variation in southwestern South America

Our results from the statistical parsimony network show that haplotypes from the southwestern South American group (the "*maura* group") separate into two or three distinct groups (Figure 10). One cluster of haplotypes, recovered from individuals in the Brazilian Pantanal floodplain and along the mid-Guaporé River Valley, was separated from a second group by one mutational step. Haplotypes from this second group consisted mostly of individuals in the eastern Andean foothills together with a single individual from the upper reaches of the Guaporé River. Further north, we recovered another group of haplotypes present in southern Peruvian Andean foothills that was separated by two steps from the second group. Based on the larger mtDNA data set (Figure 2), the easternmost group still appears monophyletic with respect to the two western groups but a separation of the latter in two distinct groups is not supported by this analysis. We detected a signature of population expansion for both eastern and western groups analyzed separately (Table 5). Assuming mutation rates of 2.1% and 4%, point estimates correspond to historical demographic expansion in years before present of 11,211 and 21,317 for the eastern group, and of 6,951 and 13,239 for the western group.

Population history and mitochondrial variation in central Andes

The statistical parsimony network recovered a complex pattern of relationships among fire-eye populations in the central part of the Andes. Our analysis shows that haplotypes from the central Andes constitute possibly four distinct groups separated from each other by several mutational steps (Figure 11). One cluster of haplotypes from *P. l. castanoptera* was recovered from individuals in southeastern Ecuador and northern Peru north of the Marañón River. This analysis corroborates the sister relationship between this northern group of *P. l. castanoptera* and the *trans*-Andean *P. l. pacifica* (Figure 2). Two other distinct clusters of haplotypes also can be identified. One comprises haplotypes of *P. l. castanoptera* collected in Huánuco and Pasco in central Peru, with one haplotype in this cluster identical to the haplotype sequenced from a single *P. l. picea* individual from the department of Junín, whereas the other cluster includes haplotypes from Zamora-Chinchipe in southeastern Ecuador. This latter cluster of haplotypes is closely allied to the cluster comprising the southernmost sampled populations rather than those from the haplotype cluster that includes samples from the same population and from geographically closer populations.

Rivers as historical barriers

For the Brazilian Atlantic Forest and the Pantanal floodplain, the results from the partial Mantel tests show that some rivers do not constitute primary barriers to diversification (Table 6). This contrasts with the pattern found in the Amazon region, where the Tocantins River has been shown to restrict gene flow in fire-eyes (see Chapter 1).

4. Discussion

Geological and Climatic Processes and Fire-eye Diversification

Identifying historical factors that underly the diversification of organisms is recognizably difficult. This issue is especially relevant in the Neotropics, where biological diversity is the highest in the world but where only few large-scale studies have been conducted to test primary factors causing population differentiation. Despite the controversy surrounding the alternative mechanisms of speciation in this region (e.g. Endler 1982; Tuomisto and Ruokolainen 1997, Patton and Silva 1998; Moritz et al. 2000), our dense geographical sampling and the phylogenetic and population genetic approaches employed here provide insights into the relationship between spatial and temporal expectations of climatic and geological scenarios that are relevant to diversification processes in this region.

Phylogenetics and biogeography of fire-eyes

Owing to weak nodal support, the basal relationships within fire-eyes could not be resolved with certainty by any of the three phylogenetic methods. This lack of resolution for the early branching events in fire-eyes might reflect rapid or simultaneous diversification that occurred in the early evolutionary history of the group. A rapid cladogenesis hypothesis is consistent with the completely unsupported basal branch, and by the fact that the three major clades are approximately equally divergent from each other. The apparently rapid differentiation of fire-eyes precludes strong inference about the geographic context of differentiation at this phylogenetic level. However, it is unlikely that fire-eyes have attained their current distribution and remained undifferentiated through gene flow over long periods. Rather, we suggest that the current geographical distribution of fire-eyes originated by rapid colonization from a single geographical origin, most likely from an Amazonian source (see below) and that several populations became evolutionarily independent (i.e. phenotypically and genetically differentiated) relatively soon after their continental spread. Thus, our results suggest that fire-eyes may have diversified in South American forests in a relatively short time and that even a fast evolving molecule like mtDNA might not have accumulated sufficient phylogenetic signal to resolve relationships among major clades. The possibility that populations classified as *P. leuconota* form a monophyletic group could not be rejected based on S-H tests (Table 2). In this case, however, the common ancestral lineage in which the male plumage characters arose must have originated and persisted for only a short period before the two major *leuconota* mtDNA lineages separated from each other.

A comparison of the fire-eye results with another circum-Amazonian avian group, the *Phaeothlypis* wood-warbler complex (Lovette 2004), shows concordance in the lack of phylogenetic resolution at the basal nodes and a temporal congruence in the deeper splitting events. Assuming a molecular clock calibration of 1.6% per million years, Lovette estimated that the separation of the five basal wood-warbler lineages was between 3.0 and 3.8 My. Adopting either the same calibration rate of 1.6% My for fire-eyes (not shown) or the BEAST 95% HPD age intervals based on the 2.1 % rate (nodes A and B, Figure 4), reveals a temporal pattern of divergence for the deepest nodes in the fire-eye phylogenetic tree slightly younger but in general agreement with the wood-warbler estimates. Relative to fire-eyes and wood-warbler complexes, divergence ages of South American forest plant taxa exhibiting geographic and ecological distributions common to these and other avian circum-Amazonian groups (compare maps in Prado and Gibbs 1993, Pennington et al. 2000 with Remsen et al. 2001, Bates 1997) were primarily older, reaching ca. 18 My for earlier splitting events (Pennington et al. 2004). However, Pennington et al.'s analysis also uncovered younger divergence events of Pleistocene age and concluded that a combination of ancient and young separation events best explains diversification in these plant taxa. Only further studies will reveal whether the generality of this temporal pattern also applies to avian circum-Amazonian groups.

Nevertheless, the fire-eye and the wood-warbler historical patterning may suggest that the initial phylogenetic divisions among lineages in circum-Amazonian avian taxa may have originated rapidly and during a similar time period, and that this likely reflects a set of underlying evolutionary processes that are common to avian groups exhibiting this distribution pattern. However, whether rapid diversification is a pattern only occurring in circum-Amazonian groups or if it represents only a general pattern resulting from a common set of processes acting in a wide-spread Neotropical avifauna, has yet to be determined. The fact that poorly supported nodes have been also found in other Neotropical forest avian groups (i.e. montane birds; Peréz-Emán 2005, Cadena et al. 2006), suggests that rapid diversification might be indeed a widespread historical phenomenon in this region.

Despite low support for early branches, several tip nodes are well-supported by all three phylogenetic methods, which allows us to make inferences about the mode and tempo of diversification in fire-eyes. We make such inferences below for two of the three main clades recovered in our phylogenetic analyses. Diversification in clade 1 is discussed in detail in the population history section based on a denser geographic sampling (see below).

Diversification in clade 2

Our phylogenetic analyses indicate that the first separation within clade 2 was between northeastern Atlantic Forest and southeastern Amazonia, with subsequent divergence across the Tocantins River. The sister relationship between the populations across the Tocantins River is supported by all tree-building methods for the mtDNA data set, but with low bootstrap and posterior probability support. Although a sister relationship between the Northeastern Atlantic Forest (subspecies *P. l. pernambucensis*) and populations on the eastern bank of the Tocantins River (subspecies *P. l. leuconota*) cannot be rejected under a maximum-likelihood framework for the mtDNA data set (Table 2), additional evidence supports the sister relationship across the river. First, the reduced data set (mtDNA plus BF5) support for this relationship was high for both Bayesian and ML analyses. Second, a haplotype network clearly places the populations across the river as sister with respect to the northeastern Atlantic Forest population (results not shown). Third, plumage analyses show diagnosable differences of female plumage in northeastern Atlantic Forest whereas populations across the Tocantins River are not distinguishable (M. Maldonado-Coelho pers. obsv.).

Our BEAST dating estimates suggest that the separation events that led to the origin of the three well-supported lineages in clade 2 are recent and occurred in a nearly simultaneous fashion. BEAST estimates suggest a diversification origin for this clade during the middle/late Pleistocene, with the divergence between Atlantic Forest and Amazonian fire-eyes followed shortly by the separation across the Tocantins River in southeastern Amazonia (Table 3). The Atlantic Forest of northeastern of Brazil is currently geographically isolated from Amazonia to the west by the semiarid Caatinga biome and from the rest of Atlantic Forest to the south by the São Francisco River. Geomorphic evidence based on travertine and speleothem deposits indicate that various wet pulses occurred in the currently semi-arid region of Caatinga during the last 0.210 My but also that moist phases extended back to 0.40 and 0.90 My (Auler et al. 2004, Wang et al. 2004). This evidence confirms earlier impressions based on palynological (de Oliveira et al. 1999) and fossil (Cartelle and Hartwig 1996, Hartwig and Cartelle 1996) data that moist forests had expanded into the dry Caatinga region during humid periods of the Pleistocene. Using the temporal framework of wet/dry cycles proposed by this geomorphic data, Carnaval and Bates (2007) found molecular divergence between frog populations in naturally isolated enclaves of moist forests within the semi-arid Caatinga and in coastal Atlantic Forest consistent with drier intervals between wet phases during the last 0.210 My. Our divergence time estimate between fire-eye populations in northeastern Atlantic Forest and southeastern Amazon is also consistent with a dry but earlier time interval (ca. 0.5 My) following one of the pluvial maxima estimated from the geomorphic data during the middle Pleistocene (0.4 My, Wang et al. 2004). Our data thus identify Pleistocene climate oscillations as the likely underlying vicariant mechanism for divergence of fire-eye populations between Amazonian and northeastern Atlantic Forest and comparisons with recent studies in this region (Carnaval and Bates 2007) suggest that vertebrate lineages (in this case birds and frogs) have responded differently to Pleistocene effects in forest distribution.

The Atlantic Forest of northeastern Brazil has been identified as an area of endemism for animals and plants (“the Pernambuco centre of endemism”; Brown 1982, Prance 1982, Costa et al. 2000, Silva et al. 2004). For birds, its biogeographical affinities seem complex as some taxa and populations appear to be more closely related to taxa/populations in Amazonian Forest while others are related to populations in southern Atlantic Forest (Teixeira and Gonzaga 1983a,b, Teixeira et al. 1986, Teixeira 1986, Vielliard 1996). Our results clearly indicate that northeastern Atlantic Forest fire-eyes share a common history with southeastern Amazonia and confirm the occurrence of a past forest connection between the two regions. Also, the presence of a well-supported clade of fire-eyes restricted to this region supports the notion that forest taxa have been evolving in isolation in northeastern Atlantic Forest. Further comparative phylogeographical studies are necessary to elucidate the historical relationships of the Pernambuco center of endemism to other Neotropical forests and to assess whether or not there are other unique evolutionary lineages in other organisms. Detail on the evolutionary dynamics of fire-eyes in southeastern Amazonia along the Tocantins River valley is provided in Chapter 1.

Diversification in clade 3

Detailed discussion of phylogeographic patterns in the sub-clade "*maura* group" is provided below (see population history), and we discuss now only the general patterns in clade 3. The basal split in clade 3 separates the population in the Xingu-Tapajós interfluvium from all western fire-eye populations. A plausible link to this vicariant event can be established with the uplift of the Guimarães Plateau, a tableland that separates the Amazonian fire-eye populations from the Paraguay River Drainage populations. A BEAST estimate indicates that this divergence occurred between 2.2 and 1.2 My, a period consistent with the uplift event that culminated with the separation of the two drainage systems that has also been cited as important for *Cercomacra* antbirds (Silva 1992). All the subsequent diversification events within *Pyraglena* clade 3 appear driven by Pleistocene climatic oscillations, since they bracket a time scale that postdates suitable elevations and habitats to fire-eyes in southern and central parts of the Andes. Fossil floras (Gregory-Wodzicki et al. 1998, Graham et al. 2001, Gregory-Wodzicki 2002) and geological evidence (Gregory-Wodzicki 2000, Hartley 2003, Gosh et al. 2006) indicate that habitats favored by fire-eyes throughout their range (cloud forest and subtropical dry forest) and altitudes at which they presently occur in the Eastern Andean Cordillera (ca. 1000-2200 m a.s.l.) were already present long ago (ca. 10 My). Geological dating suggests the Bolivian Altiplano had risen to near its modern altitude (3700 m a.s.l.) by the late Miocene (ca. 6 My, Gosh et al. 2006). At these times, BEAST estimates (nodes F, G and H in Figure 4) suggest that diversification of fire-eyes in the Central Andean region had not commenced, which refutes entirely a scenario linking their divergence to vicariance via mountain building.

Instead, diversification events driven by shifts in the distribution of vegetation belts (Van der Hammen 1974, Hooghiemstra 1995) is a more plausible causal mechanism underlying fire-eye diversification in this region. The divergence time between *trans* and *cis*-Andean fire-eye populations (between 0.59 and 0.18 My, node H in Figure 4) suggest that range expansion to the western side of the Andes from the eastern slope may have occurred during a warmer/wetter time period with subsequent isolation and commencement of differentiation during a more cold/drier period during the late Pleistocene. A past connection between opposite slopes of the Central Andes could have been facilitated by the presence of a low Andean pass in the Marañón River valley in northern Peru, which has been suggested as an important dispersal corridor for birds and other vertebrates (Chapman 1926, Parker et al. 1985, Patterson et al. 1992). Importantly, molecular divergence based on mtDNA suggests that another *trans*-Andean endemic to the Tumbesian region (the antshrike *Thamnophilus zarumae*), may also have been derived from a *cis*-Andean ancestor that dispersed across the Andes (Brumfield and Edwards 2007). Separation between this species and its eastern Andean relatives, however, is suggested to have occurred at an earlier time (between 1.1 and 3.1 My) relative to the estimated separation between fire-eye lineages on opposite Andean slopes. The comparatively recent dating of *cis* and *trans*-Andean disjunction in our study also contrasts to earlier dates reported for birds and other vertebrates [(birds - 5.5-8.2 My for curassows, Pereira and Baker 2004; 6.95-8.69 My for *Pionopsitta* parrots, Ribas et al. 2005);

(mammals - around 6.8 My for *Alouatta* monkeys, Cortes-Ortiz et al. 2003)], which suggested a direct effect of mountain uplifting in the separation of lineages east and west of the Andes in these studies. Also noteworthy is the basal split between *cis* and *trans*-Andean lineages recovered in the latter studies, in contrast with the splitting across the Andes near the tip of the phylogeny identified in our study. These contrastiong patterns emphasize that the role of the Andes as a geographical barrier during the evolutionary history of vertebrate lineages is variable.

This is not to say that the uplift of the Andes had no influence on the process of fire-eye diversification; distributions of morphologically and genetically distinct lineages fire-eyes that are restricted to different parts of the humid eastern Andean foothills and to the western slope of the Andes show a strong effect of Andean geography on population differentiation. However, our dating of divergence events suggests that all the differentiation took place more recently than would be expected if the Andean uplift had a direct causal relationship, as suggested earlier (e.g. Pereira and Baker 2004; Ribas et al. 2005, 2007). Our results suggest instead that the Andes had a dispersal rather vicariance effect on the diversification of fire-eyes; the complex topography of the region in combination with glacial and interglacial shifts in the vegetation distribution may have created opportunities for colonization and geographical differentiation in this group. In fact, divergence time estimates in *Mionectes* flycatchers west and east of the Andes suggest that at least two cross-Andean dispersal events post-date Andean uplift (Miller et al. 2008). Taken together, these findings indicate that both landscape evolution via mountain building and climate history resulting in vegetation shifts played important roles in the regional diversification of fire-eyes and other avian lineages in western South America.

Our mtDNA phylogeny also indicates that evolutionary relationships among Andean populations are more complex than suggested by current taxonomy. The subspecies *P. l. castanoptera*, which has a wide distribution in the eastern flanks of the Andes from southern Peru to central Colombia, is paraphyletic; haplotypes recovered from the southern populations of *P. l. castanoptera* (locality 75 in Figures 2 and 11) grouped with the southern subspecies *P. l. picea*, whereas some northern populations form a clade that is sister to the trans-Andean subspecies *pacifica*. This surprising result that *P. l. pacifica* is nested within the mitochondrially diverse *P. l. castanoptera* suggests a complex biogeographical scenario of diversification, in which *P. l. pacifica* likely derived from northern *P. l. castanoptera* populations that could have been geographically isolated from more southern *castanoptera* by the Marañón River valley (see population history below).

The grouping of haplotypes of *P. l. castanoptera* with the southern *P. l. picea* suggests introgressive hybridization or incomplete lineage sorting. Secondary contact with hybridization is more likely since likely intermediates between these two subspecies have been collected in the department of Huánuco, southern Peru. These intermediate birds, identified by D. Stotz in the Field Museum of Natural History (personal commucation), have the underparts prominently black, as in the more northerly *P. l. castanoptera*, but have olive feathers confined to the belly and flank regions, a character typical of the southern *P. l. picea*. However, the only sequenced individual of *P. l. picea* in this study presented no intermediate plumage (Maldonado-Coelho pers. obs.). Although we cannot rule out that this southern clade of *P. l. castanoptera* represents a north-south phylogeographic split within this subspecies, the pattern found may represent introgression of the southern *P. l. picea* into *P. l. castanoptera*, or that the *P. l. castanoptera* mtDNA has introgressed southwards.

Unfortunately, our sparse geographical sampling in this region sheds little light on this issue and further study is necessary to clarify the direction of introgression in both mtDNA and plumage traits.

Population History

Atlantic Forest - In contrast to the fire-eye populations that occur inside the Bahia refuge, the Tajima's D, Fu's Fs, R_2 and the Bayesian skyline plot detected signatures of population size changes for populations sampled south of the Bahia refuge, which may imply a scenario of population expansion into deforested areas with the onset of more humid periods following the last glacial maximum. Moreover, the presence of few high-frequency ancestral haplotypes and various recently derived low frequency haplotypes in the southernmost populations is also consistent with theoretical expectation of a signature of recently expanded populations (Figure 5, Slatkin and Hudson 1991). Although confidence intervals of Schneider and Excoffier's (1999) estimates are wide, the timing of expansion of some populations south of the Bahia refuge is in general agreement with the idea of expansion when the assumed mutation rate of 4% (Arbosgat et al. 2006).

Additional evidence indicates that fire-eye populations have had different histories in the hypothesized Bahia refugium area relative to the southern region during the last glacial maximum: i) acceptance of the isolation-by-distance model for populations inside the Bahia refuge and its lack of support for southern populations and ii) there is a decrease in nucleotide diversity in the southern Atlantic Forest relative to populations sampled inside the Bahia refugium. Evidence for environmental instability and forest replacement by more open vegetation types in southern Atlantic Forest also is provided by palynological surveys (e.g.

Behling 1997, 2002; Behling and Negrelle 2001) as well as by other phylogeographic studies of birds (Cabanne et al. 2008), mammals (Tchaicka et al. 2007) and herps (Grazziotin et al. 2006, Carnaval et al. 2009), which detected similar signals of historical demographic change in this region.

However, our findings support the notion that forests likely persisted in the southern region of Atlantic Forest as shown by i) the presence of two distinct clusters of haplotypes geographically restricted mostly to this region that could reflect isolation in one or two refugia (Cruzan and Templeton 2000, Hewitt 2000) and ii) a signature of historical demographic changes for some but not all populations in the region south of the Bahia refugium, as given by Tajima's D, Fu's Fs, and R_2 tests. Phylogeographic discontinuities in different parts of Atlantic Forest have also been detected in other recent studies; including evidence for geographic isolation and genetic differentiation in putative glacial refuges in southern Atlantic Forest (e.g. Martins et al. 2007, Tchaicka et al. 2007, Cabanne et al. 2008).

Collectively, our results are consistent with the idea that the central and northern parts of Atlantic Forest have had distinct histories, with higher forest stability during the last Pleistocene glaciation in the northern relative to the southern region of Atlantic Forest as previously suggested (Carnaval and Moritz 2008, Carnaval et al. 2009), but also underscore the idea that genetic signatures detectable in modern populations may help to gain insights into the existence of refuges, which often cannot be identified using traditional palaecological or climate modeling approaches (see discussions in Cruzan and Templeton 2000, Hewitt 2000, Carnaval and Moritz 2008).

In the Neotropical region, topographically complex regions and areas along large river courses are thought to provide suitable habitats for persistence of forest organisms during cooling periods of the Pleistocene (Brown 1987, Brown and Ab'Saber 1979, Fjeldså et al 1999, García-Moreno and Fjeldså 2000). Although current phylogeographic data from southern Atlantic Forest are too limited to identify unambiguously potential forest refugia, our data corroborate these earlier proposals by pinpointing that some areas south of the hypothesized Bahia refuge, such as the foothills of Espinhaço and Mantiqueira mountain ranges and the valleys of large rivers such as the Paraná and the Paraíba do Sul (Table 4, Figures 1 and 5), could have retained mesic characteristics during the last glacial period and, thus, could have supported forests and forest-dependent taxa such as fire-eyes. However, we further suggest that fire-eye populations were able to maintain large population sizes in these putative southern refuges as no bottleneck episodes were detected in the Bayesian Skyline Plot (Figure 9b). One possibility is that these refuges were large and suitable enough to maintain large populations of forest-dwelling organisms such as fire-eyes. This scenario could be supported by our data, given large declines in female effective population sizes were not detected. We also cannot rule out the possibility that fire-eyes (and other Atlantic Forest taxa) persisted in the matrix that supposedly replaced the humid forest during the last Pleistocene glacial period. Palynological records and the modern fragmented distribution of seasonally dry forests indicate that this form of dry-adapted vegetation may have spread widely and replaced humid vegetation types during the last glacial period in South America (Pennington et al. 2000, Naciri et al. 2006, but see Mayle 2006); this, combined with the fact that fire-eyes and several other Atlantic Forest birds do occur in some forms of dry-forests in eastern Brazil (Maldonado-Coelho pers. obs.), may suggest a scenario in which Atlantic Forest fire-eyes also persisted in the matrix surrounding humid pockets of forest and thus were able to maintain large effective population sizes.

Connection between southern and northern populations of fire-eyes in Atlantic Forest

The haplotype network uncovered an intriguing and surprising pattern of relationships among fire-eye populations within the Atlantic Forest. The southern cluster of haplotypes is closely allied to the haplotype cluster recovered for populations in the contact zone between *P. leucoptera* and *P. atra* as well as to clusters of haplotypes in the central part of Atlantic Forest (Figure 5). Interestingly, a similar pattern of sister relationship between southern and northern populations was recovered in a recent phylogeographic study of another Atlantic Forest bird species, the *Xiphorhynchus fuscus* woodcreeper (Figure 4 in Cabanne et al. 2008). Although the sister relationship among these populations of *X. fuscus* is not well supported, it is intriguing that populations existing in distant geographical areas appear to be closely allied in the same fashion as the fire-eyes.

The disjunct pattern observed in our study may involve either 1) a scenario of range expansion northward that proceeded through two independent routes, one inland and one along the coast, or 2) extinction of the intervening populations of a formerly widespread lineage, followed by a secondary colonization in the central part of Atlantic Forest from southern populations. An expansion northwards could be now evidenced by the connection between the northern and southernmost haplotype clusters and a second expansion by the connection between the southernmost haplotype cluster and the cluster of haplotypes recovered in populations from the central part of Atlantic Forest. Although we cannot fully distinguish between these alternative scenarios in light of the available evidence, an inland biogeographic connection between northern and southern Atlantic Forest may have existed through central Brazil, which is supported by contemporaneous populations of Atlantic

Forest birds in gallery forests far inland (Silva 1996), including populations of *X. fuscus* not sampled in Cabanne et al. (2008). That haplotype clusters in our study are approximately equally divergent from each other (not shown), and divergence time estimates among the *X. fuscus* clades are apparently indistinguishable (Table 4 in Cabanne et al. 2008), provide some support for two independent biogeographic connections. At any rate, our results indicate a rapid and nearly simultaneous separation among lineages of fire-eyes and woodcreepers in Atlantic Forest and that a common set of evolutionary processes likely underlie this pattern. Similar phylogeographic information from additional taxa would help determine whether consistent patterns are observed in multiple lineages.

Assuming that the second biogeographical scenario is true, southern fire-eye populations should have invaded northern Atlantic Forest prior to the establishment of a climatically stable area (i.e. the Bahia refugium) in the central part of Atlantic Forest. The pattern recovered in the network in combination with a signature of population expansion in the region of the Bahia refugium prior to the last glacial maximum as indicated in the Bayesian Skyline Plot (Figure 9), provides some support for this hypothesis, that is, that a colonization wave from the southern part of Atlantic Forest into this region may have occurred at about 0.09-0.06 My. It also indicates that the isolation-by-distance pattern detected in the Bahia refugium was established recently (during the last 0.05 My) and illustrates the potential for more complex climatic history and historical changes in the geographic distribution of Atlantic Forest populations than envisioned by earlier studies (e.g. Carnaval and Moritz 2008), with a history of instability and oscillation over a larger spatial-temporal span that resulted in multiple shifts in the distribution of forest and forest-dependent taxa.

Gene flow and introgression between P. atra and P. leucoptera

The mitochondrial data provide evidence that *P. leucoptera* and *P. atra* have not evolved entirely independently of each other. This was not unexpected given that a hybrid and a likely introgressed *P. leucoptera* individual have been reported previously (Willis and Oniki 1982). In Atlantic Forest, *P. leucoptera* extends to the middle reaches of the north bank of the Paraguaçu River, and it occurs in more deciduous and dry forests relative to more humid habitat present along the coast where it is geographically replaced by *P. atra*. Current geographic isolation between the two species seems not to exist, although the area of contact has largely been cleared (see below).

The general lack of mtDNA differentiation between *P. leucoptera* and *P. atra* could be explained by two different historical hypotheses. One alternative is that these two species diverged so recently and rapidly that there has not been enough time for lineage sorting to produce significant and complete differentiation in neutral genetic markers (Niegel and Avise 1986). A second possibility is that the haplotype sharing and the genetic similarity of *P. leucoptera* and *P. atra* are due to past or ongoing hybridization and introgression of neutral markers across population and species boundaries. Different lines of evidence argue that both incomplete sorting and hybridization could account for the observed pattern. First, two haplotypes of *P. atra* group within a lineage of *P. leucoptera* haplotypes recovered from individuals sampled at localities geographically distant from the contact zone (Figure 5), which may indicate retained ancestral polymorphisms. The hybridization hypothesis is consistent with patterns of plumage and mtDNA variation along the contact zone, where we found plumage-based introgressed males of both *P. leucoptera* and *P. atra*. Importantly, one male collected during the 18th century (Museu Paraense Emilio Goeldi #32017) and identified as *P. leucoptera*, clearly represents a hybrid between the two species. This specimen has the entire interscapular patch with a pattern typical of *P. atra*, while the two white bands on the wings are characteristic of *P. leucoptera*. Unfortunately, we were unable to precisely determine where this individual was collected, as the label indicates only "Bahia" (see also Willis and Oniki 1982). Introgression and geographically restricted gene flow are implied by the fact that haplotype sharing between these two species is mostly restricted to the parapatric zone. Moving south from the Paraguaçu River, only 2% of *leucoptera* individuals had haplotypes identical to or clustering with *P. atra* haplotypes.

Collectively, the evidence suggests that hybridization may have taken place in the history of these species and that bidirectional introgression of plumage traits as well of introgression of maternally inherited neutral markers occurs along the contact zone. However, defining the direction of the introgression in neutral markers will remain difficult until further sampling is conducted along the contact zone and in the northern range of *P. atra*. Despite an intensive survey along the Paraguaçu River valley, the hybrid zone was not found, most likely because of extensive forest clearing (S. Sampaio *pers. obsv.*).

Southwestern South America - Both haplotype network and phylogenetic analyses revealed a possible western-eastern axis of phylogeographic differentiation in the *maura* group (Figure 10). Members of an eastern and a western group come close to each other along the upper and medium reaches of the Guaporé River, where they have been collected within 250 km of one another. Their existence, together with the signature of historical demographic expansion, may represent evidence of range fragmentation followed by population expansion, with the Mamoré-Guaporé river interfluvium possibly representing an area of secondary contact. However, a recent study in the same region observed a cline in mtDNA genetic variation for Bolivian populations of *Thamnophilus* antshrikes (Brumfield 2005), and we cannot rule out the possibility that these two groups also are the extremes of a cline in genetic differentiation that we have failed to detect because of sparse sampling. In any event, our demographic expansion results are temporally coincident and consistent with the long-suspected effects of drier climates during the Pleistocene Epoch in the Brazilian Pantanal floodplain (Ab'Saber 1986, Assine and Soares 2004) and in the Andean foothills (Vuilleumier 1971, Prance 1982) and with previous phylogeographic studies of vertebrates that showed pronounced post-glacial increase in population sizes in both regions (Marquez et al. 2006, Lopes et al. 2006, Koscinski et al. 2008).

Central Andes - A detailed analysis of the geographical variation in mtDNA depicted in the network (Figure 11) not only corroborates the idea that vicariance, dispersal and apparent introgressive hybridization all have played an important role in the diversification of central Andean fire-eyes, but also reveals a complex process of geographic differentiation not entirely apparent in the phylogenetic analysis (see diversification on clade 3 above). Although the geographical sampling in this region was relatively sparse, it is striking that some lineages separated by hundreds of kilometers are more closely allied to each other relative to populations geographically closer or to samples collected at the same localities. Genetic distances between geographically distant central and northern Andes of *P. l. castanoptera* are *ca*. 0.5% divergent, whereas divergences among nearer *P. l. castanoptera* populations in northern Peru or from the same locality reach *ca*. 1.3 %. The documentation of representatives of two distantly related haplotype groups in the same locality south of the Marañón River valley suggests that this region might constitute an area of secondary contact.

It is also intriguing that representatives of the divergent haplotype clusters on the opposite sides of the network have been recorded south and north of the Marañón River valley and that within each of these groups no identical haplotypes were shared across this geographic barrier. We suggest that this complex pattern of geographic variation in mtDNA documented in our study likely originated through a series of multiple independent dispersal events following isolation and divergence mediated by a combined historical barrier to dispersal effect imposed by the Marañón River valley (Vuilleumier 1975; Parker et al. 1985) and shifts in the montane forest distribution driven by past climatic oscillations (Van der Hammen 1974, Hooghiemstra 1995). If this scenario were true, it would represent further evidence of the effect of the complex topography of the Andes on changes in geographic distributions and in promoting diversification (see also Cadena et al. 2006, Dingle et al. 2006). More specifically, it would reveal an undocumented interchangeable effectiveness and permeability of the Marañón River valley during respectively dry and wet periods as a historical barrier to gene flow in forest organisms (see García-Moreno et al. 1998; Miller et al. 2007, Weir et al. 2008).

The role of rivers on the diversification of fire-eyes

Andes and Pantanal - Although several studies have shown that the Marañón River valley represents an important geographic barrier to both Páramo and cloud-forest birds based on distributional (Vuilleumier 1975; Parker et al. 1985) and phylogenetic (García-Moreno et al. 1998; Miller et al. 2007, but see Weir et al. 2008) evidence, our study shows that this river has not been an impermeable barrier to gene flow in fire-eyes (see above, Figure 11). The Apurímac River in southern Peru has also been considered a major geographic barrier to distribution limits (Weske 1985, but see Schuchmann and Zuchner 1997) and coincides with phylogenetic breaks (García-Moreno et al. 1998, Weir et al. 2008) of some Central Andean bird species and subspecies. We are unable to determine whether this river is a barrier to gene flow in fire-eyes as we lack samples of *P. l. picea* from the Cordillera Vilcabamba (AMNH specimens examined by M. Maldonado-Coelho) on the eastern bank of the this river. The effect of this barrier on the genetic structure of *P. l. picea* and the geographic barrier that caused the divergence between the clade including northern subspecies *P. l. picea*, *P. l. castanoptera* and *P. l. pacifica* from the southern Andean clade, await further investigation.

In the Pantanal basin, the Paraguay River does not impose a strong barrier for fire-eyes, as we recovered shared haplotypes among populations in opposite banks of this river. Although we lack other phylogeographic studies in this region, distributional limits of other birds also do not correlate with this river (Hayes 1995).

Atlantic Forest and Amazon - Along the Atlantic coastal forests, large rivers have been suggested to form geographical barriers to dispersal in many forest organisms. Major rivers have shown to correlate with distribution limits of birds (i.e. São Francisco, Doce and Paraíba do Sul Rivers; Silva and Straube 1996; Vielliard 1996, Silva et al. 2004), mammals (Costa et al. 2000) and with phylogenetic breaks in a lizard (Pellegrino et al. 2005). In this study, phylogeographic breaks in *P. leucoptera* did not correlate with any river barriers in central and southern Atlantic Forest. Likewise, genetic breaks in the Lesser Woodcreeper (*Xiphorhynchus fuscus*, Cabanne et al. 2007; 2008) did not coincide with these large river valleys. However, based on a previous work of Lacerda et al. (2007), Cabanne et al. (2008) suggested that one large river in the central part of the Atlantic Forest (the Jequitinhonha River) could have acted as a barrier to the genetic differentiation in *Thamnophilus* antshrikes. This claim is, however, incorrect given is the presence of a phylogeographic leak in the upper reaches of this river (localities 13 and 15 in Lacerda et al. 2007). Instead, the genetic break apparently occurs along the Araçuaí River, the major southern tributary of the Jequitinhonha River.

In northern Atlantic Forest, *P. leucoptera* and *P. atra* form a narrow contact zone along the Paraguaçu River where likely introgression of mitochondrial (Figure 5) and plumage (Maldonado-Coelho pers. obsv.) characters was observed. Assuming a model of allopatric differentiation, one possible and yet untested speciation scenario is that the ancestor of both forms was isolated across the lower Paraguaçu River, where differentiation by drift or divergent selection may have taken place. Later, population expansion northward following the onset of forest re-expansion during recent times, likely after the last glacial period, could have resulted in a zone of secondary contact as currently observed. This model would be validated if populations of *P. leucoptera* and *P. atra* along the coast exhibited a signature of demographic stability while populations in newly colonized headwater areas presented evidence of demographic expansion. Although Fu's F_s, Tajima's D and Ramos-Onsins and Rozas' R_2 show no evidence of demographic changes for all the populations along the coast in the area of parapatry, a signature of demographic expansion in the headwaters of the Paraguaçu River (locality 27) was detected by Tajimas's D test, which would provide partial support to this model.

It is intriguing that the deeper phylogenetic events in fire-eyes correspond to the position of the modern course of large rivers in the Amazon basin and in Atlantic Forest. For example, our results shown that the geographical distribution of members of clade 2 are bounded by the Xingu River to the west in the Amazon basin and by the São Francisco River in northeastern Atlantic Forest (Figures 1 and 2). It is conceivable that an “ancient” barrier effect of these two large rivers has been of primary importance in the origin of this clade, followed by further divergence within the region bounded by these rivers. Although we lack precise dating estimates of the placement of the modern course of the Xingu River, geomorphological evidence indicates that the São Francisco River once was a tributary of the Tocantins River in the Amazon basin and shifted its course southwards to its modern position in northeast Atlantic Forest (Barreto et al. 2002), probably during the Pleistocene (K. Suguio pers. com.). Importantly, confidence intervals of the BEAST relaxed clock estimate assuming a mutation rate of 2.1% per My (1.79-2.48 My) cannot reject the possibility that clade 2 originated during or since the formation of the modern course of the São Francisco River (Barreto et al. 2002).

Regional differences in the “River effect”- Factors that could determine whether river valleys act as effective barriers to population differentiation in some regions (e.g. Amazonia) but not in others (e.g. southern Atlantic Forest) for fire-eyes (and possibly to other vertebrates) can be related to factors such as their spatial stability as a barrier (i.e. geological history), strength (i.e. river width), spatial distribution of habitats (i.e. presence of suitable habitat in the headwaters) and natural history of organisms (i.e. habitat breadth). We suggest that one reason why Amazonian rivers represent stronger barriers to the dispersal for fire-eyes than most Atlantic Forest rivers is due to the fact that they are significantly wider (e.g. 3.0 and 1.3 km for the Madeira and Tocantins rivers, Ayres and Clutton-Brock 1992) than most Atlantic Forest rivers (Maldonado-Coelho pers. obs.). A second factor is the presence of suitable habitat (i.e. montane evergreen or semideciduous forests) and of fire-eyes (and other Atlantic forest birds) in the headwaters of most Atlantic Forest Rivers, where rivers are less wide and hence constitute weaker barriers to dispersal. In contrast, Amazonian fire-eyes seem not to occur in the upper reaches of the large southeastern Amazonian rivers (Xingu and Tocantins), where streams that constitute the headwaters are either bordered by gallery forests situated within the cerrado phytogeographic domain or are bordered by dry forests, habitats in which Amazonian fire-eyes do not occur (M. Maldonado-Coelho and L. F. Silveira pers. obsv.).

A digression on the causes and evolution of the circum-Amazonian pattern of geographic distribution

The circum-Amazonian pattern of distribution is defined as one in which sister taxa are found in montane forested areas on the humid slopes of the Andes and the coastal range of Venezuela or the Tepui region as well as in lowland forests south and east of the Amazon basin (Remsen et al. 1991). However, extensive non-overlapping parts of the distributions of several circum-Amazonian species also occur, which led Remsen et al. to further distinguish between a “complete” pattern and a “partial” pattern (see also Bates 1997). What explains the variations in circum-Amazonian distribution patterns? What are the historical and ecological factors underlying the origin and maintenance of these patterns? Answers to these questions and a clearer picture of the ecological and evolutionary determinants of the circum-Amazonian pattern of geographic distribution will become fully apparent only with further studies. However, we feel that a discussion at this stage on the potential causes underlying this distribution pattern, although highly speculative, may provide insights into its origins and maintenance, and highlight approaches for future research.

Because fire eyes and several other taxa exhibiting the circum-Amazonian distribution pattern occur in the southern part of lowland Amazonia basin or in areas encircling this region, we argue that circum-Amazonian distributions possibly reflect evolutionary interactions between birds and their enemies (i.e. competitors, predators or pathogens), that is, the origin of this distribution pattern might be largely unrelated to factors of the physical environment (i.e. niche conservatism) at the regional scale. It was suggested that the distribution of fire-eyes was shaped by their dependence on foraging following antswarms and their interactions with potential competitors over this foraging resource (Willis 1981). Because fire-eyes are behaviorally subordinate over antswarms, it has been hypothesized that they are geographically excluded in western Amazon by more aggressive and dominant antbird species (antbirds of the genera *Rhegmatorhina*, *Gymnopithys* and *Pithys*; Willis 1981). Support for Willis' insight can be found in a recent analysis of species density maps for ant-swarm antbird followers, which show a higher co-occurrence of dominant antbird species in western Amazonia (Brumfield et al. 2007). One alternative hypothesis to competitive exclusion is niche conservatism, that is, that fire-eye populations preferred elevations or habitats that originated prior to the geographical contact with competitors. It could be argued that this hypothesis is plausible as fire-eyes populations in the southwestern lowlands and along the Andean slopes belong to distinct evolutionary mtDNA lineages with respect to the ones from the Amazon basin that, in turn, could not expand their ranges into western and the northern regions of the Amazon basin due to the strong effect of larger rivers as historical barriers to dispersal. Although this hypothesis is plausible based on the evidence that larger Amazonian rivers seems to represent historical barriers to dispersal for fire-eyes (see Chapter 1), it is unlikely that larger rivers are impediments to range expansion into western Amazonia for members of clade 3 in Figure 2 (except the Amazonian *P. l. similis*). The fact that populations belonging to this clade exist in the lowland headwater regions of the Madeira River in western Amazonia (subspecies *P. l. maura*), but have not been able to colonize western Amazonia, minimize the possibility that niche conservatism is driving the circum-Amazonian distribution in fire-eyes. Also, one could argue that if members of a clade have distinct elevational distributions, it is less parsimonious to assume evolutionary stasis in elevational distributions. Members of clade 3 are present in the Amazonian basin (subspecies *P. l. similis*) and in the lowlands southward (subspecies *P. l. maura*), are restricted to the Andean foothills (ca. 1000-2300 m) from northern Bolivia to southern Colombia (subspecies *P. l. marcapatensis*, *P. l. picea* and *P. l. castanoptera*), and shift back to lowlands in the pacific Andean slope (subspecies *P. l. pacifica*). This demonstrates that elevational distributions differ among lineages of fire-eyes, but also implies that they do change over time. In other words, it is tempting to infer that physiological constraints probably have not have been of historical importance involving the circum-Amazonian distribution in fire-eyes.

Geographical exclusion due to competition over ant-swarms does not explain the absence of other circum-Amazonian species with distinct ecologies in some parts of the Amazon basin or in its entirety. The plain antvireo (*Dysithamnus mentalis*) is one of the forest taxa showing a complete circum-Amazonian pattern of distribution (Remsen et al. 1991), but is not a regular ant-swarm follower (Maldonado-Coelho pers. obsv.). This taxon exists in the lowlands around the upper reaches of the large southeastern Amazonian rivers as the Tocantins and Madeira Rivers as well as in the lowlands of the northeastern coast of Brazil to the eastern bank of the Tocantins River (Zimmer and Isler 2003). Because populations of the plain antvireo are present in the lowlands but do not exist in the central parts of the Amazonian basin, it is suggestive that its biogeographic history has been influenced by biotic interactions rather than as consequence of characteristics of the physical environment.

Although a shared common evolutionary history to the members of the circum-Amazonian distribution pattern can be inferred based on their overlapping ranges, the fact that extensive non-overlapping portions to their distributions also exist indicates that differences in ecology and evolutionary history also have to be invoked (Bates 1997). We agree with this statement, but further suggest that if the causes underlying the circum-Amazonian pattern were solely or mostly determined by the conditions of the physical environment, such broad discrepancies in the geographic ranges would not be so apparent. Instead, we suggest forest habitats around the periphery of the Amazon could represent long-term ecological opportunities for colonization and adaptation, and that biotic interaction, such as interspecific competition with taxa in lowland Amazonia and other forest regions, may act as a sorting mechanism with considerable variation in its strength among taxa at the regional scale. This would explain, for example, variation in the extent of lowland Amazonian forest present in the geographical range of circum-Amazonian taxa. This is not to say that the factors of the physical environment such as climate were irrelevant in shaping the evolutionary history and distribution of these taxa: the fact that local distributions of circum-Amazonian taxa seem to be tightly associated with individual tolerances to local environmental conditions (Maldonado-Coelho pers. obsv.) as well as the high likelihood that climate oscillations have had an important role in geographic differentiation and in present-day disjunct distributions observable in fire-eyes and other circum-Amazonian taxa (Remsen et al. 1991, Bates 1997), offer evidence to the contrary. Rather, we argue that it appears unlikely that the physical environment has played out a larger role than ecological interactions in the *origin* of circum-Amazonian distributions.

The geographical origin of circum-Amazonian taxa is difficult to identify unambiguously in light of available information for most taxa, but we suspect that at least two taxa might have been derived from distinct regional stocks. Willis (1981) considered "fire-eyes to be primitive or moderately modified former Amazonian birds that cannot compete well with crowded present-day ant-following guilds there." He proposed that fire-eyes could have been derived from an Amazonian ancestor since they are behaviorally alike to antbirds of the genera *Myrmeciza* and *Percnostola* (and also to the Atlantic Forest endemic *Rhopornis*). Although *Rhopornis* was lacking in the survey of Brumfield et al (2007), their phylogenetic analysis of representatives of the antbird assemblage confirmed that fire-eyes are nested in a clade with taxa represesntative of *Myrmeciza* and *Percnostola* gnera restricted to the Amazon basin. This could imply an Amazonian ancestry of fire-eyes followed by subsequent colonization of peripheral forest regions. However, sister relationships of other circum-Amazonian taxa likely reside outside the Amazon basin. For example, all congeners of the circum-Amazonian plain antvireo (genus *Dysithamnus*) are endemic to the periphery of Amazonian lowlands areas. This suggest that the geographical origin as well as secondary expansions have taken place from within the circum-Amazonian areas for this group and indicates that circum-Amazonian taxa may have originated from ancestors belonging to distinct geographical and ecological settings.

Conclusions

Our phylogenetic reconstructions and Bayesian dating estimates suggest that fire-eyes represent a rapid diversification in South America. It is conceivable that the origin of the three major clades recovered in our study trace back to older events, such as the formation of the modern course of the Amazon, Tapajós, Xingu and São Francisco Rivers, with subsequent diversification fostered by more recent events, such as the creation of the modern Tocantins River course (Chapter 1) and by Pleistocene climatic oscillations creating opportunities for range expansion and geographic isolation in the Andes, Pantanal, and Atlantic Forest. However, as demonstrated here and in Chapter 1, the role of large rivers as historical barriers to dispersal is apparently stronger in the Amazon basin relative to western South America (western lowlands and Andes) and Atlantic Forest. In contrast, climatic oscillations seem less important in creating opportunities for geographic differentiation within the Amazon compared to the other regions. Also, favorable climate conditions for population expansions following the last glacial maximum apparently extended across all of South America, as shown by consistent timing of expansions (Figure 12). If this scenario is correct, then fire-eyes have a complex history of diversification, involving large-scale geological and climatic processes acting over regional and continental scales during the last ~ 2.5 My. We suggest that factors underlying the circum-Amazonian distribution pattern could be dependent of evolutionary change in relation to the physical environment (e.g. climate) at a regional scale; however, it is conceivable that adaptations to local ecological conditions influenced by the balance of evolutionary relationships of circum-Amazonian taxa with other organisms are of larger importance.

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Table 1. Primers designed for PCR amplification and cycle sequencing.

Gene	Primer	Sequence (5’∏ 3’)
ND2	ND2pyrintH1	GGTGGGTGAGTTGGGTAATG
ND2	ND2pyrintL2	CATCGAGGCCACAACAAAAT
ND2	ND2pyrintH3	GGCAATGATTGTTGCTGTTG
ND2	ND2pyrintL4	CTCCATTAACGGGCTTTCTG
ATPase	ATPasepyrH	CATAGGCTTGAATTATGGCGAC
ATPase	ATPasepyrL	GCCTTTTAAGCTAGAGAAAGAGG

Table 2. Results of Shimodaira-Hasegawa tests for comparison of alternative phylogenetic hypotheses. The likelihood of the maximum-likelihood estimate of phylogeny for the four combined mitochondrial genes (-lnL=1406.443) was compared with those of trees recovered in maximum-likelihood analyses in which alternative topologies were enforced.

Enforced topology	Constrained tree -lnL	*p*-value
Monophyly of *P. leuconota*	14907.496	0.402
Monophyly of *P. l. castanoptera*	14959.883	0.006
Monophyly of *P. leucoptera* and *P. atra*	14968.974	0.002
Monophyly between *P. l. pernambucensis* and *P. l. leuconota*	14906.863	0.420

Table 3. Estimates of divergence dates (TMRCA) and the 95% highest posterior density values inferred for each node in Figure 4. Dates are in million years (My) before present.

Node	Mean (My)	95% Highest posterior density	
		Lower (My)	Upper (My)
A	2.5	1.81	3.27
B	0.49	0.26	0.77
C	0.34	0.15	0.56
D*	2.20 (2.12)	1.58 (1.79)	2.8 (2.48)
E	1.66	1.20	2.20
F	1.06	0.72	1.46
G	0.67	0.41	0.97
H	0.37	0.18	0.59

* numbers between brackets represent the TMRCA for the divergence time and 95% highest posterior density values between clades 2 and 3 in Figure 4.

Table 4. Sample size, number of haplotypes, and historical demographic analyses (Tajima's D, Fu's F_s and Ramos-Onsins and Rozas' R_2 tests) for populations of Atlantic Forest fire-eyes (*Pyriglena leucoptera* and *P. atra*) with sample size larger than five individuals. Locality numbers as in Figure 1. Samples of some populations geographically close were pooled to increase sample size in the analyses.

No.	Geographic region and locality	Sample size	Number of haplotypes	Tajimas's D	Fu's F_s	R_2
	Atlantic Forest					
1	Iguacu region	19		-1.47*	-3.46**	0.09
10	Morro Grande	28		-1.71*	-6.25***	0.06***
14	Morro do Diabo	8		-1.54*	-1.53*	0.34
16	Itatiaia	8		1.44	0.97	0.28
19	Cantagalo	8		0.45	-0.73	0.21
20	Itacolomi	7		-0.73	-1.45	0.14
21	Arcos	7		1.24	0.69	0.23
24	Santa Tereza	8		-0.44	-0.48	0.19***
25	Serra Piedade	14		-1.24	-2.41	0.10
26	Sooretama	5		1.12	1.22	0.25
31	Monte Pascoal	6		1.12	2.51	0.26
32	Porto Seguro	9		0.69	0.70	0.21
28	Mata Escura	6		-0.93	-1.91	0.18
29	Duas Barras	9		-1.00	-1.25	0.15
30	Limoeiro	10		0.50	1.45	0.19
33	Fazenda Santana	7		0.19	1.14	0.18
35	Ouricana	9		0.01	0.72	0.16
36	Sao Roque do Paraguaçu	8		-0.08	-1.58	0.23
37, 38, 39	Paraguaçu Headwaters	21		-2.13***	-0.87	0.13
43	Campina	10		0.02	1.52	0.18
44	Lontra	7		-1.36	-0.24	0.24
	Bahia refuge	64	21	-1.34	-9.35**	0.07
	South of the Bahia refuge	127	43	-2.46***	-27.78***	0.02***
	Pernambuco Refuge	14	8	0.62	-4.50**	0.18
	Southwestern South America					
	Western group	17	9	-0.94	-4.56**	0.10
	Eastern group	22	8	-1.59*	-3.61**	0.08*
	Central Andes					
	Oxapampa	9	3	0.156***	-3.106***	0.194
	Marañón River Valley	12	6	-0.54	-2.979**	0.155

*$P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. The Fu's test was considered as significant at the 5% level if its P value was below 0.02 (Fu 1997).

Table 5. Population estimates of Θ_0, Θ_1, τ and estimated time since the population expansion in y.b.p for fire-eye populations in South America, assuming two mitochondrial mutation rates (2.1% and 4.0%). Samples south of the Bahia refugium, from the Pernambuco refugium, from southern Andean foothills and from western lowlands were pooled into their respective groups to estimate global population expansion. Sampling localities numbered as in Figure 1.

Biogeographic Region and Sampling Locality	Θ_0	Θ_1	τ	Time of expansion	
				2.1%	4.0%
Atlantic Forest					
Bahia refuge	0.00 (0.00-1.659)	6.891 (3.64-99,999)	4.539 (0.938-8.254)	45,563 (9,436-83,038)	23,977 (4,962-43,602)
South of the Bahia refuge	0.401 (0.00-3.42)	2.32 (0.175-99,999)	2.889 (0.00-5.725)	29,056 (0-50,725)	15,254 (0-30,228)
Foz do Iguaçu (Locality 2)	0.594 (0.00-3.746)	2.245 (0.00-99,999)	1.832 (0-4.549)	18,430 (0-45,774)	9,693 (0-24,074)
Morro do Diabo (Locality 14)	0.00 (0.00-0.00)	0.186 (0.00-99,999)	3.0 (0.570-3.500)	30,181 (5,734-35,211)	15,847 (3,015-18,518)
Morro Grande (Locality 10)	0.004 (0.00-2.34)	3.37 (0.297-99,999)	3.13 (0.672-5.543)	31,480 (6,758-55,750)	16,527 (3,548-29,268)
Santa Tereza (Locality 24)	0.00 (0.00-0.004)	99,999 (4.342-99,999)	0.877 (0-2.736)	8,822 (0-27,526)	4,640 (0-14,453)
Headwaters of Paraguaçu			3.00 (0.410-3.127)	30,173 (4,123-31,450)	15,840 (2,164-16,511)
Pernambuco refuge	0.005 (0.00-4.437)	5.953 (0.370-99,999)	2.281 (0.00-6.008)	22,347 (0.00-60,362)	12,049 (0.00-31,746)
Southwestern South America (the "maura group")					
Western group	0.021 (0.00-0.977)	12.310 (0.00-99,999)	2.119 (0.406-3.939)	21,317 (4,084-39,627)	11,211 (2,148-20,341)
Eastern group	0.009 (0.00-0.946)	99,999 (1.451-99,999)	1.316 (0.00-2.727)	13,239 (0.00-27,364)	6,951 (0.00-14,428)
Central Andes					
Oxapampa (Locality 75)	0.004 (0.0-0.004)	99999,00 (99869,00-99999,00)	0.543 (0.262-1.230)	5,461 (2,635-12,371)	2,867 (1,383-6,494)
Marañón River Valley (Localities 77, 78 and 79)	0.012 (0.00-0.026)	6.187 (1.67-99999,00)	1.52 (0.586-4.682)	15,287 (0,589-47,090)	8,026 (3,094-24,722)

Table 6. Role of South American rivers in the diversification of fire-eyes. The Mantel tests were performed for three river basins in this study**. *r* = partial correlation between the corrected genetic matrix and the binary matrix (the river barrier) after controlling for geographic distance.

River	Geographic Region	Historical Role	*r*
Paraíba do Sul	Atlantic Forest	none	7.84
Doce	Atlantic Forest	none	17.45
Jequitinhonha	Atlantic Forest	none	28.78
Tocantins**	Amazon Forest	primary	89.36*

*$P<0.0001$; ** data from Chapter 1.

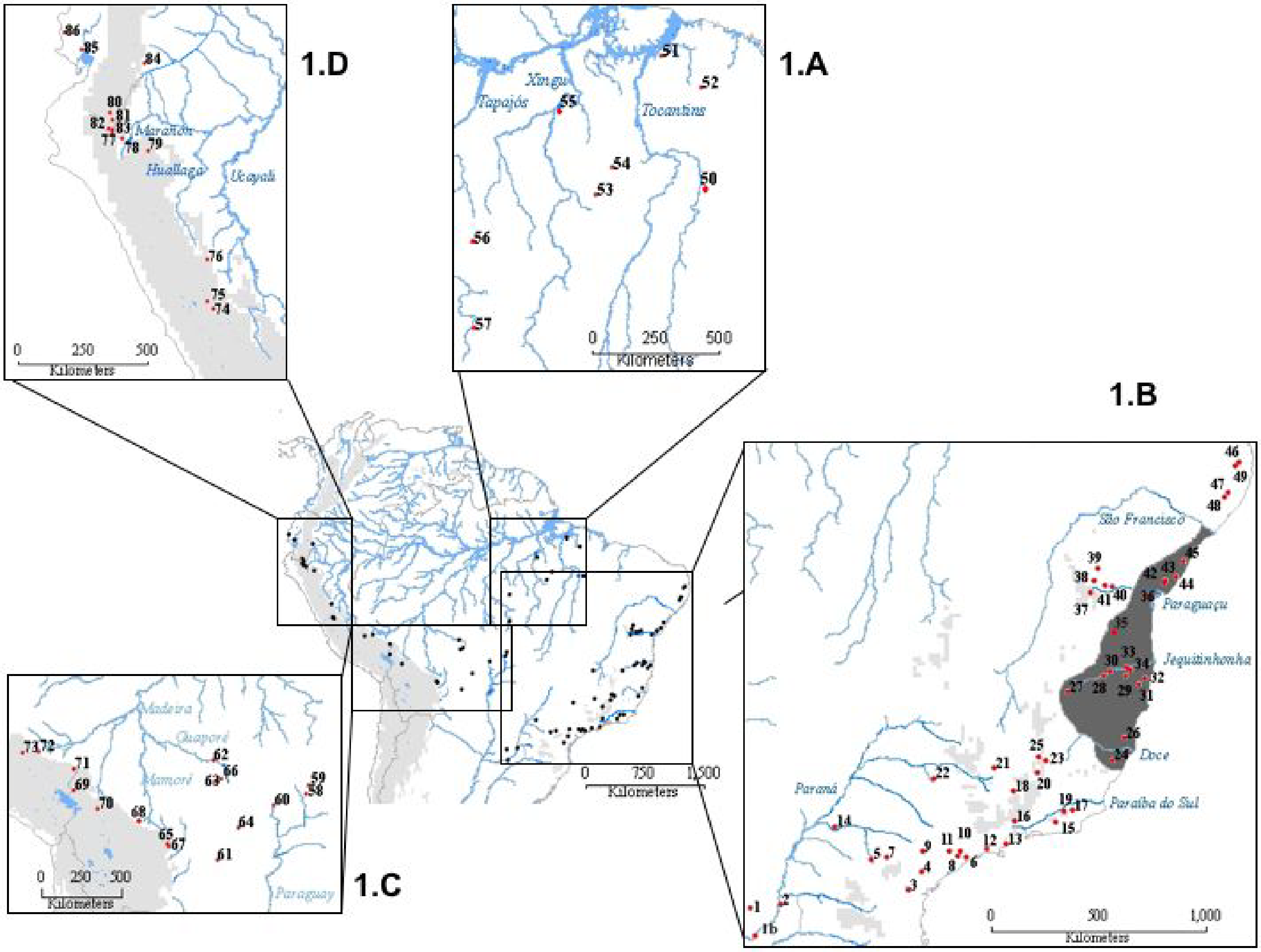

Figure 1. Sampling localities of fire-eye antbirds (genus *Pyriglena*) across South America. 1.A) Southeastern Amazon; 1.B) Atlantic Forest. The dark grey shaded area depicts the Bahia Pleistocene refuge (after Carnaval and Moritz 2008); 1.C) Southwestern South America (the "*maura* group"); 1.D) Central Andes. Rivers and River names are depicted in light blue. Light shaded gray indicates areas above 1000 m a.s.l.

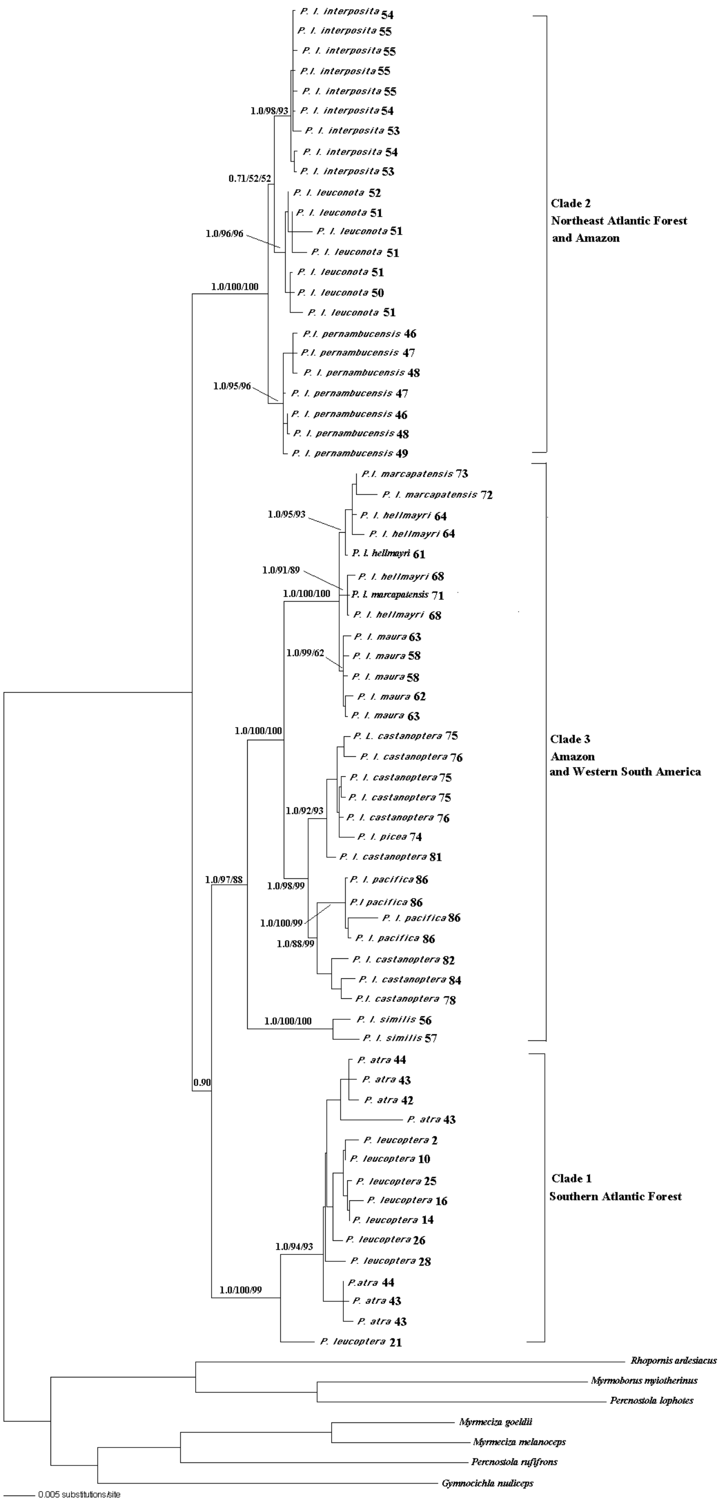

Figure 2. Maximum-likelihood phylogram showing relationships among fire-eye populations and taxa based on combined analyses of 3213 aligned base pairs of four mitochondrial. Numbers following taxa names represent localities as in Figure 1. Numbers on branches indicate Bayesian posterior probabilities and bootstrap values obtained under maximum-likelihood and maximum-parsimony. Lower clades represent outgroups used to root the fire-eye phylogenetic tree.

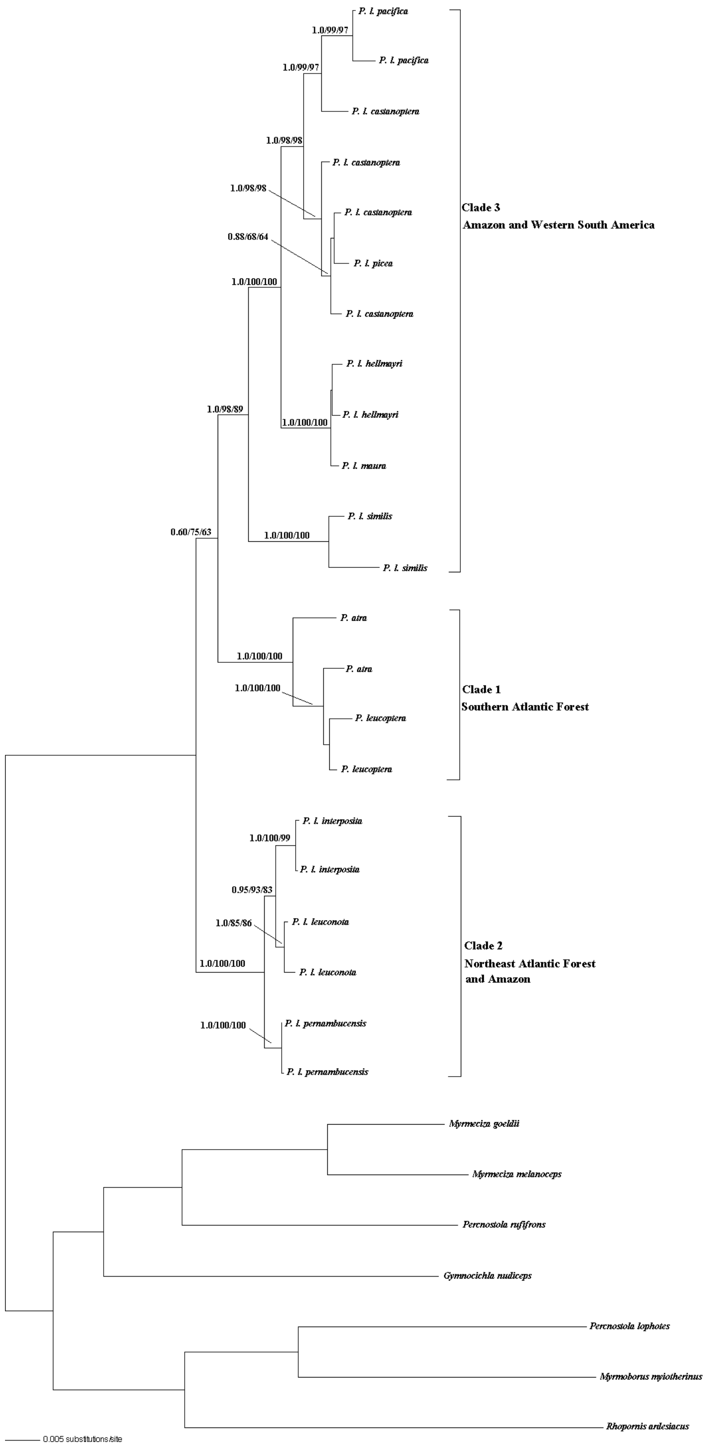

Figure 3. Maximum-likelihood phylogram showing relationships among fire-eye populations and taxa based on combined analyses of 3748 aligned base pairs of four mitochondrial and one nuclear gene. Numbers on branches indicate Bayesian posterior probabilities and bootstrap values obtained under maximum-likelihood and maximum-parsimony. Lower clades represent outgroups used to root the fire-eye phylogenetic tree.

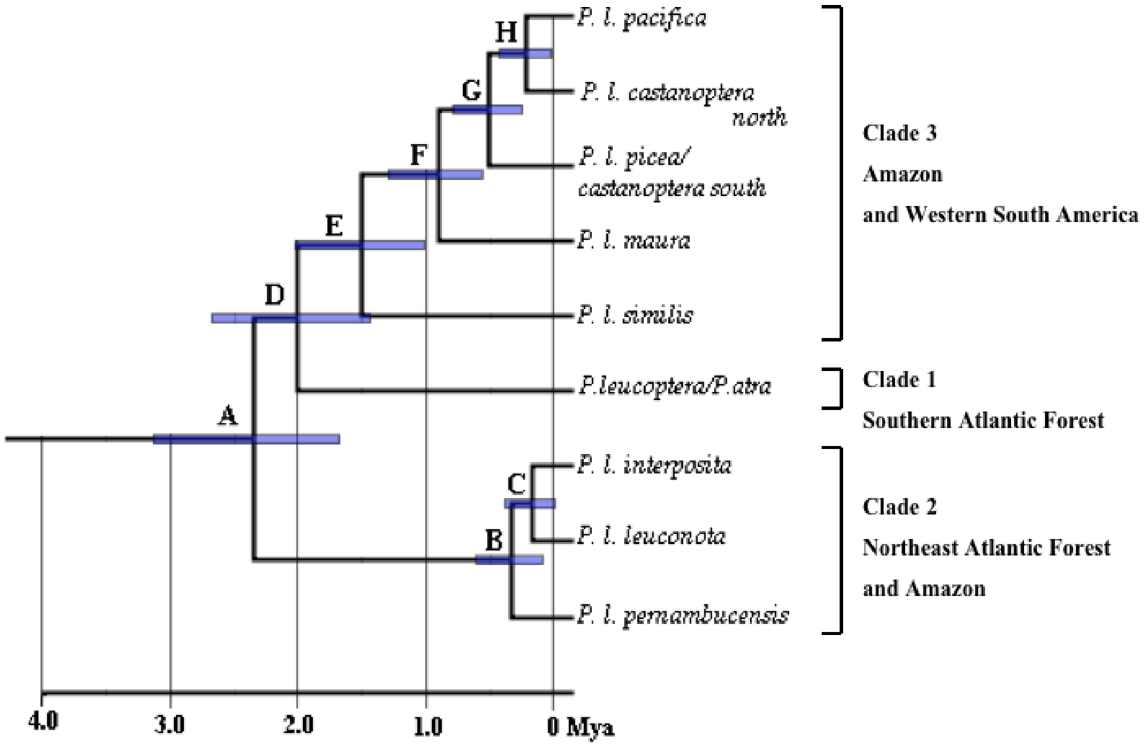

Figure 4. Bayesian chronogram of fire-eyes diversification inferred using the 2.1% mutation rate. The blue bars represent the 95% credibility intervals for the TMRCAs estimated in BEAST. See Table 3 for Bayesian estimation of TMRCA's values for each node.

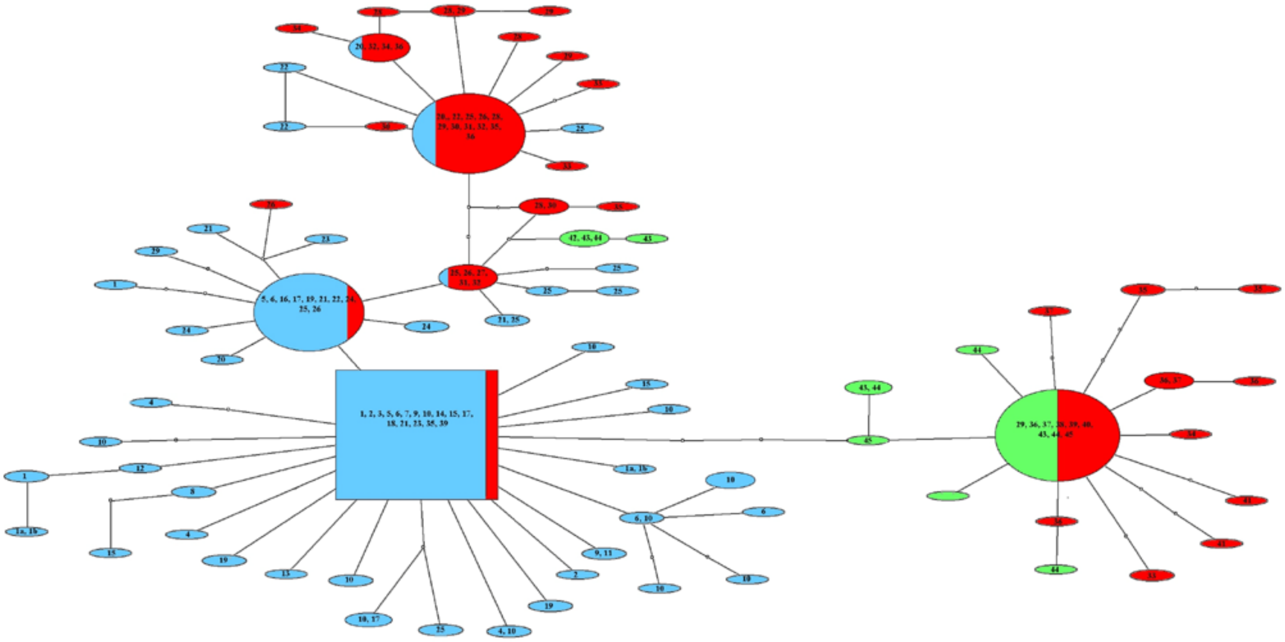

Figure 5. Statistical parsimony network among the haplotypes of the Atlantic Forest *P. leucoptera* and *P. atra* populations based on ND2 sequences. Each circle represents a different haplotype with size proportional to its relative frequency. The numbers correspond to sampling localities labeled in Figure 1B. Colours correspond to i) blue: area south of the Bahia refuge; ii) red: area inside the Bahia refuge; iii) green: haplotypes recovered in *P. atra* individuals. See text for details.

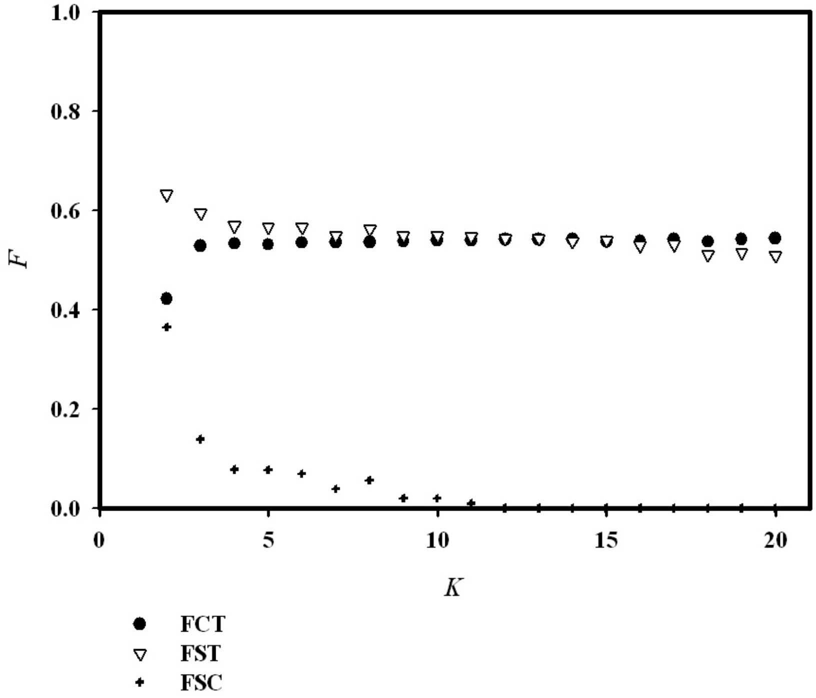

Figure 6. Fixation indices (*F*) as a function of grouping number (*K*). F_{CT}, genetic differences among groups of populations, F_{ST} genetic differences among populations and F_{SC} genetic differentiation among population within groups obtained with the SAMOVA.

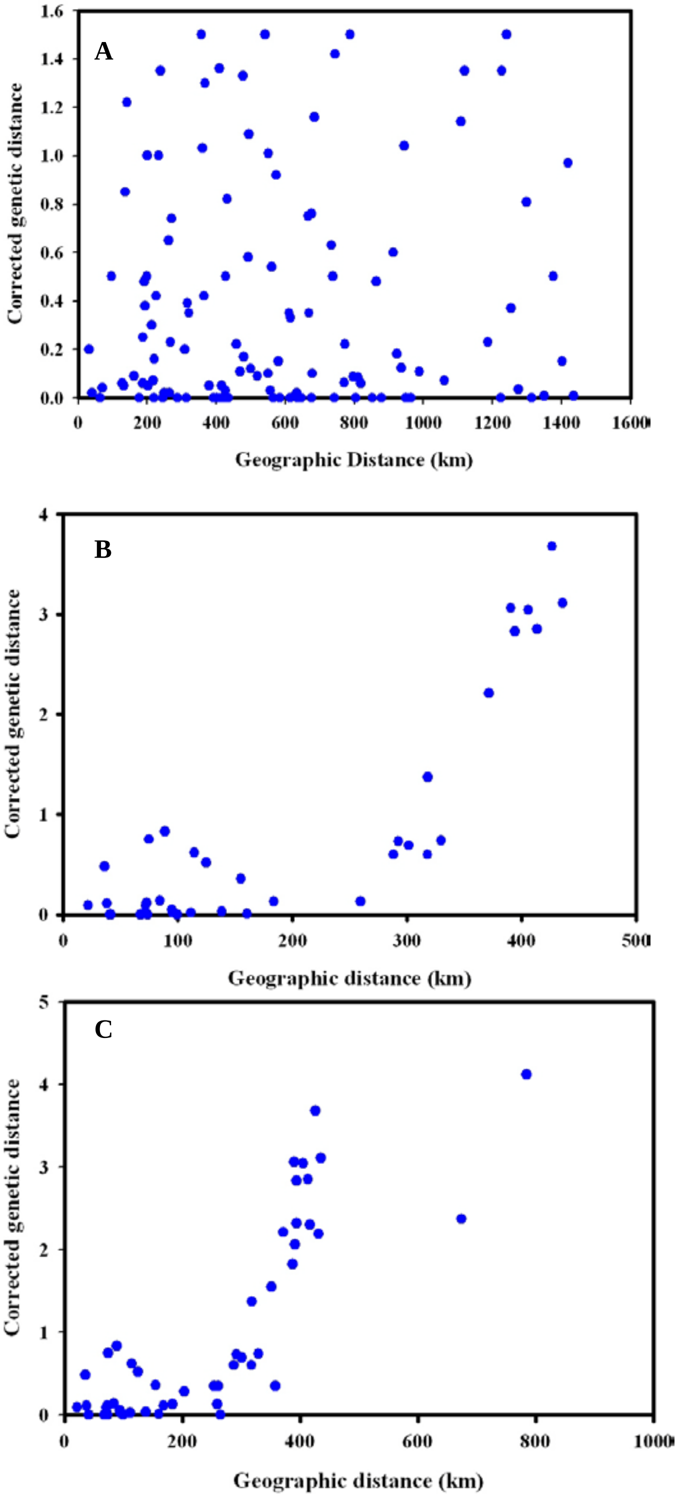

Figure 7. Relationship of geographic distances and corrected genetic distances. A) south of the Bahia refuge, B) inside the Bahia refuge and C) inside the Bahia refuge including the *P. leucoptera* population along the contact zone with *P. atra* (see text for details).

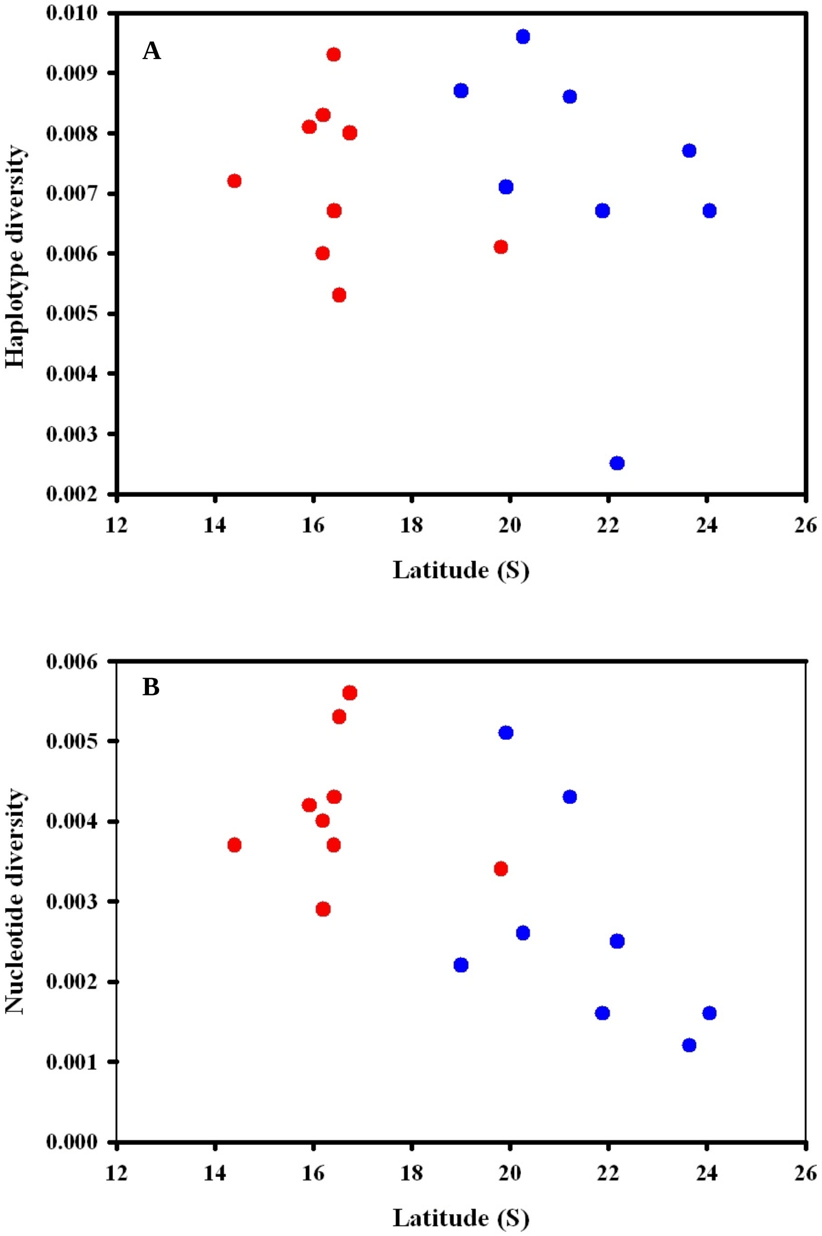

Figure 8. Relationship of (A) haplotype diversity and (B) nucleotide diversity with latitude in Atlantic Forest. Red and blue dots represent localities sampled inside and outside the Bahia refuge, respectively.

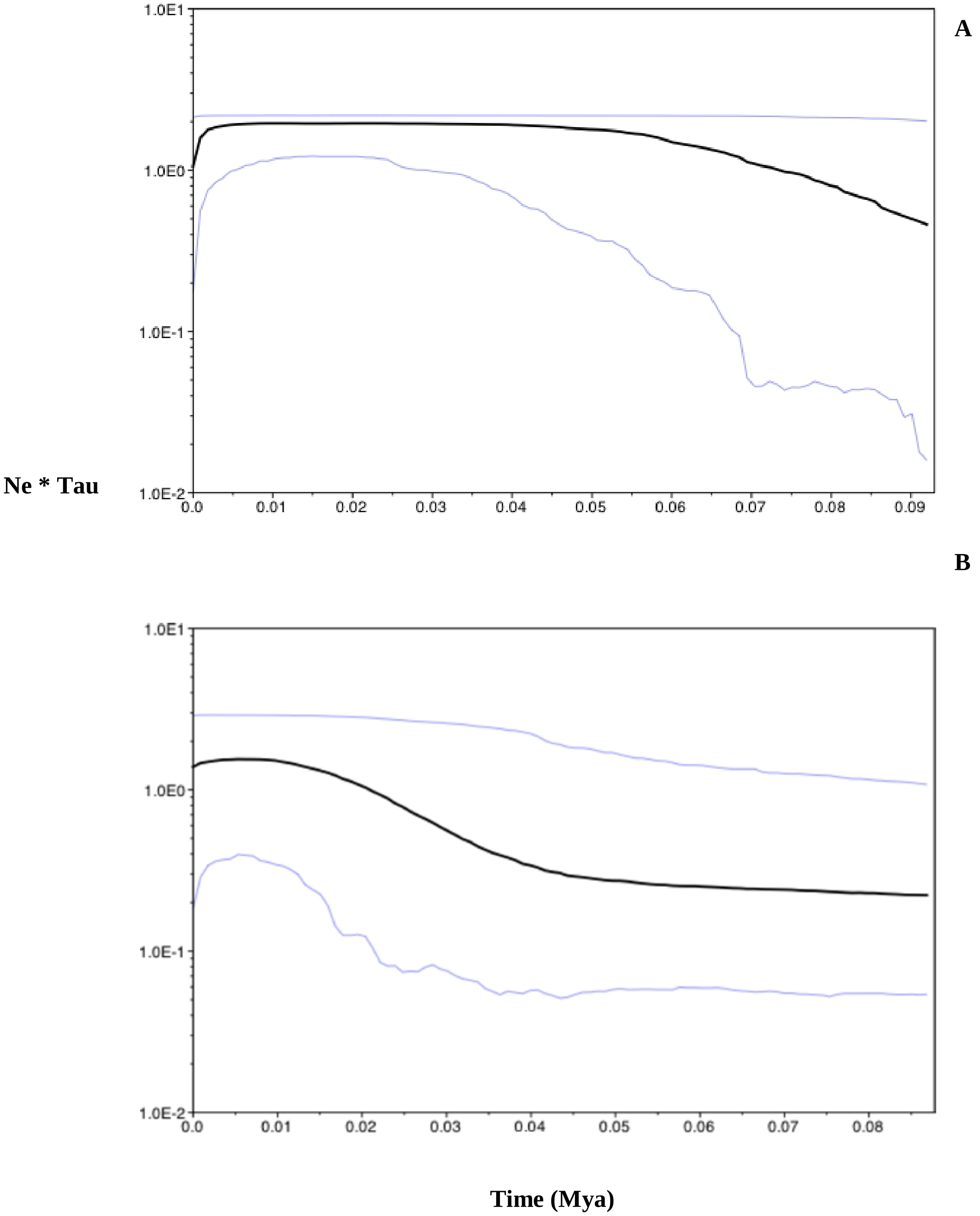

Figure 9. Bayesian Skyline Plots depicting the demographic history of fire-eye (*Pyriglena leucoptera*) populations A) inside and B) south of the Bahia refuge in Atlantic Forest, with time axis scaled to the mutation rate of 2.1% per MY. The solid black line represents the median value for the log of the effective population size and the blue lines represent the upper and lower 95% credible intervals. Time zero is the present, with values indicating time increasing towards the past.

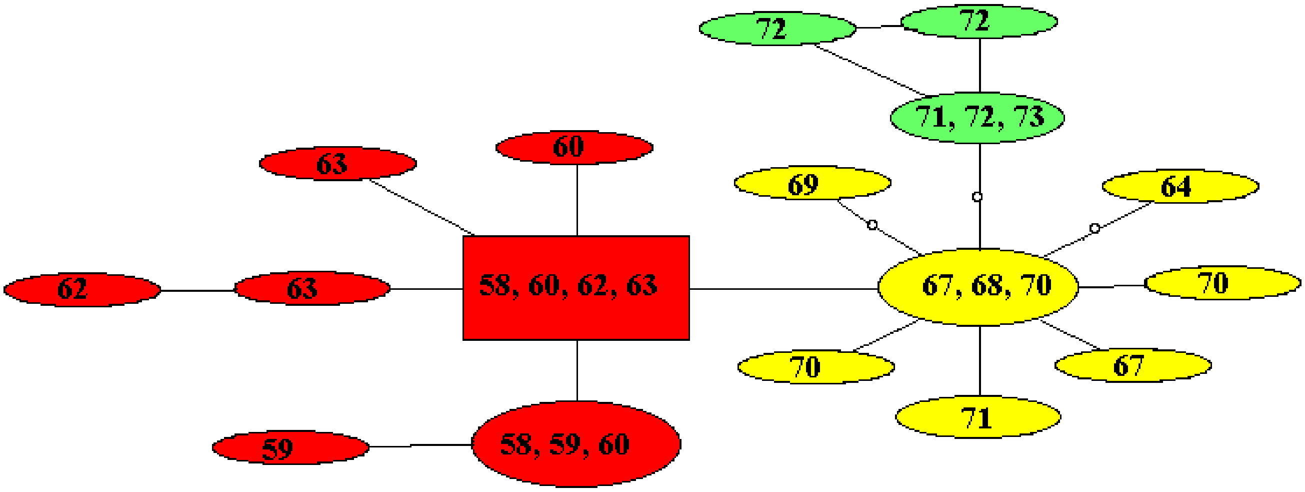

Figure 10. Statistical parsimony network among the haplotypes of southwestern South American fire-eye populations (the "*maura*" group) based on ND2 sequences. Each circle represents a different haplotype with size proportional to its relative frequency. The numbers correspond to sampling localities labeled in Figure 1C. Colours correspond to i) red: lowlands of Brazil and Bolivia; ii) yellow: lowlands of Bolivia and foothills of Bolivian and Peruvian Andes; iii) green: foothills of Peruvian Andes. See text for details.

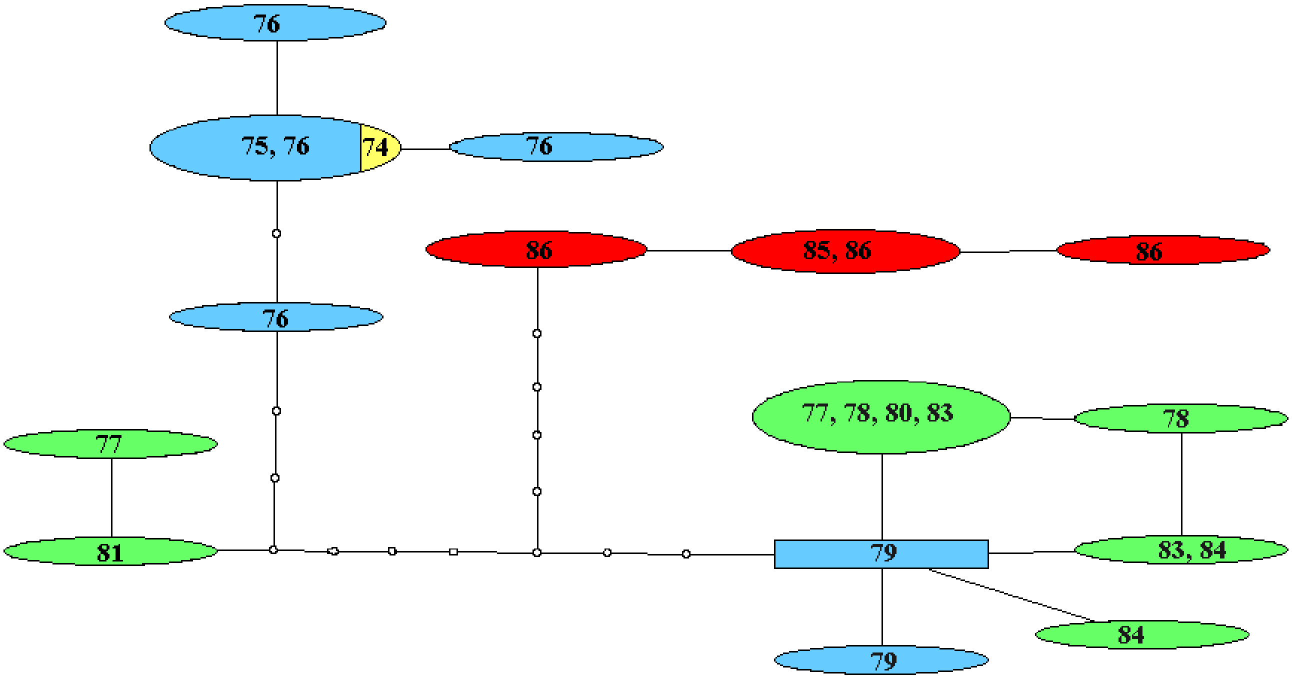

Figure 11. Statistical parsimony network among the haplotypes of Central Andean fire-eye populations based on ND2 sequences. Each circle represents a different haplotype with size proportional to its relative frequency. The numbers correspond to sampling localities labeled in Figure 1D. Colours correspond to i) red: *trans*-Andean *P. l. pacifica*; ii) blue: south bank of the Marañón River valley; iii) green: north bank of Marañón River valley and iv) yellow: *P. l. picea*. See text for details.

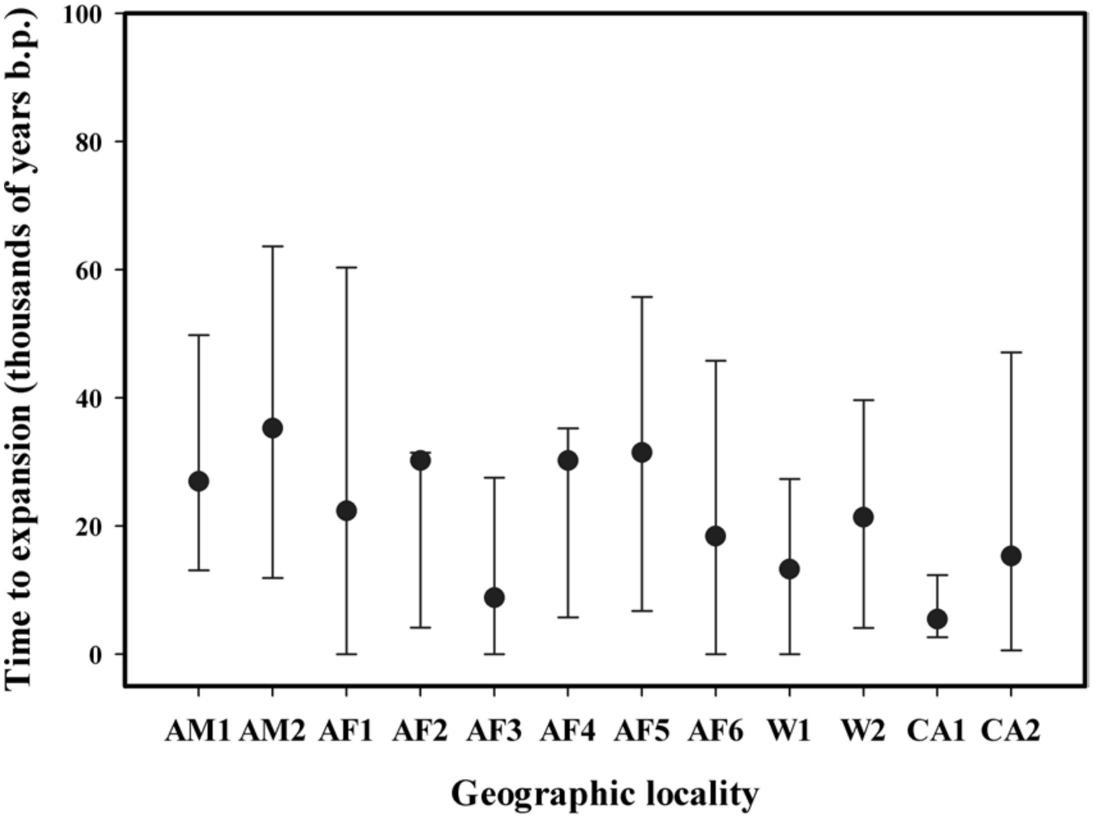

Figure 12. Estimated time since the population expansion in y.b.p using the generalized non-linear least-squares approach of Schneider and Excoffier (1999) for fire-eye populations in South America, assuming the 2.1% mitochondrial mutation rate. Bars represent 95% confidence intervals. Geographic localities are in Amazonian basin (AM1 = Serra dos Carajás; AM2 = Santana do Araguaia), Atlantic Forest (AF1 = Pernambuco refuge; AF2 = Headwaters of Paraguaçu; AF3 = Santa Tereza (24); AF4 = Morro do Diabo (14); AF5 = Morro Grande (10); AF6= Foz do Iguaçu (2)), Western South America (W1 = eastern group in Figure 10, W2 = western group in Figure 10) and Central Andes (CA1= Oxapampa (75), CA2=populations in the Marañón River Valley (77, 78 and 79). Number between brackets represent sampling localities numbered as in Figure 1.

Appendix 1

Localities, sample size and geographic coordinates for samples of Pyriglena included in phylogeographic and phylogenetic analyses. Voucher numbers will be provided in future publications.

Number	Taxon	Country	Locality	Sample Size	Lat.	Lon.
1	*Pyriglena leucoptera*	Paraguay	Cord. de Caaguazú, Department of Caaguazú	7	-26.117	-55.733
1b	*Pyriglena leucoptera*	Argentina	Missiones, Department of San Ignacio	5	-27.250	-55.540
2	*Pyriglena leucoptera*	Brazil	Iguaçu National Park, State of Paraná	9	-25.934	-54.478
3	*Pyriglena leucoptera*	Brazil	Quatro Barras, Corvo, State of Paraná	1	-25.333	-49.131
4	*Pyriglena leucoptera*	Brazil	Serra do Mar State Park, Núcleo Caboclos, State of São Paulo	3	-24.587	-48.595
5	*Pyriglena leucoptera*	Brazil	Monte Alegre Farm, Telêmaco Borba, State of Paraná	4	-24.056	-50.693
6	*Pyriglena leucoptera*	Brazil	Serra do Mar State Park, Núcleo Curucutu, State of São Paulo	3	-23.985	-46.743
7	*Pyriglena leucoptera*	Brazil	Pinhalão, State of Paraná	1	-23.967	-50.050
8	*Pyriglena leucoptera*	Brazil	Juqitiba, State of São Paulo	1	-23.932	-47.087
9	*Pyriglena leucoptera*	Brazil	Buri, State of São Paulo	2	-23.717	-48.567
10	*Pyriglena leucoptera*	Brazil	Morro Grande, State of São Paulo	31	-23.700	-46.983
11	*Pyriglena leucoptera*	Brazil	Piedade, State of São Paulo	2	-23.711	-47.419
12	*Pyriglena leucoptera*	Brazil	Boracéia, State of São Paulo	2	-23.633	-45.867
13	*Pyriglena leucoptera*	Brazil	Ubatuba, State of São Paulo	1	-23.435	-45.070
14	*Pyriglena leucoptera*	Brazil	Morro do Diabo State Park, State of São Paulo	9	-22.699	-52.247
15	*Pyriglena leucoptera*	Brazil	Gaupimirim, State of Rio de Janeiro	2	-22.521	-43.010
16	*Pyriglena leucoptera*	Brazil	Itatiaia National Park, State of Rio de Janeiro	7	-22.489	-44.726
17	*Pyriglena leucoptera*	Brazil	Bela Vista Farm, Cordeiro, State of Rio de Janeiro	4	-22.034	-42.302
18	*Pyriglena leucoptera*	Brazil	Mata da Cambraia, Itumirim, State of Minas Gerais	5	-21.217	-44.783
19	*Pyriglena leucoptera*	Brazil	Fazenda Henrique Bohn, Cantagalo, State of Rio de Janeiro	4	-22.057	-42.662
20	*Pyriglena leucoptera*	Brazil	Itacolomi State Park, Ouro Preto, State of Minas Gerais	8	-20.435	-43.764
21	*Pyriglena leucoptera*	Brazil	Faroeste Farm, Arcos, State of Minas Gerais	7	-20.264	-45.556
22	*Pyriglena leucoptera*	Brazil	Barreiro Rico, State of São Paulo	1	-20.683	-48.100
23	*Pyriglena leucoptera*	Brazil	Santa Bárbara, State of Minas Gerais	4	-19.960	-43.414
24	*Pyriglena leucoptera*	Brazil	Augusto Ruschi Biological Reserve, Santa Tereza, State of Espírito Santo	7	-19.925	-40.613
25	*Pyriglena leucoptera*	Brazil	Serra Piedade, Caeté, State of Minas Gerais	13	-19.815	-43.678
26	*Pyriglena leucoptera*	Brazil	Sooretama Biological Reserve, Sooretama, State of Espírito Santo	6	-19.009	-40.116
27	*Pyriglena leucoptera*	Brazil	Onofre Sandina Farm, Leme do Prado, State of Minas Gerais	4	-17.065	-42.502
28	*Pyriglena leucoptera*	Brazil	northern bank of Jequitinhona River, Mata Escura, Jequitinhonha, State of Minas Gerais	5	-16.434	-41.003
29	*Pyriglena leucoptera*	Brazil	southern bank of Jequitinhona River, Duas Barras Farm, State of Minas Gerais	6	-16.421	-40.069

Number	Taxon	Country	Locality	Sample Size	Lat.	Lon.
30	*Pyriglena leucoptera*	Brazil	northern bank of Jequitinhona River, Limoeiro Farm, Almenara, State of Minas Gerais	11	-16.206	-40.703
31	*Pyriglena leucoptera*	Brazil	Monte Pascoal National Park, Itamaraju, State of Bahia	5	-16.745	-39.533
32	*Pyriglena leucoptera*	Brazil	Pau Brazil National Park, Porto Seguro, State of Bahia	9	-16.503	-39.283
33	*Pyriglena leucoptera*	Brazil	northern bank of Jequitinhona River, Fazenda Santana, Salto da Divisa, State of Minas Gerais	7	-16.045	-40.041
34	*Pyriglena leucoptera*	Brazil	northern bank of Jequitinhona River, Fazenda Palmeiras, Itapebi, State of Bahia	3	-16.156	-39.872
35	*Pyriglena leucoptera*	Brazil	Serra da Ouricana, Boa Nova, State of Bahia	8	-14.506	-40.349
36	*Pyriglena leucoptera*	Brazil	southern bank of Paraguaçu River, São Roque do Paraguaçu, State of Bahia	7	-13.023	-39.098
37	*Pyriglena leucoptera*	Brazil	southern bank of Paraguaçu River, Andaraí, State of Bahia	7	-12.950	-41.539
38	*Pyriglena leucoptera*	Brazil	northern bank of Paraguaçu River, Lençóis, State of Bahia	10	-12.433	-41.363
39	*Pyriglena leucoptera*	Brazil	northern bank of Paraguaçu River, Bonito, State of Bahia	2	-11.913	-41.215
40	*Pyriglena leucoptera*	Brazil	northern bank of Paraguaçu River, Boa Vista do Tupim, State of Bahia	3	-12.663	-40.607
41	*Pyriglena leucoptera*	Brazil	northern bank of Paraguaçu River, Ibiquera, State of Bahia	1	-12.648	-40.939
42	*Pyriglena atra*	Brazil	Serra do Curió, State of Bahia	1	-12.511	-38.461
43	*Pyriglena atra*	Brazil	Campina, State of Bahia	12	-12.448	-38.408
44	*Pyriglena atra*	Brazil	Lontra, State of Bahia	11	-12.255	-37.971
45	*Pyriglena atra*	Brazil	Jandaíra, State of Bahia	6	-11.624	-37.648
46	*Pyriglena leuconota pernambucensis*	Brazil	Timbaúba, State of Pernambuco	3	-7.505	-35.318
47	*Pyriglena leuconota pernambucensis*	Brazil	Serra do Espelho, Jaqueira, State of Pernambuco	2	-8.727	-35.793
48	*Pyriglena leuconota pernambucensis*	Brazil	Ibateguara, Engenho Ceimba, Usina Serra Grande, State of Alagoas	10	-8.973	-35.939
49	*Pyriglena leuconota pernambucensis*	Brazil	Mata do Estado, State of Pernambuco	2	-7.617	-35.500
50	*Pyriglena leuconota leuconota*	Brazil	Porto Franco, State of Maranhão	1	-6.341	-47.407
51	*Pyriglena leuconota leuconota*	Brazil	Moju, State of Pará	6	-1.885	-48.765
52	*Pyriglena leuconota leuconota*	Brazil	Paragominas, State of Pará	1	-2.997	-47.353
53	*Pyriglena leuconota interposita*	Brazil	Ourilândia do Norte, State of Pará	2	-6.749	-51.081
54	*Pyriglena leuconota interposita*	Brazil	Serra dos Carajás, State of Pará	3	-5.800	-50.500
55	*Pyriglena leuconota interposita*	Brazil	right bank of Tapajós River, Altamira, State of Pará	2	-3.650	-52.367
56	*Pyriglena leuconota similis*	Brazil	ca 30 km SW Castelo dos Sonhos, Jamanxin Farm, Altamira, State of Pará	3	-8.399	-55.386
57	*Pyriglena leuconota similis*	Brazil	Loanda Farm, Sinop, State of Mato Grosso	1	-11.434	-55.367
58	*Pyriglena leuconota maura*	Brazil	Vale da Bênção, Chapada dos Guimarães, State of Mato Grosso	7	-15.417	-55.833
59	*Pyriglena leuconota maura*	Brazil	Pirizal, Nossa Senhora do Livramento, State of Mato Grosso	2	-14.917	-55.683
60	*Pyriglena leuconota maura*	Brazil	Cáceres, State of Mato Grosso	3	-16.067	-57.683
61	*Pyriglena leuconota hellmayri*	Bolivia	San Jose, Department of Santa Cruz	2	-19.165	-60.878
62	*Pyriglena leuconota maura*	Bolivia	W Bank Rio Paucerna, 4 km upstream from Rio Itenez, Velasco, Department of Santa Cruz	3	-13.533	-61.100
63	*Pyriglena leuconota maura*	Bolivia	Parque Nacional Noel Kempff Mercado 60 km ESE of Florida, Velasco, Department of Santa Cruz	4	-14.683	-61.017

Number	Taxon	Country	Locality	Sample Size	Lat.	Lon.
64	*Pyriglena leuconota hellmayri*	Bolivia	126 km ENE San Jose de Chiquitos, Mina Don Marie, Department of Santa Cruz	1	-17.333	-59.683
65	*Pyriglena leuconota hellmayri*	Bolivia	Prov. Florida, 23.2 km E Samaipata, Department of Santa Cruz	4	-18.278	-63.674
66	*Pyriglena leuconota maura*	Bolivia	50 km ESE Florida, Arroyo del Encanto, Velasco, Department of Santa Cruz	1	-14.567	-60.667
67	*Pyriglena leuconota hellmayri*	Bolivia	ca 37 km SE Samaipata, Chuchial, Department of Santa Cruz	1	-18.383	-63.617
68	*Pyriglena leuconota hellmayri*	Bolivia	Prov. Chapare, San Onofre, ca. 43 km W. Villa Tunari, Department of Cochabamba	4	-17.146	-65.767
69	*Pyriglena leuconota hellmayri*	Bolivia	Prov. B. Saavedra, 83 km by road E. Charazani, Cerro Asunta Pata, Department of La Paz	1	-15.183	-69.000
70	*Pyriglena leuconota hellmayri*	Bolivia	Prov. Nor. Yungas, near the Rio Elena, Department of La Paz	3	-16.250	-67.667
71	*Pyriglena leuconota marcapatensis*	Peru	Abra de Maruncunca, 10 km SW San Juan del Oro, Department of Puno	1	-14.000	-69.000
72	*Pyriglena leuconota marcapatensis*	Peru	Cuzco, Consuelo	3	-13.000	-71.000
73	*Pyriglena leuconota marcapatensis*	Peru	Cuzco, San Pedro	1	-13.083	-71.917
74	*Pyriglena leuconota picea*	Peru	Utcuyacu, Department of Junín	1	-11.200	-75.467
75	*Pyriglena leuconota castanoptera*	Peru	ca 9km SSE of Oxapampa, Santa Cruz, Department of Pasco	8	-10.917	-75.667
76	*Pyriglena leuconota castanoptera*	Peru	Playa Pampa, ca 8 km NW Cushi on trail to Chaglla, Department of Pasco	2	-9.472	-75.695
77	*Pyriglena leuconota castanoptera*	Peru	ca 3km NNE San Jose de Lourdes, Department of Cajamarca	2	-5.067	-78.967
78	*Pyriglena leuconota castanoptera*	Peru	Nuevo Peru, 16 km NE junction Rios Tabacomas and Chinchipe, Department of Cajamarca	2	-5.283	-78.652
79	*Pyriglena leuconota castanoptera*	Peru	ca 24 km ENE Florida, Department of San Martin	3	-5.686	-77.754
80	*Pyriglena leuconota castanoptera*	Ecuador	Numbala-Bajo, Zamora-Chinchipe Province	1	-4.367	-79.050
81	*Pyriglena leuconota castanoptera*	Ecuador	Panguri, NE of San Francisco del Vergel Ecuador, Province of Zamora-Chinchipe	2	-4.617	-78.967
82	*Pyriglena leuconota castanoptera*	Ecuador	La Chonta, Rio Mayo	1	-4.949	-79.089
83	*Pyriglena leuconota castanoptera*	Peru	Cordillera del Condor, Picorana, Department of Cajamarca	7	-4.983	-78.967
84	*Pyriglena leuconota castanoptera*	Ecuador	west slope of Cordillera del Cutucu on trail from Lagrono to Yaupi-Yapitya, Morona Santiago Province	1	-2.667	-77.850
85	*Pyriglena leuconota pacifica*	Ecuador	Guayas, Cerro Colonche	1	-2.200	-80.033
86	*Pyriglena leuconota pacifica*	Ecuador	Machalilla National Park, Cerro San Sebastian	5	-1.583	-80.667

CHAPTER 3

Geographic variation in songs of Atlantic Forest fire-eye antbirds: assessing the influence of stochastic and deterministic processes

1. Introduction

Investigations on the variation of mating signals throughout the range of a taxon are fundamental for an understanding of evolutionary forces underlying the process of geographic divergence. Particularly important are studies on the geographic divergence of signals that are involved in mate recognition and in reproductive isolation and that might, therefore, contribute to the speciation process (Foster and Endler 1999, Irwin et al. 2001, Pröhl et al. 2006, Price 2008). Bird vocalizations, for example, because of their importance to mate selection (Baker and Baker 1988, Searcy and Yasukawa 1996, Patten et al. 2004) and species recognition (Payne 1986, Baker and Baker 1990, Martens 1996), have been suggested to play a key role in the development of pre-mating reproductive barriers during the speciation process (Grant and Grant 1997, Irwin and Price 1999, Irwin et al. 2001, Haavie et al. 2004, Patten et al. 2004, Edwards et al. 2005, Price 2008).

In this study, we examine in detail how vocalizations vary across the range of two suboscine species, the Atlantic Forest fire-eye antbirds *Pyriglena atra* and *P. leucoptera*, and we test whether different hypotheses for the origin of song divergence could explain their current vocal variation. We first define the hypotheses tested according to the highlighted processes and then describe our study system.

Stochastic factors - The first set of hypotheses underscores the importance of stochastic processes, as their role in the evolution of geographic variation in songs has gained support (Irwin 2000, Koetz et al. 2007, Podos and Warren 2007, Irwin et al. 2008). One process is song drift. Song drift is sampling variation that occurs between successive generations or sampling errors during the learning process or during colonization of new areas; this new variation in learned song characteristics (Grant and Grant 1995, Baker 1996, Podos and Warren 2007) can become fixed in different populations due to chance events (Lynch 1996, Podos et al. 2004b, Price 2008). The effect of drift on song variation may also result from the same processes that drive variation in neutral molecular traits; that is, songs may evolve in response to random changes in the genetic loci underpinning their expression (Koetz et al. 2007, Podos and Warren 2007). As such, songs can diverge as a consequence of random process due to the cessation of gene flow when populations are geographically isolated or due to accumulation of small differences when gene flow is more frequent among neighbor populations (i.e. isolation-by-distance). If random drift is driving vocal divergence in fire-eyes in the absence of selection, we should expect a correspondence between neutral genetic and vocal variation. Alternatively, if the concordance between patterns of variation in vocal traits and presumably neutral genes is low, it suggests that something other than drift must be affecting variation in this character. For example, if divergence in vocal traits exceeds that of neutral genes, it would indicate that geographic variation in selection pressures, environmental heterogeneity or cultural evolution (i.e. song learning) may be playing a role in promoting population differentiation. On the other hand, a pattern of variation in neutral genes exceeding that of vocal traits may imply selective constraints on among-population character divergence (see below).

Even if drift is unimportant, song features may still vary geographically due to stochastic factors. Acoustic features such as songs play a key role in mate choice and male-male competition and are, therefore, likely to evolve rapidly in response to sexual selection (Lande 1981, West-Eberhard 1983, Schluter and Price 1993, Andersson 1994). Stochasticity can occur during the evolution of sexually selected traits because, under Fisherian runaway selection, the direction and extent of trait change can be highly unpredictable (Lande 1981, Kirkpatrick 1982). In oscine songbirds, empirical evidence indicates that females choose more complex vocal patterns (Hasselquist et al. 1996, Searcy and Yasukawa 1996, McGraw et al. 2001, Parker et al. 2003, but see Hill and McGraw 2004). Spatial variation in female preferences for mating signals (Lande 1981, Kirkpatrick 1982, Endler 1992, Endler and Houde 1995, Searcy et al. 1997) could potentially lead to rapid and prominent geographic differentiation in sexually selected traits (Irwin 2000, Price 2008), without major genome-wide differentiation (West-Eberhard 1983, Zink 1996, Uy and Borgia 2000, Panhui et al. 2001).

Sexual selection may also be a driving force of geographic variation in songs of Neotropical antbirds. For example, song duetting in the warbling antbird (*Hypocnemis cantator*) possibly results from intersexual conflict whereby females respond to male songs to prevent themselves from being displaced from the partnership by another female (Seddon and Tobias 2006, Tobias and Seddon 2009a). The same behavior may apply to other antbirds that have female songs that overlap male songs. Moreover, previous studies with antbirds have shown that rate of mate switching can be high (Morton et al. 2000) and that occasional polygamy can occur (Tobias and Seddon 2009a, but see Fleischer et al. 1997); these are both indicators that sexual selection may be operating in antbird mating systems (Anderson 1994, Anderson and Iwasa 1996). In fact, sexual selection has been invoked to explain sexual dimorphism in songs and plumage in antbirds (Seddon et al. 2008, Tobias and Seddon 2009b). All this evidence suggests that patterns of geographic variation in songs of male antbirds may be driven by sexual selection exerted by females, and so one would predict that males should exhibit more geographic variation in songs than females.

Alternatively, sexual selection could also act distinctively on songs of males and females throughout a species' geographic range. For example, geographic variation in plumage traits of antbirds is often notable in females whereas males exhibit no or only subtle differences among allopatric forms, a pattern referred to as "heterogynism" (Hellmayr 1929). No mechanism has been proposed to explain this pattern and whether the same selective forces underlying geographical variation in plumage also lead to acoustic diversification in antbirds is unknown; if they do, one would expect more accentuated geographical variation in songs of female antbirds than in songs of males. Larger geographic variation in songs of females relative to variation in songs of males may imply that evolutionary forces are operating in a similar fashion in vocal and plumage sexual signals.

Deterministic factors - The second set of hypotheses highlights the importance of processes responsible for song divergence among populations in specific and predictable ways. It is widely acknowledged that directional selection can promote divergence in song traits among populations and taxa that occur in habitats that are spatially heterogeneous in signal transmissions and background noise (Ryan and Brenowitz 1985, Handford and Lougheed 1991, Badyaev and Leaf 1997, Brown and Handford 2000, Slabbekoorn and Smith 2002a), despite substantial gene flow (Slabbekoorn and Smith 2002b). Indeed, several studies suggest that intraspecific divergence may result when the distribution of a species covers multiple habitats in which transmission properties select for different accoustic designs (Slabbekoorn and Smith 2002b, Nicholls and Goldzien 2006, Ruegg et al. 2006, Dingle et al. 2008). For example, birds with slow-paced and low-frequency songs are typical of denser habitats, supposedly because songs with those features attenuate and degrade less rapidly than faster and higher-frequency songs, due to reflections and reverberations on tree branches and leaves (Morton 1975, Marten and Marler 1977, Wiley 1991, Slabbekoorn and Smith 2002b, Slabbekoorn et al. 2002). Higher frequency songs also attenuate faster than lower frequency songs, and attenuation by absorption is greater as temperature increases and is reduced with increasing humidity (Wiley and Richards 1982, Slabbekoorn 2004). Consequently, birds living in denser habitats should use songs with (i) lower frequency, (ii) longer elements and (iii) slower pace (i.e. songs with longer intervals between notes) relative to more open habitats, whereas hot and dry environments should favor lower-frequency songs.

Another important source of environmental selection on acoustic signals is ambient noise (Morton 1975, Ryan and Brenowitz 1985). In this context, songs of populations in regions with similar rainfall patterns may converge as a consequence of habitat-dependent ambient noise characteristics, because acoustic competition by abiotic ambient noise can lead populations in habitats with similar ambient noise to spectrally occupy less competitive parts of the frequency range (Slabbekoorn and Smith 2002b, Dingle et al. 2008). Thus, if vocal variation of fire-eyes has evolved as a response to environmental selection, we should expect a strong correlation between ecological variables of the habitat, such as forest cover and the climatic setting, and the spectral and temporal elements of their vocalizations. Moreover, if habitat-dependent selection pressures have shaped acoustic characteristics of fire-eye populations, similarity of songs across the fire-eyes' range should reflect similarity in acoustic transmission properties of the environment.

Geographic variation in song traits can also be shaped and limited by constraints. This is because over the evolutionary history of a lineage, voice diversification is expected to be tempered by limits of mechanical possibility (Podos 1997, 2001, Podos et al. 2004a,b, Ballentine 2006). One example is song frequency, which varies negatively with body size (Ryan and Brenowitz 1985, Palacios and Tubaro 2000), implying that upper limits on syrinx size constrain the production of songs with low fundamental frequency. Selection on beak form and function also may drive, as a secondary consequence, patterns of song frequency and vocal performance. For example, birds with longer, deeper and wider beaks increase the volume of the vocal tract and consequently produce songs with significantly lower frequency patterns (Palacios and Tubaro 2000, Ballentine 2006, Huber and Podos 2006). Thus, we predict that fire-eyes with both larger body and beak sizes will sing songs with lower frequencies. If song variation is driven by morphology, we expect that fire-eye populations with similar morphologies (i.e. in size and beak dimensions) experience similar constraints on song production and should produce similar songs.

We also should expect that the balance between evolutionary forces should change when one contrasts patterns of geographic variation in a trait of a species that occupies regions with distinct histories (Kaneshiro 1989, Irwin 2000, Tregenza et al. 2001). For example, acoustic traits can be highly correlated with geographic distance in a region ecologically stable over the last thousand years due to the influence of isolation by distance, whereas isolation into refugia during glaciations and subsequent range expansions could not only lead to differentiation in acoustic traits but also to eliminate the influence of geography (i.e. distance) in ecologically unstable areas. Thus, we predict a shift in the influences of isolation by distance on loudsong variation of fire-eyes between the southern and the northern Atlantic Forest, as these regions seemingly present different evolutionary histories (see study system below).

Finally, we investigate here the likely influences of hybridization and introgression on the variation of vocalizations along the zone of contact between two Atlantic Forest fire-eye species. The resulting potential for evolutionary change and the ecological, phenotypic and genetic consequences of hybridization and introgression have been of interest for some time (Grant and Grant 1992, Seehausen 2004, Baack and Rieseberg 2007). It is common wisdom that populations from hybrid zones can present higher phenotypic variability, as they often include hybrid and backcrossed (i.e. introgressed) individuals with novel or extreme phenotypes relative to pure parental populations (Parsons et al. 1993, Grant and Grant 1994, Rieseberg et al. 1999, Chiba 2005). For quantitative traits, this is possibly due to the increased and combined effects of heterozygosity and new linkage relations in hybrid populations or even due to nongenetic environmental variance if developmental stability is diminished in hybrids (Barton and Gale 1993). Importantly, introgression of genes through hybridization can potentially increase genetic variation and phenotypic variability in the neighboring (i.e. parapatric) recipient populations.

We examine the potential of introgression as a source of enhanced vocal variation along the parapatric zone between the Atlantic Forest fire-eye species, *Pyriglena atra* and *P. leucoptera* (see study system below). These two species can be discriminated clearly on the basis of male plumage, but less so by songs (Willis and Oniki 1982). Although poorly distinguishable vocally along the parapatric zone, populations of these two species distant from the zone of contact can be discriminated in a multivariate space (see results). On the basis of male plumage pattern, introgressed individuals of both species are found in populations along the parapatric zone (pers. obs.; see also Willis and Oniki 1982). Moreover, a study of mitochondrial (mtDNA) variation throughout the range of these species has uncovered an elevated level of haplotype sharing along, and restricted to, the parapatric zone in northern Atlantic Forest. Although the hybrid zone has not been found, this pattern of phenotypic and genetic variation, combined with the presence of likely hybrids (of unknown geographic origin) in museums (pers. obs.; see also Willis and Oniki 1982), has been interpreted as reflecting hybridization and introgression among populations of these two species (see Chapter 2).

Hybridization is a rare phenomenon in suboscine birds, supposedly due to the absence of learning and the effectiveness of prezygotic behavioral isolation mechanisms such as acoustic signals (Graves 1992, Cadena et al. 2007). In fact, acoustic divergence accompanying geographic isolation and genetic differentiation is thought to lead to prezygotic isolation in this group (Seddon and Tobias 2007). Thus, fire-eyes provide a very good opportunity to investigate acoustic change as a consequence of hybridization and introgression. Whether hybridization and introgression can lead to more variable vocal phenotypes is unknown in suboscine birds. If we assume that suboscine voices are heritable (Zimmer and Isler 2003), we should expect enhancement of vocal variation in populations containing hybrids and introgressed individuals. Thus, if introgression of vocal traits along the parapatric zone has occurred during the history of fire-eye populations and if it is geographically restricted as for plumage traits and mtDNA, we should expect that vocal variability in populations along the parapatric zone will be larger relative to parental populations in other parts of the range.

Here, we adopt an integrative approach to assess the influence of stochastic and deterministic processes on the geographic variation and divergence of fire-eye vocalizations across their range in the Brazilian Atlantic Forest. Although these hypotheses are not mutually exclusive because they may very well work in concert, we argue that by contrasting their predictions with the patterns of variation in songs, one can provide important insights into the ecology and evolution of songs of a poorly studied group as the Neotropical suboscine antbirds. More specifically, we aim to (i) describe general patterns of geographic variation in songs from several localities for both males and females, (ii) test the predictions derived from the stochastic and deterministic hypotheses by combining acoustic, molecular, morphological and ecological variation and (iii) assess if song variability is enhanced in populations along the parapatric zone of *P. atra* and *P. leucoptera*.

The system - The fire-eye antbirds (genus *Pyriglena*) form a species complex endemic to South America; species occur in a broad variety of forest habitats. Males and females sing duets and both sexes engage in nest-building, incubation, and feeding of nestlings and fledglings (Willis 1981). In the Atlantic Forests, three species can be found: *Pyriglena leuconota*, which occurs north of the São Francisco River, and *P. atra* and *P. leucoptera*, which occur in parapatry along the Paraguaçu River in the northern Brazilian Atlantic Forest (Figure 1). *Pyriglena atra* and *P. leucoptera* constitute good models to examine the influence of stochastic and deterministic factors on vocal variation and divergence for several reasons.

(i) Fire-eyes belong to one of the most numerically significant and vocally diverse groups of Neotropical birds, the suboscine thamnophilid antbirds. Recent studies of geographic variation of members of this group have revealed a previously unrecognized high level of vocal diversity (Isler et al. 1998, 1999, 2001, 2002). Thus, results of this study may have implications for a better understanding of vocal geographical variation and evolution in other members of the antbird assemblage.

(ii) Fire-eyes have a wide distribution in Atlantic Forest and occur across a range of habitat types, from coastal lowland and montane moist forests to dry inland forests, and thus are a useful model to examine the relationships between vocalizations and characteristics of the habitat. These Atlantic Forest types are floristically and structurally different and possess distinct levels of leaf deciduousness that are strongly correlated with rainfall and temperature regimes. Coastal forests typically have higher precipitation and trees maintain their leaves year round; deciduous and dry inland forests have significantly lower precipitation and lose some of their leaves during the dry season (Oliveira-Filho and Fontes 2000, Maldonado-Coelho pers. obs.).

(iii) Unlike oscine songbirds, fire-eye females (as with other antbird females) sing frequently (Zimmer and Isler 2003), which allows investigation of both male and female vocal geographic variation.

(iv) Fire-eye songs are relatively simple (Figure 1) and vocal landmarks can be easily established and compared among populations. Moreover, available evidence suggests that the evolution of antbird vocal signals has been influenced by the acoustic environment (Nemeth et al. 2001, Seddon 2005), sexually selective forces (Tobias and Seddon 2009b) and by patterns of genetic differentiation (Isler et al. 2005), implying a great potential for acoustic environments, spatial differences in mating preferences, and population history to shape geographic variation of fire-eye songs at large geographic scales.

(v) Songs of Atlantic Forest fire-eyes can be interpreted in terms of a historical scenario. Atlantic Forest fire-eye populations are genetically structured with a pattern of phylogeographic variation uncovered in a mitochondrial DNA (mtDNA) study likely congruent with events of geographic vicariance, isolation by distance and range expansion (Chapter 2). Results from this phylogeographic study showed that populations that occur in the northern Atlantic Forest region follow a pattern of isolation by distance that suggests long-term spatial stability in a putative Pleistocene forest refuge (hereafter Bahia refuge). In contrast, mitochondrial variation of southern populations shows no correspondence with geographic distance among populations and strong evidence of population expansion, likely reflecting isolation in one or more local refuges followed by population expansion episodes; these patterns may reflect heterogeneity in the historical stability of forests in the northern and southern parts of Atlantic Forests, as suggested by previous analyses (Carnaval and Moritz 2008, Carnaval et al. 2009, Chapter 2). (vi) Finally, the presence of hybrids and plumage-introgressed individuals along the parapatric zone between these species implies historical role of hybridization that shape the evolutionary history of these two forms and allows us to investigate the influence of gene flow and introgression on geographic patterns of vocal variation in fire-eyes.

2. Methods

Sampling - We tape-recorded vocalizations at 38 locations across the ranges of *P. leucoptera* and *P. atra* in Atlantic Forest (Table 1, Figure 1). Because the birds were not marked, we adopted three strategies to avoid recording the same bird twice. First, we always tried to voucher our genetic and vocal samples by collecting the singing individuals of *P. leucoptera*; as *P. atra* is a endangered taxon, only blood samples were obtained. Second, we marked territories where we had recorded individuals that we could not collect. Third, we moved continuously and away from the previous recording location. To increase sample sizes at localities we sampled, and to augment the geographic scope of the study, we obtained recordings made by other researchers (see Acknowledgments). At some localities, we used playbacks to attract birds closer in order to record vocalizations of better quality or to increase sample size. Fire-eyes did not alter their song when responding to playbacks (i.e. when compared to unstimulated songs; not shown).

Vocal analyses - Throughout this study, we followed song nomenclature described by Willis (1981) and Willis and Oniki (1982), the most complete and detailed work on natural history of *Pyriglena* vocalizations. Although three types of songs are described for Pyriglena (Willis 1981), we restricted our analysis to loudsongs because this is the more frequently delivered song type by fire-eyes and because it is the only one we were able to sample satisfactorily. Loudsongs are a series of loud and clear notes, which possibly are used in mate attraction and territory defense.

Only good quality recordings were used. We attempted to minimize problems caused by the sound environments in which recordings took place, such as reverberation and attenuation, and only used undistorted spectrograms for the analyses. Spectrograms were made using the program Raven 1.2.1 (Charif et al. 2004) on default settings. We defined and measured 22 loudsong characters (hereafter loudsong elements, Table 2) in 238 male and 92 female loudsongs. For some loudsong elements measured (numbers 3 to 6 below), we included the first four notes and the last note. The measurements consisted of: (1) number of notes (note defined as an unbroken trace on a spectogram), (2) pace (defined as the number of notes per second and computed as the ratio between the time interval including the beginning of the first note to the beginning of the last note and the total number of notes excluding the last one), (3) length of the note (measured as the time interval between the beginning and the end of a note), (4) space of the note (measured as the time interval between the end of a note and the beginning of the following note), (5) high frequency of the note, and (6) maximum power frequency of the note (frequency at which maximum power occurs in the note; see Charif et al. 2004). Measurement units are presented in the scatterplots and more complete descriptions of antbird vocal measures are described elsewhere (Isler et al. 1998, 1999).

A discriminant function analysis showed that fire-eyes have sex-specific loudsong features (results not shown) and, therefore, males and females were analyzed separately during all vocal analyses. Females of several species of antbirds sing in coordinated duets with males (Zimmer and Isler 2003), and a recent study suggested that duetting in the warbling antbird (*Hypocnemis cantator*) may result from females jamming the songs of their own mates as a strategy to avoid cuckoldry or divorce (Tobias and Seddon 2009a). As jamming and jamming avoidance can potentially influence the temporal structure of the song (Tobias and Seddon 2009a), caution should be taken when analyzing songs of duetting antbirds. We performed a series of discriminant function analyses in fire-eye populations where we had a large number of vocalizations, in order to test whether loudsongs of males singing in coordinated duets differed from solo loudsongs; in all surveyed populations, loudsongs delivered in duets did not differ from solo loudsongs (results not shown). Thus, duet and solo loudsongs were combined in all subsequent analyses.

Morphological analyses - In order to assess the geographic patterns of morphological variation and to test the effect of body size on loudsong frequency in Atlantic Forest fire-eyes, we measured six morphometric characters from a total of 228 males and 150 females, including individuals collected in this study and individuals housed in several Brazilian and North American museums. The characters measured represent body and bill dimensions that represent overall avian body size and bill dimensions (e.g. Rising and Somers 1989, Huber and Podos 2006). We measured six traits with dial calipers (to 0.01 mm): bill depth (at its base), bill width (at its base), bill length (from the anterior end of the nostril to the tip), tail length, tarsus length and wing chord length (unflattened, from bend of wing to longest primary).

Ecological variables - We examined the correspondence between acoustic variation and seven ecological variables (Table 8) based on a 1-km resolution grid extracted from Worldclim (Hijmans et al. 2004), using the software DIVA-GIS. These variables include the remotely-sensed Normalized Difference Vegetation Index (NDVI), rainfall (e.g. annual precipitation) and temperature (e.g. temperature annual range, Table 8), which are factors thought to shape acoustic signal design (Slabbekoorn and Smith 2002b, Slabbekoorn 2004, Ruegg et al. 2006). NDVI represents the difference between the reflectance values in the near infrared and visible light spectrum, normalized over the sun on both readings (Parra et al. 2004). We performed analyses employing six different NDVI indices described in Parra et al. (2004); as all NDVI values produced similar results in correlation analyses, we used only the NDVI that reflects annual seasonality (see Parra et al. 2004 for calculation) in the Mantel tests (see below). We used NDVI data from one year (October 1982 - October 1983) in these analyses.

Statistical analyses - We performed analyses on two data sets for both loudsongs and morphometric variables. The first data set included all populations from which we obtained loudsong recordings or morphometric measurements. We used these data sets to assess general patterns of geographic variation in loudsongs and morphometrics because of their broad geographic scope. The second data set included only populations that were sampled genetically; the Mantel correlations were then restricted to these populations (see below). We used principal components analyses (PCA) on the correlation matrix of individual loudsong measurements to summarize predominant patterns of covariation among the 22 loudsong variables. PCA reduces the original dimensions of the multivariate data set to a set of new and fewer dimensions composed of linear combinations of the original variables. This analysis is adequate when the original variables are likely correlated, because it reduces these variables to new uncorrelated and independent variables. Principal component scores (PC scores) of individual loudsongs were used to calculate populations means and standard errors, which were used for further analyses. We plotted population mean score values *versus* latitude to explore broad patterns of geographic variation in loudsongs. Variation in the six morphometric variables was tested for geographic variation in the same fashion as loudsongs. Plots of principal components were visually inspected and statistical differences among the scores of geographical PC groups were assessed by nested ANOVAs with species as the highest nesting hierarchical factor.

We determined if loudsong elements increased in variation toward the parapatric zone by (i) plotting coefficients of variation (CVs) as a function of latitude and (ii) comparing the distribution of variance ratios (*F*) between populations along the parapatric zone and populations outside the parapatric zone within a fire-eye species (both analyses restricted to males). Thus, *P. atra* populations sampled along the parapatric zone were compared to *P. atra* populations distant from the parapatric zone. For *P. leucoptera*, only the populations in the geographic neighborhood of the parapatric zone were included in this analysis; because inclusion and comparison of loudsongs from populations isolated by large distances would inflate the variance of loudsongs. The range of variation in CVs of loudsong elements between populations along the parapatric zone and populations distant from the parapatric zone was tested using Mann-Whitney *U*-test. The Bonferroni procedure was employed to adjust probabilities when necessary.

We used partial Mantel tests (Legendre and Legendre 1998, Manly 2007) to decompose the relative influences of stochastic and deterministic processes on the loudsong variation of fire-eyes. Partial Mantel tests are appropriate in this study because the ability to reject hypotheses is increased when one simultaneously takes into account the influence of many potential explanatory factors that might or might not be intercorrelated, and that can contribute alone or in combination to the pattern of geographic variation in a given response variable (Thorpe 1996). Variables were first statistically normalized to have zero mean and unit variance and then the dissimilarity matrices of loudsong elements, morphometric characters and environmental variables were calculated using Euclidean distances between all pairwise population mean values of PC1 and PC2 scores for each of these variables. Straight-line geographic distances between sites were estimated using DIVA-GIS. The mtDNA ND2 was used to represent neutral DNA variation and to estimate genetic distances among Atlantic Forest populations in the same fashion as described in Chapter 2. We did not distinguish between *P. atra* and *P. leucoptera* as distinct historical groups in partial Mantels because a detailed geographical study of mtDNA variation revealed that they reflect no diagnosable units (Chapter 2). Significance values of the Mantel correlations were obtained by 10,000 permutations.

We used coefficients of variation (CV) to assess the geographic variation in loudsong elements in females versus males. The direction and magnitude of differences were tested using the Wilcoxon paired-sample test based on CVs of loudsong characters.

Statistical analyses were performed using SPSS (SPSS Inc., Chicago, IL, USA) and FSTAT (Goudet 1995).

3. Results

Geographic variation in loudsongs - Patterns of geographic variation in male and female loudsongs were quantified in PC analyses, which were used to summarize the variation in 22 loudsong variables. In general, males and females showed a similar pattern of geographic variation in loudsongs. For males, the three first PCs explained 49.5%, 22.7% and 13.6% of the variation in loudsong variables, while for females, the three first PCs explained 41.3%, 18.3% and 9.6% of the variation. PC1, which is largely an axis of loudsong complexity, increased with spectral parameters (higher frequency and maximum frequency) and number of notes, and decreased with temporal parameters (note length and space length) in both sexes. PC1 is higher northwards and to the east and lower southwards and to the west (i.e. inland, Figure 2, Tables 2 and 3). Geographic variation in most male and female individual loudsong elements showed a gradual change along the latitudinal axis in Atlantic Forest (Figures 3-6). One exception was the length and space of notes, which followed a distinct and more complex pattern (Figure 3). In males, these temporal elements of loudsongs increase in variation from outside (in the northernmost *P. atra* population) to the parapatric zone, where there are larger mean values and variation (see parapatric zone below), decrease in variation moving south and away from the parapatric zone and then increase in a clinal fashion southwards. Females show a somewhat similar pattern, except that the northermost population of *P. atra* had more similar values relative to the parapatric zone.

Although loudsong variation varied clinally throughout most of the range of fire-eyes in Atlantic Forest, a graph of PC2 against latitude reveals an abrupt change in loudsong complexity at about 20°S for males (Figure 7). Geographic variation in male loudsongs is further clarified when PC1 is plotted against PC2, in which positive values of PC2 for *P. leucoptera* essentially represent the southernmost populations (except three *P. atra* populations), whereas negative values represent all populations north of 20°S (Figure 8). Inspection of factor scores reveals a trend for southern male populations to have loudsongs with more widely spaced notes and a lower number of notes than male populations north of 20°S (see Figures 4 and 6). The distribution of factor scores along PC1 and PC2 axes differ significantly between the male groups north and south of 20°S (t-test = - 6.12, df = 225, $p < 0.001$ and t-test =12.64, df = 225, $p < 0.001$ for PC1 and PC2, respectively).

Differentiation in a larger number of geographically distinct vocal groups was, however, recovered for females (Figures 7 and 8). The first group, as in males, consisted of populations south of about 20°S, while the second group was geographically distributed aproximately between 14°S and 20°S. The third group encompassed *P. leucoptera* populations in the parapatric zone with *P. atra* and finally, the fourth group was composed of two populations of *P. atra*, including the northermost sampled population. These groups are significantly different in the distribution of factor scores along PC1 and PC2 (nested ANOVA, $F = 31.91$, $p < 0.001$for PC1 and $F= 6.38$, $p <$

0.001 for PC2). *Post-hoc* tests indicate that only the group pair three-four was not significantly different in PC1, whereas only groups one-two and two-four were significantly different in PC2 (Tukey's tests; not shown).

Loudsong variation in the parapatric zone - Fire-eye populations sampled along the parapatric zone exhibited increased variability in PC1, PC2 and individual loudsong element means and standard error scores (Figures 2-7). Geographic variation in the coefficients of variation (CV) (Figures 9, 10) shows that, although populations from the parapatric zone did not exhibit significantly different levels of variation relative to populations distant from the parapatric zone (Mann-Whitney's *U*-tests; not shown), a trend of increased CVs toward the parapatric zone is apparent for some populations (e.g. frequency of all five loudsong notes, Figure 10). For *P. atra*, variance of populations in the parapatric zone relative to the single population sampled outside of it was larger in 20 loudsong elements (although only eight comparisons were significantly different by *F*-ratio tests; Table 4). However, the pattern was different for the southern species *P. leucoptera*, as only 14 loudsong elements showed larger variance in the contact zone populations whereas the group of non-parapatric populations had higher variance in eight loudsong elements (although only three comparisons were significantly different by *F*-ratio tests; Table 4).

Loudsong differences between P. atra *and* P. leucoptera - The ordination of loudsong characters along the first two PCs show that *P. atra* loudsongs are nested within *P. leucoptera* loudsongs (Figure 8). Inspection of plots for males and females also reveals that overlap in loudsongs between these two species is due mostly to loudsong similarities between *P. atra* and northern populations of *P. leucoptera*, as more southerly populations of the latter species presented a stronger segregation along both PC axes. However, the northernmost female population of *P. atra* was clearly separated from all other populations by the first two PCs, whereas the same pattern was not observed for males. Separation in male loudsongs betweeen the two species is better visualized by the ordination of PC1 and PC3 (Figure 11). Although some *P. leucoptera* populations from the parapatric zone overlap with *P. atra*, there is a trend for the latter species to segregate with high values of both PC1 and PC3. The loudsong element correlated with PC3 is note length (Table 2).

These patterns suggest that hybridization and introgression along the parapatric zone may have influenced loudsong variation of *P. atra* and *P. leucoptera*. To further evaluate effects of these processes on loudsongs, we performed three distinct analyses using the distribution of PC scores: (i) we compared loudsong variation between all samples of *P. leucoptera* and all samples of *P. atra*; (ii) we compared loudsongs of *P. atra* and *P. leucoptera*, but including only *P. leucoptera* loudsongs recorded along the parapatric zone; and (iii) we contrasted the variation in the northernmost population of *P. atra* (population 1 in Figure 1) against a second group composed of *P. atra* populations along the parapatric zone and a third group of *P. leucoptera* populations along the parapatric zone. Results of these analyses suggests that when comparing all populations of *P. atra* and *P. leucoptera*, the distribution of factor scores is significantly different along PCs 1 and 3 (Table 5); however, this difference is likely due to the inclusion of the more geographically distant and vocally distinct southern *P. leucoptera* populations in the analysis, as comparisons restricted to the parapatric zone showed no significant differences along PC scores between loudsongs of northern *P. leucoptera* and *P. atra*.

Geographic variation in males versus females - CVs indicate that fire-eye females do exhibit significantly higher geographic variation in loudsong parameters relative to males. Variation in females was larger for 16 of the 22 loudsong characters measured (Wilcoxon paired-sample test $T_{0.05\ (1),\ 22} = 75$, $p < 0.05$; Table 6). The only loudsong elements with higher geographic variation in males relative to females were the lengths of the first, second and third notes and in the space between the first and the second note. Although females showed higher variation than males in most loudsong elements, only a small number of them differed greatly.

Geographic variation in morphometrics - PCs reveal that differences across populations in Atlantic Forest involved shape as well as size. For males, the first three PCs explained 29.4%, 23.2% and 17.0% of the variation (Table 7). PC1 was largely a shape axis, with negative loadings on both bill width and bill depth in northern Atlantic Forest fire-eye populations and positive loadings for wing length, tail length and tarsus length in southern populations. PC2 was largely a size axis, with all characters exhibiting positive loadings. PC3 was also a shape axis, with positive loadings for bill length and negative loadings for tail length. PC1 and PC2 are positively correlated with latitude, whereas PC3 did not show such a relationship (Figures 12-14, Table 7). For females, the first and second axis explained 25.7% and 23.3% of the variation. PC1 is largely a size axis, with all characters loading positively, whereas PC2 is largely a shape axis, with some characters loadings positively (bill depth and bill width) and others loading negatively (wing length and tail length). PC1 (but not PC2) is positively correlated with latitude (Figures 12 and 13, Table 7).

In the following correlation analyses, we used PC2 and PC1, respectively, for males and females as vectors of body size, since they were essentially a measure of body size in the morphometric PC analyses (Table 7). In order to obtain a vector of bill dimensions while minimizing the influence of body size, we regressed all three bill variables (log-transformed) against the first axis of a principal component analysis that included tarsus length, tail length and wing chord as a vector of body size for males and females (PC1 accounts for 59% and 46% of the variation for males and females in these three measures). The resulting residual values were used in a subsequent PC analysis to obtain a vector of bill dimensions, in which bill width and bill depth were correlated with each other in one PC and uncorrelated with bill length (which loaded heavily in another PC); thus, we used these two bill dimensions separately in the subsequent Mantel correlation analyses. In the Mantel analyses, Euclidean distances among populations were estimated using these PC scores.

Mantel Correlations - Mantel tests show that for the total range of fire-eyes there is a significant relationship between genetic distances inferred from the mtDNA variation and geographic distance ($r = 0.59$, $p < 0.001$; see Chapter 2). Mantel correlations for males and females show that loudsongs were also strongly correlated with geography either including or excluding populations from the parapatric zone (Table 8). However, when other factors (e.g. genetics and body size) were used as covariates, partial Mantel tests showed that there was still a strong and statistically significant correlation between loudsongs and geography for males but that this relationship was weaker for females (Table 8). Correlation coefficients across the total range between loudsongs and genetic, ecological and body size distances were also significant in both sexes. When controlling for all other factors, however, partial Mantel tests show that there remains a significant partial correlation only between loudsongs and body size in males when excluding populations from the parapatric zone, and loudsongs and genetics in females. In males, further analyses revealed that loudsong PC1 and morphological PC1 (a vector of body size) were negatively and significantly correlated (Spearman's rank correlation $r = -0.53$, $p = 0.03$); however, although there was a trend of a negative relationship between body size and loudsong note frequency as expected by theory (e.g. wing length vs. frequency of note 1; Spearman's rank correlation, $r = -0.32$, $p = 0.20$), the pattern was best explained by the strong correlation between body size and temporal loudsong elements (e.g. morphological PC1 vs. space of note 2; Spearman's rank correlation, $r = 0.63$; $p = 0.005$, see also the group outside of Bahia refuge below).

In the Bahia refuge group, a strong and significant Mantel correlation between geography and genetic distance was also observed ($r = 0.84$, $p < 0.001$). In this group, the same pattern observed for the entire range described above was also detected in Mantel tests for fire-eye female populations, except that we did not detect a significant partial correlation between loudsongs and geography when other factors were partialled out (Table 8). In males, although there is significant correlation between loudsongs and geographic, genetic, body size and bill dimension (width and depth) distances, there were no significant partial correlations between loudsongs and these factors in partial Mantel tests (Table 8). However, although not significant, partial Mantel correlations between loudsong and genetic distances were stronger in both males and females when parapatric zone populations were excluded from the analysis.

In the group of populations outside the Bahia refuge, the Mantel test revealed that, in contrast to the other two groups above, there was no significant relationship between geographic distance and genetic distance ($r = 0.09$, $p > 0.05$). In males, Mantel correlations showed that loudsongs were significantly correlated with both genetic distance and body size, and partial Mantel tests revealed that these correlations were still significant even after controlling for the effect of other factors (Table 8). However, further analyses showed that no correlations between frequencies of individual loudsong notes with body size metrics (Spearman's rank correlations, not shown) were observed in this group. Instead, and as for the entire range of fire-eyes, there was a positive association between body size and both note length and note space (Spearman's rank correlations; not shown). For females, correlation coefficients between loudsongs and genetic, ecological and body size distances were significant, but loudsongs remained significantly correlated only with ecological distances in partial Mantel tests (Table 8). Additional analyses testing the association between female loudsong elements and environmental variables showed that both note space and note length were negatively correlated with NDVI (Spearman's rank correlations; not shown), that is, populations in habitats with denser forest cover presented loudsongs that were faster paced and with shorter notes relative to populations in more open habitats. These results are opposite to expectations from theory.

Three other results were also noteworthy in our study. (i) Strong correlations and strong partial correlations between loudsongs and genetic distance were always detected in females independent of the geographical group whether populations from the parapatric zone were included or not. (ii) The correlations and partial correlations between loudsong distances and genetic distances were stronger for females relative to males when considering geographical groups of the entire range; in the Bahia refuge, the strength of Mantel correlations depended on whether populations from the parapatric zone were included in the analysis. (iii) Since genetic distance and geography are not significantly correlated for the group of populations outside the Bahia refuge, using geography as a covariate has less influence on the partial Mantel correlations in this group than in the two other groups.

4. Discussion

Loudsong variation in the parapatric zone - For a shift in variation of a trait to be attributed to the processes of hybridization and introgression, it should be shown that the two parental species or populations differ in this trait. In both sexes, results from the multivariate analysis showed that loudsongs between the northernmost population of *P. atra* and southern *P. leucoptera* were distinguishable.

Larger variances of loudsong elements within the parapatric zone compared to the populations outside it for *P. atra* indicates that the increased loudsong variability in the parapatric zone may have arisen through introgressive hybridization between the two fire-eye species. Our results also reveal an increased variance in several individual loudsong elements for both males and females at 18-20° S. Indeed, geographic variation in PC2 scores in both sexes indicate a major transition zone of loudsong variation (i.e. a steep cline) at 18°-20° S. This region has been shown to be an area of secondary contact between fire-eye populations following range expansion from putative Pleistocene refuges (see Chapter 2). This might indicate that hybridization and introgression were relatively recent in southern Atlantic Forest and that these were most likely the underlying mechanisms of the higher variability in loudsong characteristics of populations at 18°-20° S.

How much variance arises in a hybrid population is contingent on the genetic basis of the character. Although the genetic architecture of vocalizations in suboscine birds is unknown, patterns of geographic covariation in antbird songs and genetic markers (Cohn-Haft 2000, Isler et al. 2005) provide support to earlier suggestions that learning may not be involved during song development and that vocal variation in this group may have a strong genetic basis (Kroodsma 1984, 1989, Kroodsma and Konishi 1991). Thus, even if the increased variance in loudsongs within the parapatric zone and in the contact zone in southern Atlantic Forest were a consequence of reduced developmental stability of hybrid and introgressed individuals (e.g. in features of the vocal tract) and, hence, not directly related to the heritable nature of loudsongs, it is plausible that this pattern may have arisen due to additive genetic variance and linkage disequilibrium (Slatkin and Lande 1994, Templeton 2006).

Convergence of loudsongs in the parapatric zone - Our results indicated that songs of *P. leucoptera* and *P. atra* converged along the parapatric zone. What are the likely mechanisms driving this pattern? Song convergence can arise due to (i) adaptive interspecific territoriality in the presence (Cody 1969, Rainey and Grether 2007) or absence (Tobias and Seddon 2009c) of song learning and copying, (ii) non-adaptive song learning and copying (Irwin and Price 1999, Haavie et al. 2004), (iii) adaptation to habitats with similar acoustic transmission properties (Wiley and Richards 1982) and (iv) production of hybrids and back-crossed individuals with intermediate vocal traits that have a strong genetic component (de Kort et al. 2002). Although we cannot rule entirely out that fire-eye loudsongs have converged via social selection in sympatry, as suggested for *Hypocnemis* antbirds, and that this trait has subsequently spread into the parapatric populations through gene flow (Tobias and Seddon 2009c), there is no indication that the sound environment is shaping loudsong variation of fire-eyes in northern Atlantic Forest (see Mantel results). Our results allow us to draw two conclusions. First, the fact that introgression of plumage traits and mtDNA have been detected along the parapatric region (Maldonado-Coelho unplub. data) suggests that introgression may have driven the convergence of loudsongs of *P. atra* and *P. leucoptera* in the parapatric zone. Further sampling in the northernmost range of *P. atra* should provide definitive loudsongs diagnostic of this species and allow inference of the direction of vocal introgression.

Vocal geographic variation in females versus males - Our results provide no support that sexual selection mediated by female choice lead to larger geographic variation in male fire-eye loudsongs relative to loudsongs of females. Although male fire-eye loudsongs differ across populations as do female loudsongs, sexes differ in the magnitude of geographic variation as would be predicted by theory. This was evidenced by the coefficients of variation in individual song elements and in the multivariate space, which showed a more prominent variation for females. Rather, geographic variation in fire-eye loudsongs in Atlantic Forest parallels variation in plumage of females (but not males, unpubl. data) and is concordant with the phenomenon described long ago for antbirds, that is, females of closely related populations and species are often more strongly differentiated in plumage than males (including fire-eyes, Hellmayr 1929). To our knowledge, this is the first analysis to demonstrate heterogynism in vocal characters of a thamnophilid antbird.

Why do sexes differ in the amount of loudsong geographic variation and what are the likely mechanisms underlying this pattern? We suggest that three not mutually exclusive possibilities may account for the pattern (but see Mennill and Rogers 2006 for different views). One possibility is that selective constraints on male loudsongs have shaped this pattern. For example, females could exhibit low geographical variance in preference for male loudsongs. Thus, geographical variation in male loudsongs could be the net result of the opposite forces of female selection that would tend to spread to fixation the preferred loudsong type across the fire-eye's range and of mutation and drift, that create and spread to fixation new loudsong variants in local populations. Intersexual pressures on female loudsongs, on the other hand, could be weak or absent and hence new loudsong variants that arise by mutation would tend to spread to fixation by drift more often than in males.

A second alternative is that, because antbird vocalizations seem to be genetically determined, larger population differences in females would arise if genes affecting antbird vocal characters evolve at a faster rate in this sex than in males. If so, one possibility is that antbird vocal traits are expressed by Z-linked genes as suggested for songs and other sexually selected traits in birds (Price 2002, Wright et al. 2004), and genes expressed on the heterogametic sex would evolve faster that those expressed on the homogametic sex due to favorable or neutral recessive mutations that arise and become fixed more frequently in females (Orr 1997, Kirkpatrick and Hall 2004, Qvarnström and Bailey 2009). This is because the W chromosome is degenerate in most birds (Mizuno et al. 2002), and hence, recessive mutations on the Z chromosome would be masked in males, but expressed in females. Thus, Z-linked mutations arising in distinct populations could provide a mechanism for heterogynism not only for vocal but also for plumage characters in antbirds. The third possibility is that females are less likely to disperse across zones of secondary contact relative to males (i.e. due to Haldane's rule), and higher mobility of males would have a greater homogenizing effect in this sex's loudsongs. However, this hypothesis seems less likely as a general mechanism since many geographically isolated populations of antbirds with no evidence of hybridization do exhibit heterogynism in plumage traits. Future studies should be designed to clarify the underlying mechanisms behind sex differences in spatial variation on behavioral and morphological traits.

Mantel correlations and the influence of genetic divergence on loudsong geographical variation - The greater correspondence between loudsong and mtDNA variation in males (but also in females) inside the Bahia refuge when populations from the parapatric zone were removed from analysis, may be explained by differences in the amount of introgression of mtDNA and loudsongs between *P. leucoptera* and *P. atra*. On the one hand, mitochondrial haplotypes recovered in the parapatric zone clustered in a distinct group relative to haplotypes recovered in populations south of the parapatric zone (Chapter 2). Loudsongs from the parapatric zone, on the other hand, were clustered together with loudsongs from populations south of the parapatric zone. This suggests that introgressive hybridization may have decoupled the association between vocal characters and mtDNA variation that was observed in males elsewhere across the fire-eyes' range in Atlantic Forest. For populations inside the Bahia refuge, a gradual transition in mitochondrial haplotype frequencies across populations south of the parapatric zone could explain clinal variation in loudsong elements (e.g. in temporal and spectral song elements).

In males, it is also possible that introgression influences the pattern of correspondence between loudsongs and mtDNA over the entire range in Atlantic Forest. For example, high variability in male loudsongs at 18°-20° S combined with steep transitions in haplotype frequency (Chapter 2) imply that this region might represent an area of secondary contact in southern Atlantic Forest; although the extension and nature of this contact zone in Atlantic Forest is still unknown, we suggest that asymmetry in the amount of introgression of vocal characters and mtDNA could also have disrupted the association between male loudsong and mitochondrial variation at this scale. Although it is difficult to explain the contrasting pattern between females and males on the association between loudsongs and mtDNA over the entire range, given available information, we suggest that two non-mutually exclusive hypothesis could account for this discrepancy. First, a larger amount of introgression in male songs relative to females could occur in fire-eyes, mainly if male loudsongs are associated with an increased reproductive success mediated by female choice or behavioral dominance as has been found for sexually selected traits in avian hybrid zones (Pearson and Rowher 2000, McDonald et al. 2001, Stein and Uy 2006). Second, Haldane's rule predicts sex-linked differential introgression in hybrid zones (Carling and Brumfield 2008, Saetre and Saether 2010), and because females are the heterogametic sex (ZW) in birds, female hybrids are expected to be less viable and more sterile than male hybrids (Graves and O'Neill 1997, Orr 1997, Price 2008). Thus, female-linked traits such as mtDNA and loudsongs would show reduced patterns of introgression compared to male-linked characters such as male-loudsongs and could provide an explanation for the greater association between vocalizations and mtDNA in females than in males across the total range of fire-eyes in Atlantic Forest.

Thus, it seems that genetic differentiation explains part of the overall loudsong geographic structuring in both sexes in Atlantic Forest, which provides support for the role of stochastic factors in promoting vocal divergence in fire-eyes. Additional support for this conclusion is that part of the variation was clinal, which may be explained by gradual changes in the amount of introgression among geographically adjacent populations. Moreover, the association of vocal geographic clusters recovered in the multivariate analysis and mtDNA genetic groups found in a phylogeographic study (Chapter 2), suggest that these vocal groups could have originated due to genetic divergence following geographic isolation into Pleistocene forest refugia.

Alternative factors do not seem to explain satisfactorily the overall loudsong variation for males and females. Although partial Mantel correlations reveal that loudsongs sampled across the entire range (for males) and south of the Bahia refuge (for both sexes) were significantly correlated either with body size or with ecological factors, the associations do not correspond to theoretical expectations. If body size played a decisive role in the evolution of fire-eye vocalizations, we would expect a negative correspondence between this trait and loudsong frequency. Contrary to this prediction, we found that temporal elements (note width and note space) of loudsongs were the mostly strongly and the only significantly correlated vocal characters with the vector of body size. This association was unexpected and we have no clear explanation for this. One possibility, however, is that variation in temporal elements of male loudsongs is neutral and correlated with patterns of genetic differentiation, as indicated by partial Mantel tests. Turning to the ecological hypothesis, it is predicted, among other things, that song elements should be shorter and that repeated and identical song elements should be more spaced in denser habitats (Morton 1975, Wiley 1991, Slabbekoorn 2004, van Dongen and Mulder 2006). Our results were the opposite of these expectations as note length and note width were negatively correlated with NDVI values. One potential drawback with our study is that the vocal traits in fire-eyes could have been evolved in response to past acoustic environments that differ from contemporaneous habitat conditions. Although forest disturbance is well known to have occurred throughout Atlantic Forest over the past centuries, we suspect that, at a large scale, forest disturbance may not pose a problem to our study. This is because some aspects of forest structure that may affect acoustic transmission, such as forest leaf deciduoness and their climatic correlates (e.g. rainfall and temperature) may not have changed drastically at the biome scale. The influence of climate is perhaps more pervasive in morphometric traits, which vary in concert with the climate setting in Atlantic Forest, as body size in fire-eyes is positively correlated with temperature and latitude (i.e. follows the Bergman’s rule).

The ecological variables adopted in our study are only a few of numerous possible sources of selective factors that could promote vocal divergence. Future studies should evaluate the influence of other sources of background noise on song divergence, including the influence of spatial variation in animal acoustic communities (e.g. Ryan and Brenowitz 1985, Luther 2009).

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Table 1. Localities, sample size and geographic coordinates for vocal samples of Brazilian Atlantic Forest fire-eyes included in this study.

Number	Taxon	Locality	Sample Size		Lat.	Lon.
			Male	Female		
1	*Pyriglena atra*	Santa Luzia do Itanhi, State of Sergipe	8	3	-11.260	-37.430
2	*Pyriglena atra*	Valentim, State of Bahia	0	1	-12.020	-37.920
3	*Pyriglena atra*	Jangada, State of Bahia	1	0	-12.090	-38.570
4	*Pyriglena atra*	Projeto Subaumirim, State of Bahia	1	1	-12.110	-37.890
5	*Pyriglena atra*	Lontra, State of Bahia	2	0	-12.255	-37.971
6	*Pyriglena atra*	Projeto Taimbé, State of Bahia, Brazil	0	1	-12.300	-38.420
7	*Pyriglena atra*	Cachoeira, State of Bahia	3	1	-12.360	-37.930
8	*Pyriglena atra*	Mata Fome, Icatu, State of Bahia	2	1	-12.060	-38.380
9	*Pyriglena atra*	Terra Nova, State of Bahia	2	0	-12.390	-38.610
10	*Pyriglena atra*	Pojuca, State of Bahia	1	0	-12.420	-38.370
11	*Pyriglena atra*	Fazenda Caboclo, State of Bahia	0	1	-12.820	-38.370
12	*Pyriglena atra*	Campina, State of Bahia	2	1	-12.448	-38.408
13	*Pyriglena atra*	Santo Amaro, State of Bahia	2	1	-12.530	-38.720
14	*Pyriglena atra*	Fazenda Jordão, Monte Gordo, State of Bahia	1	0	-12.640	-38.130
15	*Pyriglena atra*	Fazenda Araripe, Saubara, State of Bahia	1	0	-12.820	-38.810
16	*Pyriglena leucoptera*	northern bank of Paraguaçu River, Boa Vista do Tupim, State of Bahia	5	3	-12.663	-40.607
17	*Pyriglena leucoptera*	southern bank of Paraguaçu River, Andaraí, State of Bahia	10	6	-12.950	-41.539
18	*Pyriglena leucoptera*	southern bank of Paraguaçu River, São Roque do Paraguaçu, State of Bahia	5	2	-13.023	-39.098
19	*Pyriglena leucoptera*	Serra do Timbó, State of Bahia	7	3	-13.030	-39.610
20	*Pyriglena leucoptera*	Serra da Ouricana, Boa Nova, State of Bahia	11	1	-14.506	-40.349
21	*Pyriglena leucoptera*	Fazenda Duas Barras, State of Minas Gerais	4	0	-16.420	-40.070
22	*Pyriglena leucoptera*	Pau Brazil National Park, Porto Seguro, State of Bahia	9	6	-16.503	-39.283
23	*Pyriglena leucoptera*	Monte Pascoal National Park, Itamaraju, State of Bahia	6	3	-16.745	-39.533
24	*Pyriglena leucoptera*	Sooretama Biological Reserve, Sooretama, State of Espírito Santo	10	1	-19.009	-40.116
25	*Pyriglena leucoptera*	Serra Piedade, Caeté, State of Minas Gerais	9	3	-19.815	-43.678
26	*Pyriglena leucoptera*	Santa Tereza, State of Espírito Santo	4	0	-19.925	-40.613
27	*Pyriglena leucoptera*	Parque Natural do Caraça, State of Minas Gerais	4	0	-20.130	-43.500
28	*Pyriglena leucoptera*	Fazenda Faroeste, Arcos, State of Minas Gerais	6	3	-20.264	-45.556
29	*Pyriglena leucoptera*	Itacolomi State Park, Ouro Preto, State of Minas Gerais	20	12	-20.435	-43.764
30	*Pyriglena leucoptera*	Fazenda Henrique Bohn, Cantagalo, State of Rio de Janeiro	12	7	-22.057	-42.662
31	*Pyriglena leucoptera*	Itatiaia National Park, State of Rio de Janeiro	21	8	-22.489	-44.726
32	*Pyriglena leucoptera*	Gaupimirim, State of Rio de Janeiro	2	3	-22.521	-43.010
33	*Pyriglena leucoptera*	Morro do Diabo State Park, State of São Paulo	13	2	-22.699	-52.247

34	*Pyriglena leucoptera*	Ubatuba, State of São Paulo	5	1	-23.435	-45.070
35	*Pyriglena leucoptera*	Fazenda Monte Alegre, Telêmaco Borba, State of Paraná	19	9	-24.056	-50.693
36	*Pyriglena leucoptera*	Iguaçu National Park, State of Paraná	26	8	-25.934	-54.478
37	*Pyriglena leucoptera*	Itatiba do Sul, State of Rio Grande do Sul	1	0	-27.370	-52.450
38	*Pyriglena leucoptera*	Lagoa do Jacaré, State of Rio Grande do Sul	2	0	-29.330	-49.820

Table 2. Factor scores from a principal component analysis of loudsong variables and Spearman’s rank correlations of principal components against latitude and longitude for male fire-eyes of Atlantic Forest.

Loudsong element	PC1	PC2	PC3	PC4
Number of notes	0.41	-0.57	0.26	0.66
Pace	-0.06	-0.10	0.40	0.91
Length of note 1	-0.50	0.32	0.61	-0.14
Space of note 1	-0.51	0.70	-0.37	0.21
Frequency of note 1	0.90	0.30	0.18	0.06
Maximum frequency of note 1	0.83	0.42	0.02	0.1
Length of note 2	-0.58	0.45	0.61	-0.07
Space of note 2	-0.57	0.68	-0.40	0.16
Frequency of note 2	0.92	0.30	0.18	0.03
Maximum frequency of note 2	0.88	0.40	-0.03	0.06
Length of note 3	-0.55	0.50	0.62	-0.05
Space of note 3	-0.56	0.69	-0.39	0.13
Frequency of note 3	0.92	0.30	0.17	0.02
Maximum frequency of note 3	0.87	0.41	-0.04	0.04
Length of note 4	-0.53	0.44	0.67	-0.01
Space of note 4	-0.57	0.68	-0.38	0.15
Frequency of note 4	0.91	0.35	0.14	-0.03
Maximum frequency of note 4	0.85	0.45	-0.06	0.01
Length of note 5	-0.55	0.44	0.60	-0.09
Space of note 5	-0.58	0.63	-0.39	0.21
Frequency of note 5	0.87	0.36	0.11	-0.09
Maximum frequency of note 5	0.82	0.47	-0.11	-0.09
Spearman’s rank correlation - latitude	-0.72**	0.45*	0.33	-0.11
Spearman’s rank correlation - longitude	-0.62**	0.39*	-0.34*	-0.05

*p<0.05;**p<0.001

Table 3. Factor scores from a principal component analysis of loudsong variables and Spearman's rank correlations of principal components against latitude and longitude for female fire-eyes of Atlantic Forest.

Loudsong element	PC1	PC2	PC3	PC4
Number of notes	0.50	-0.32	0.13	0.02
Pace	0.03	-0.05	0.29	-0.10
Length of note 1	-0.37	0.54	-0.48	-0.16
Space of note 1	-0.69	0.49	0.34	-0.06
Frequency of note 1	0.86	0.39	-0.08	0.10
Maximum frequency of note 1	0.51	0.33	0.19	0.08
Length of note 2	-0.45	0.60	-0.55	-0.13
Space of note 2	-0.76	0.44	0.40	-0.08
Frequency of note 2	0.87	0.87	0.39	-0.03
Maximum frequency of note 2	0.67	0.49	0.20	0.04
Length of note 3	-0.50	0.55	-0.60	-0.06
Space of note 3	-0.76	0.44	0.43	0.00
Frequency of note 3	0.91	0.34	-0.01	0.11
Maximum frequency of note 3	0.79	0.36	0.21	0.08
Length of note 4	-0.31	0.10	-0.07	0.93
Space of note 4	-0.75	0.43	0.44	0.02
Frequency of note 4	0.88	0.41	0.00	0.07
Maximum frequency of note 4	0.61	0.21	0.13	-0.74
Length of note 5	-0.54	0.48	-0.39	0.04
Space of note 5	-0.72	0.43	0.42	0.06
Frequency of note 5	0.61	0.43	-0.05	0.09
Maximum frequency of note 5	0.75	0.44	0.16	0.06
Spearman's rank correlation - latitude	-0.81**	0.32	0.39*	-0.55*
Spearman's rank correlation - longitude	-0.68**	0.42*	0.45*	-0.33

*p<0.05; **p<0.001

Table 4. Distribution of variance of loudsong elements among fire-eye populations sampled along the parapatric zone and fire-eye populations sampled in regions distant from the parapatric zone. In this analysis, we defined as parapatric populations of *P. atra* and *P. leucoptera* as populations 2-19 and 16-19 in Figure 1, respectively. Populations 1 and 20-24 were chosen to represent non-parapatric populations of *P. atra* and *P. leucoptera*, respectively.

	Pyriglena atra				*Pyriglena leucoptera*			
Loudsong element	Parapapatric populations	Non-parapatric populations	*F* ratio	Group exhibiting large variance	Parapatric populations	Non-parapatric populations	*F* ratio	Group exhibiting large variance
Number of notes	1.56	0.55	2.84	parapatric	2.24	1.30	1.72	parapatric
Pace	0.51	0.54	1.05	parapatric	0.11	0.06	1.85*	parapatric
Length of note 1	809.56	276.70	2.9	parapatric	695.23	532.67	1.30	parapatric
Frequency of note 1	34667.50	17135.13	2.0	parapatric	27684.99	17261.96	1.60	parapatric
Maximum frequency of note 1	34483.59	12236.79	2.8	parapatric	27686.19	24870.06	1.11	parapatric
Space of note 1	369.91	222.79	1.65	parapatric	27	36	1.33	non-parapatric
Length of note 2	305.99	55.13	5.5*	parapatric	279.16	360.33	1.29	non-parapatric
Frequency of note 2	34912.04	8903.27	3.92*	parapatric	25046.74	20243.16	1.24	parapatric
Maximum frequency of note 2	30851.47	14917.55	2.06	parapatric	27506.03	21498.93	1.28	parapatric
Space of note 2	278.91	113.13	2.46	parapatric	181.42	230.31	1.27	non-parapatric
Length of note 3	400.56	71.41	5.60*	parapatric	299.62	362.97	1.21	non-parapatric
Frequency of note 3	35663.14	8720.98	4.1*	parapatric	29203.26	20436.87	1.43	parapatric
Maximum frequency of note 3	26605.78	8388.29	3.17	parapatric	30239.73	28358.50	1.48	parapatric
Space of note 3	195.56	134.69	1.45	parapatric	94.60	229.83	2.43*	non-parapatric
Length of note 4	612.92	74.27	8.27*	parapatric	237.71	388.49	1.64	non-parapatric
Frequency of note 4	37096.37	7659.84	4.84*	parapatric	28477.35	18430.29	1.55	parapatric
Maximum frequency of note 4	26824.56	5885.14	4.55	parapatric	27241.79	18423.85	1.48	parapatric
Space of note 4	261.69	135.13	1.94	parapatric	131.87	257.64	1.96*	non-parapatric
Length of note 5	500.14	82.27	6.1*	parapatric	229.72	408.09	1.78	non-parapatric
Frequency of note 5	35745.43	14603.55	2.45	parapatric	28245.79	15851.40	1.73	parapatric
Maximum frequency of note 5	32017.29	10037.43	3.20	parapatric	28705.94	16195.00	1.77	parapatric
Space of note 5	111.22	164.55	1.48	non-parapatric	236.88	193.28	1.22	parapatric

Table 5. Distribution of factor scores along the first four principal components for *P. atra* and *P. leucoptera*.

Comparison and statistical test employed	Sex	Principal Components			
		PC1	PC2	PC3	PC4
Between all populations of *P. atra* and all populations of *P. leucoptera*; t-test	Male	5.19***	-1.80	3.98***	1.42
	Female	4.47***	0.49	-1.95	0.61
Between all populations of *P. atra* and populations of *P. leucoptera* along the contact zone only; t-test	Male	0.20	0.82	-0.82	2.04*
	Female	0.23	1.65	-0.09	-0.99
Among (1) the northernmost population of *P. atra*, (2) populations of *P. atra* along the contact zone only, (3) populations of *P. leucoptera* along the contact zone only; nested ANOVA	Male	1.26	0.38	4.9*a	2.55
	Female	0.75	2.53	0.004	0.96

***p < 0.001; **p < 0.01; *p < 0.05

a - 1-2 and 2-3 group comparisons were significantly different (Tukey *post-hoc* test, p < 0.05)

Table 6. Among-population coefficients of variation (CV) and global means ± SE for loudsong elements of fire-eyes males and females in Atlantic Forest.

	Male			Female			Sex exhibiting
Loudsong element	Mean	SE	CV	Mean	SE	CV	larger variation
Number of notes	6.8	0.09	15.6%	4.9	0.23	24.0%	Female
Pace	1.8	0.02	10.4%	1.4	0.04	16.9%	Female
Length of note 1	146.1	1.65	14.0%	138.8	3.29	12.1%	Male
Frequency of note 1	2645.1	11.66	6.0%	2897.9	37.14	6.5%	Female
Maximum frequency of note 1	2513.1	11.29	5.4%	2663.1	41.07	7.9%	Female
Space of note 1	125.3	1.89	19.0%	131.2	4.67	18.2%	Male
Length of note 2	144.7	1.43	13.4%	143.1	3.44	12.2%	Male
Frequency of note 2	2656.7	11.56	5.9%	2884.2	36.41	6.4%	Female
Maximum frequency of note 2	2522.2	11.23	5.3%	2703.7	31.59	6.0%	Female
Space of note 2	128.0	1.89	18.8%	131.3	4.95	19.2%	Female
Length of note 3	148.4	1.49	14.1%	144.2	3.22	10.9%	Male
Frequency of note 3	2645.3	11.60	6.0%	2859.0	36.11	6.2%	Female
Maximum frequency of note 3	2503.6	11.11	5.0%	2706.5	30.57	5.5%	Female
Space of note 3	128.2	1.87	18.2%	130.0	5.89	22.2%	Female
Length of note 4	151.0	1.48	13.6%	158.6	10.97	35.3%	Female
Frequency of note 4	2616.7	10.97	5.6%	2824.0	31.11	5.6%	Equal
Maximum frequency of note 4	2476.2	10.34	4.7%	2670.3	28.51	5.4%	Female
Space of note 4	126.6	1.89	19.1%	129.3	5.22	20.6%	Female
Length of note 5	154.7	1.50	13.6%	155.0	4.51	14.8%	Female
Frequency of note 5	2516.3	10.65	5.7%	2690.3	30.08	5.7%	Equal
Maximum frequency of note 5	2369.5	9.87	4.9%	2540.1	25.14	5.1%	Female
Space of note 5	130.6	1.84	18.0%	129.5	5.11	20.1%	Female

Table 7. Factor scores from a principal component analysis of morphological variables and Spearman’s rank correlations of principal components against latitude for male and female fire-eyes of Atlantic Forest.

	Males			Females		
Variable	PC1	PC2	PC3	PC1	PC2	PC3
Wing length	0.67	0.4	-0.13	0.58	-0.40	0.21
Tail length	0.61	0.40	-0.41	0.57	-0.44	-0.37
Tarsus length	0.40	0.48	0.25	0.62	-0.22	-0.13
Bill length	0.05	0.39	0.86	0.42	0.20	0.82
Bill depth	-0.6	0.66	-0.11	0.49	0.64	-0.27
Bill width	-0.67	0.54	-0.19	0.30	0.74	-0.13
Spearman’s rank correlation	0.40**	0.32*	-0.72	0.57**	-0.12$^{\mathrm{n.s.}}$	

* $p < 0.05$; ** $p < 0.01$;

Table 8. Correlation and partial correlation coefficients of Mantel tests between loudsong distance and geographic distance, genetic distance, ecological distance* and morphological distance (body size and bill dimensions) among the populations of fire-eyes in Atlantic Forest. Mantel tests were performed separately for each sex and for the total range, populations inside the Bahia refuge, and populations outside the Bahia refuge. Values in brackets represent Mantel correlations for male and female populations sampled over the total range and inside the Bahia refuge, excluding the parapatric zone.

Group		*R*		*P*	
	Correlation	Males	Females	Males	Females
Total range	Loudsong *vs* geography	0.58 (0.68)	0.63 (0.69)	<0.0001 (<0.0001)	<0.0001 (<0.0001)
	Loudsong *vs* genetic	0.42 (0.45)	0.70 (0.72)	<0.0001 (<0.0001)	<0.0001 (<0.0001)
	Loudsong *vs* ecology	0.14 (0.09)	0.33 (0.38)	0.03 (0.29)	<0.0001 (<0.0001)
	Loudsong *vs* body size	0.26 (0.32)	0.48 (0.46)	<0.001 (<0.0001)	<0.0001 (<0.0001)
	Loudsong *vs* bill culmen length	-0.06 (-0.18)	0.07 (0.02)	0.38 (0.03)	0.38 (0.82)
	Loudsong *vs* bill width and depth	-0.03 (-0.02)	-0.04 (-0.06)	0.63 (0.34)	0.65 (0.55)
	Partial correlation				
	Loudsong *vs* geography	0.42 (0.54)	0.18 (0.27)	<0.0001 (<0.0001)	0.03 (0.008)
	Loudsong *vs* genetic	0.07 (0.05)	0.35 (0.36)	0.28 (0.56)	<0.0001 (<0.001)
	Loudsong *vs* ecology	-0.12 (-0.17)	0.05 (0.12)	0.65 (0.05)	0.55 (0.26)
	Loudsong *vs* body size	0.12 (0.17)	0.06 (-0.03)	0.05 (0.04)	0.45 (0.76)
	Loudsong *vs* bill culmen length	0.00 (-0.03)	0.05 (0.13)	0.92 (0.72)	0.49 (0.20)
	Loudsong *vs* bill width and depth	0.03 (0.04)	-0.00 (0.00)	0.68 (0.65)	0.91 (0.96)
Bahia refuge	*Correlation*				
	Loudsong *vs* geography	0.55 (0.30)	0.85 (0.74)	<0.0001 (0.40)	<0.0001 (0.06)
	Loudsong *vs* genetic	0.57 (0.61)	0.88 (0.78)	<0.0001 (0.06)	<0.0001 (0.05)
	Loudsong *vs* ecology	-0.00 (0.23)	0.72 (0.71)	0.98 (0.52)	<0.0001 (0.70)
	Loudsong *vs* body size	0.32 (0.08)	0.54 (0.07)	0.03 (0.83)	<0.001 (0.90)
	Loudsong *vs* bill culmen length	0.07 (-0.03)	0.54 (0.08)	0.65 (0.0.93)	<0.001 (0.87)
	Loudsong *vs* bill width and depth	0.34 (0.53)	0.58 (-0.24)	0.03 (0.11)	<0.001 (0.63)
	Partial correlation				
	Loudsong *vs* geography	0.22 (-0.05)	0.00 (-0.40)	0.15 (0.90)	0.97 (0.38)
	Loudsong *vs* genetic	0.13 (0.45)	0.31 (0.42)	0.37 (0.20)	0.04 (0.34)
	Loudsong *vs* ecology	-0.16 (0.05)	0.26 (-0.11)	0.28 (0.90)	0.08 (0.83)
	Loudsong *vs* body size	-0.08 (0.24)	-0.01 (-0.28)	0.60 (0.51)	0.93 (0.53)
	Loudsong *vs* bill culmen length	0.12 (-0.27)	0.00 (0.31)	0.43 (0.50)	0.96 (0.48)
	Loudsong *vs* bill width and depth	0.29 (0.30)	-0.02 (0.04)	0.06 (0.40)	0.88 (0.94)
Outside of Bahia refuge	*Correlation*				
	Loudsong *vs* geography	0.20	0.12	0.09	0.41
	Loudsong *vs* genetic	0.39	0.57	0.002	<0.001
	Loudsong *vs* ecology	0.13	0.31	0.30	0.004
	Loudsong *vs* body size	0.39	0.40	<0.001	0.007
	Loudsong *vs* bill culmen length	0.01	0.02	0.91	0.88
	Loudsong *vs* bill width and depth	-0.02	0.12	0.90	0.45
	Partial correlation				
	Loudsong *vs* geography	0.06	-0.29	0.64	0.05
	Loudsong *vs* genetic	0.25	0.26	0.04	0.08
	Loudsong *vs* ecology	-0.03	0.34	0.82	0.02
	Loudsong *vs* body size	0.32	0.25	0.01	0.09
	Loudsong *vs* bill culmen length	-0.07	-0.16	0.56	0.28
	Loudsong *vs* bill width and depth	0.06	0.19	0.65	0.21

*climatic variables used were temperature annual range, annual precipitation, temperature seasonality, precipitation seasonality, precipitation of the wettest month, precipitation of the wettest quarter and precipitation of the coldest quarter

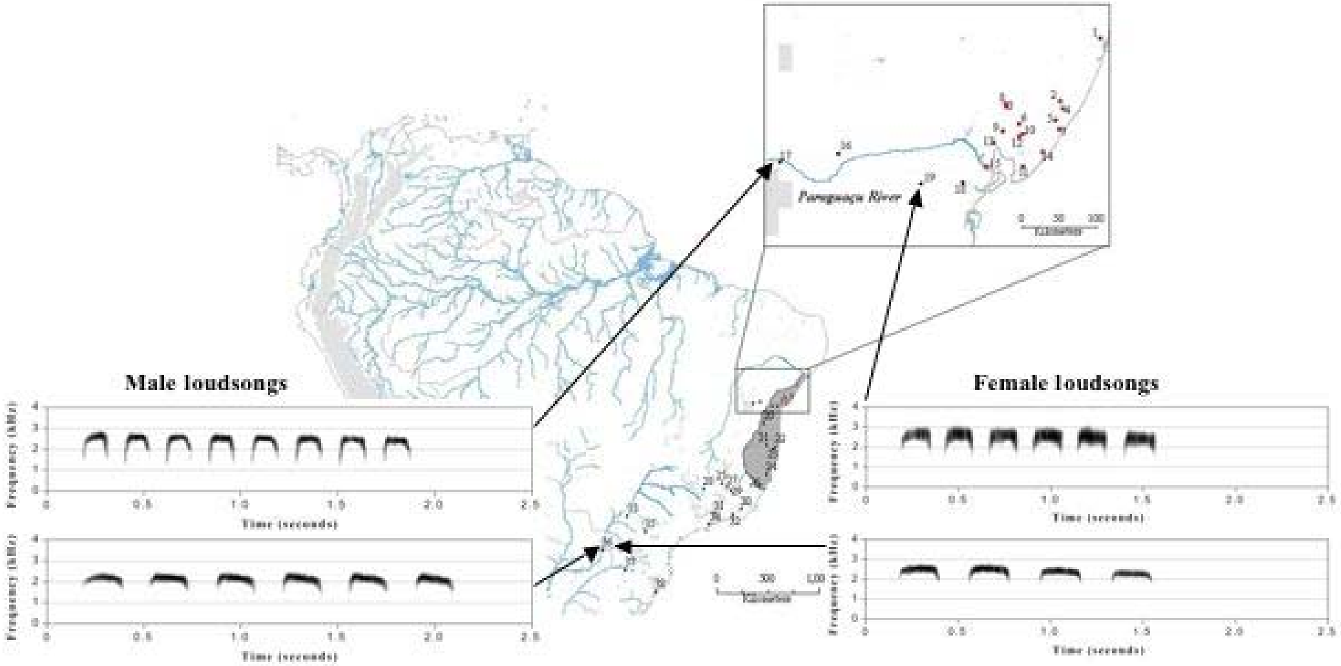


Figure 1. Recording localities and examples of loudsongs of fire-eye antbirds (genus *Pyriglena*) across Atlantic Forest, Brazil. Black circles and red squares represent vocalizations sampled inside *P. leucoptera* and *P. atra* ranges, respectively. The dark grey shaded area depicts the Bahia Pleistocene refuge (after Carnaval and Moritz 2008) and the inset depicts the parapatric area between the two species along the Paraguaçu River valley. The populations 2-19 were considered as part of the parapatric area in the analyses. Spectrograms depict male (left) and female (right) loudsongs in the northernmost and southernmost sampling localities. Note that northern loudsongs have more notes, higher frequency, shorter notes and shorter space between notes relative to southern loudsongs. Spectrograms show frequency (kHz) in the *y* axes and time (seconds) in the *x* axes. Light shaded gray indicates areas above 1000 m a.s.l.

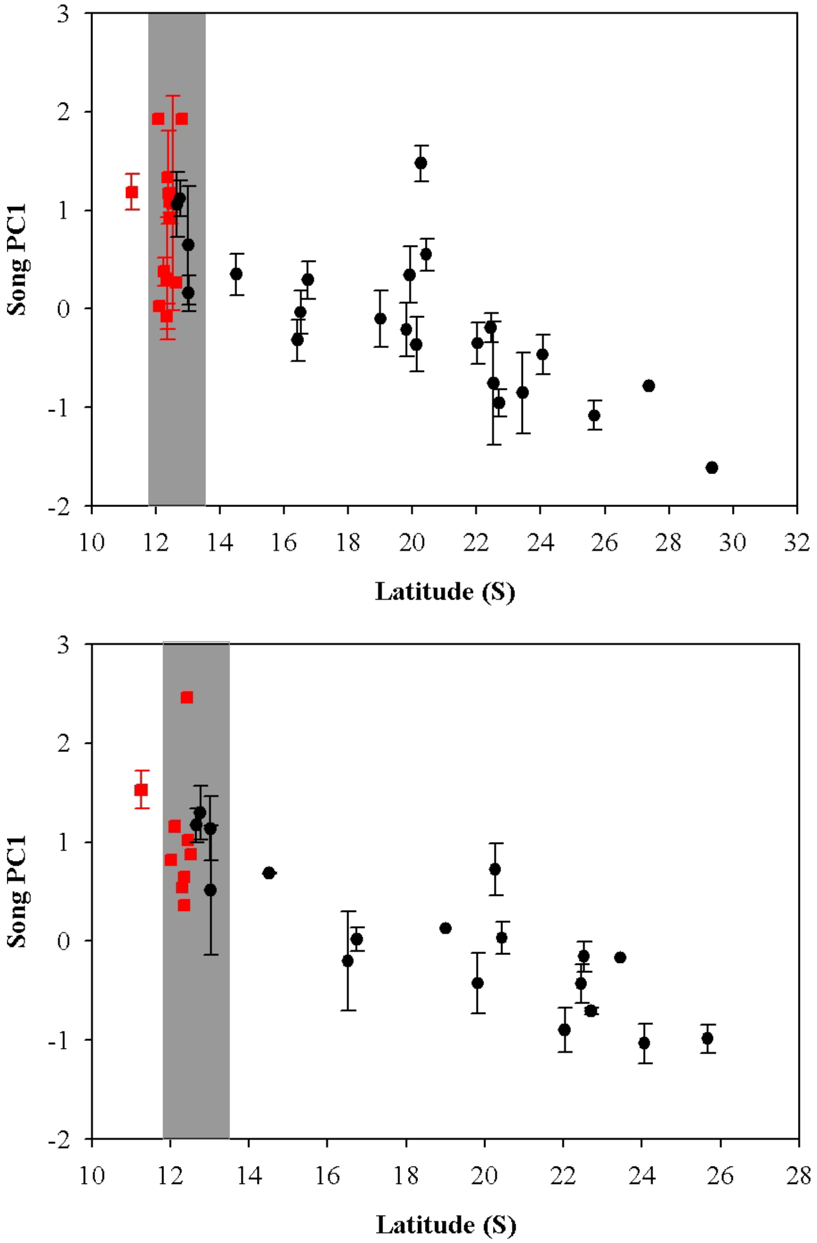


Figure 2. Latitudinal variation in loudsong PC1 scores among male (top figure) and female (bottom figure) fire-eye populations. Error bars represent standard error of the mean, using individuals as replicates. Localities without error bars had only one individual sampled. Red squares show populations of *Pyriglena atra* and black dots show populations of *P. leucoptera*. Shaded area depicts the parapatric area between the two species.

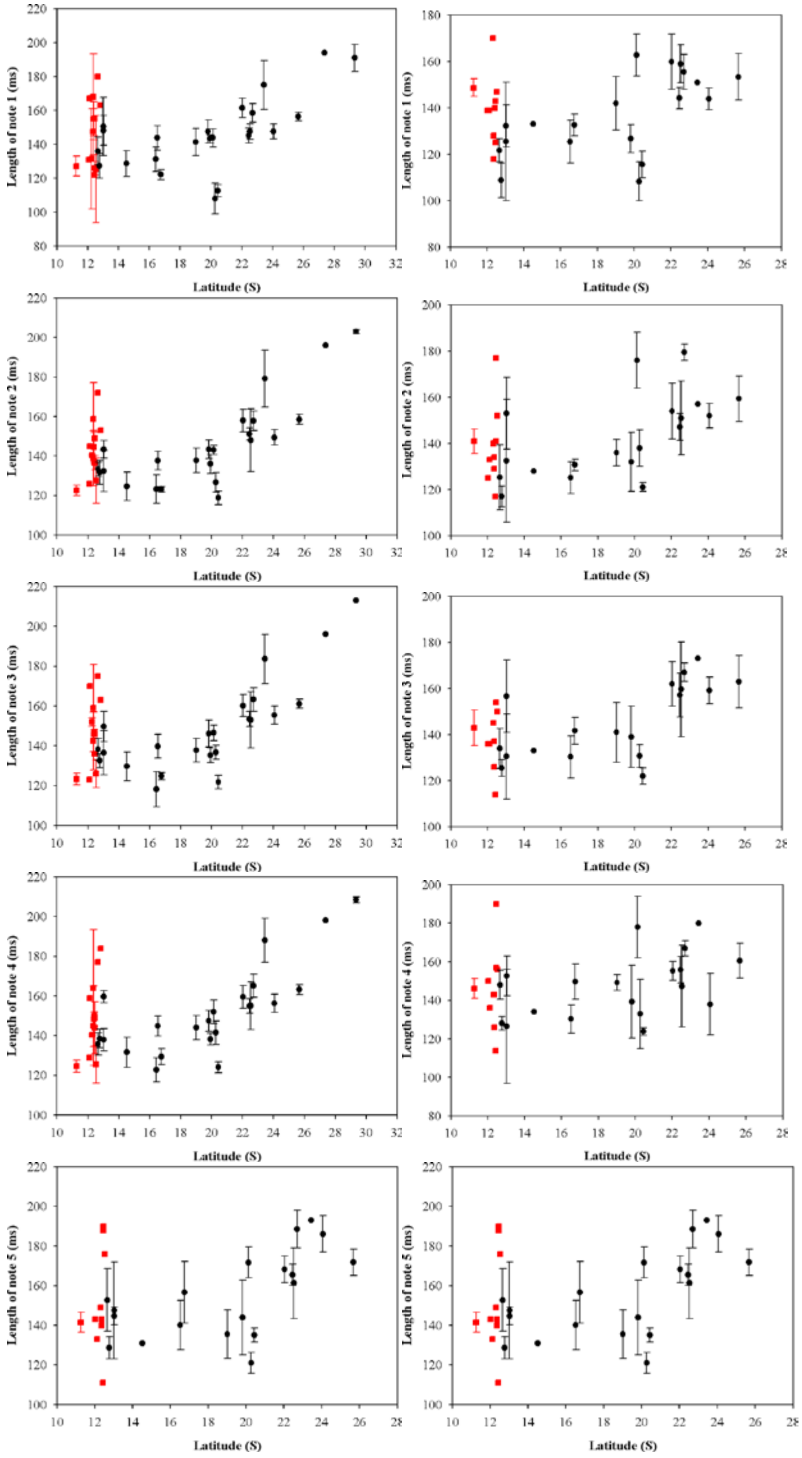


Figure 3. Latitudinal variation in note length of loudsongs for male (left column) and for female (right column) fire-eyes in Atlantic Forest. Error bars represent standard error of the mean, using individuals as replicates. Localities without error bars had only one individual sampled. Red squares show populations of *Pyriglena atra* and black dots show populations of *P. leucoptera*. See Figure 1 for location of the parapatric area between the two species.

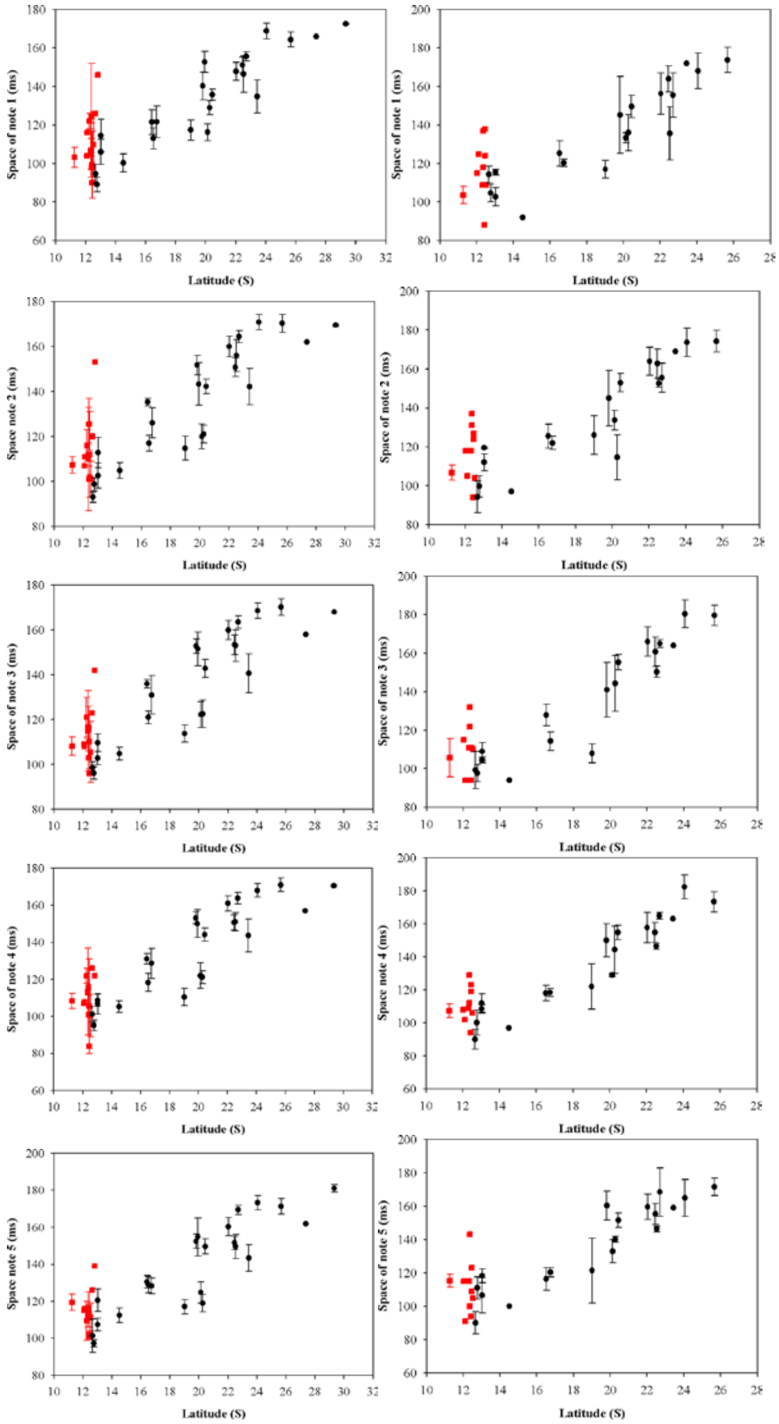


Figure 4. Latitudinal variation in note space of loudsongs for male (left column) and for female (right column) fire-eyes in Atlantic Forest. Error bars represent standard error of the mean, using individuals as replicates. Localities without error bars had only one individual sampled. Red squares show populations of *Pyriglena atra* and black dots show populations of *P. leucoptera*. See Figure 1 for location of the parapatric area between the two species.

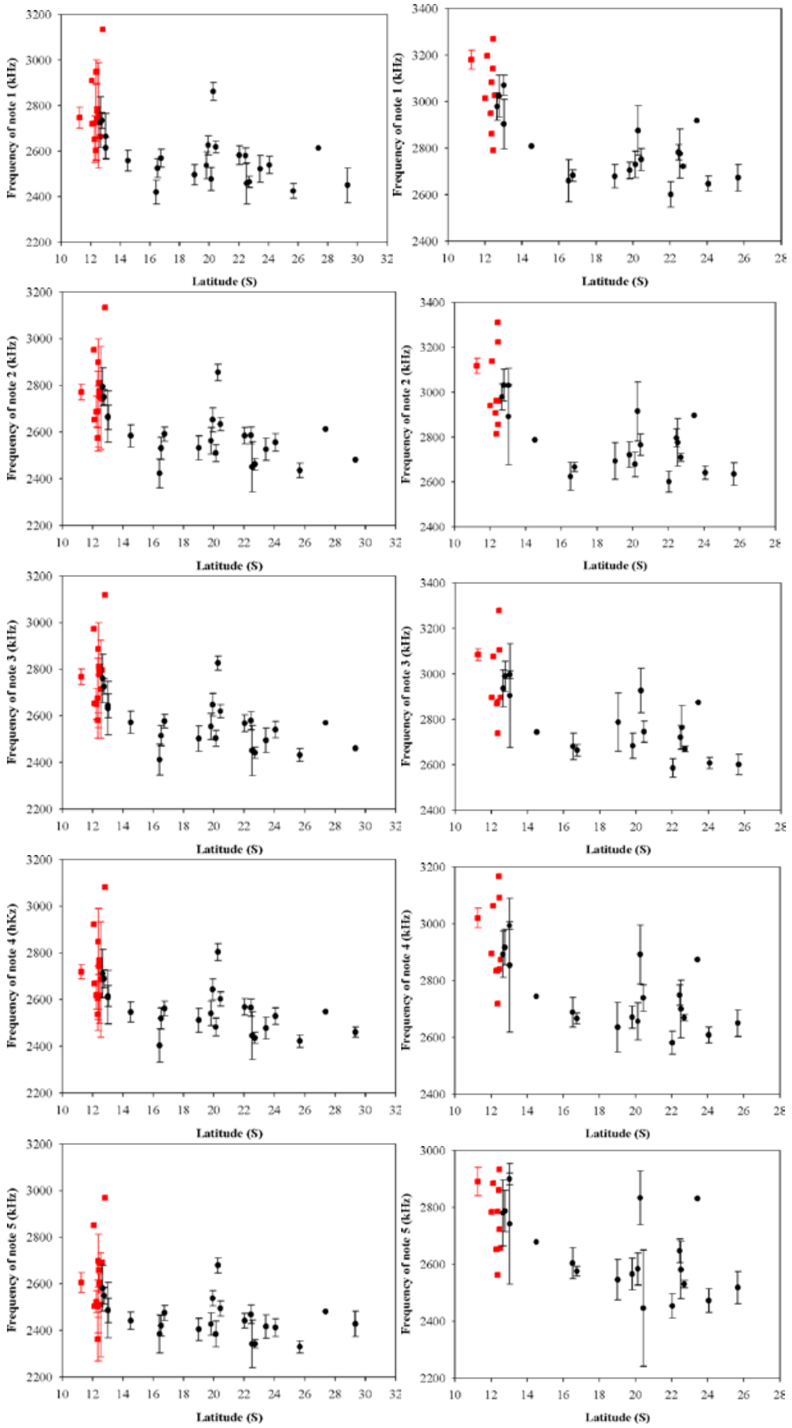


Figure 5. Latitudinal variation in note frequency of loudsongs for male (left column) and for female (right column) fire-eyes in Atlantic Forest. Error bars represent standard error of the mean, using individuals as replicates. Localities without error bars had only one individual sampled. Red squares show populations of *Pyriglena atra* and black dots show populations of *P. leucoptera*. See Figure 1 for location of the parapatric area between the two species.

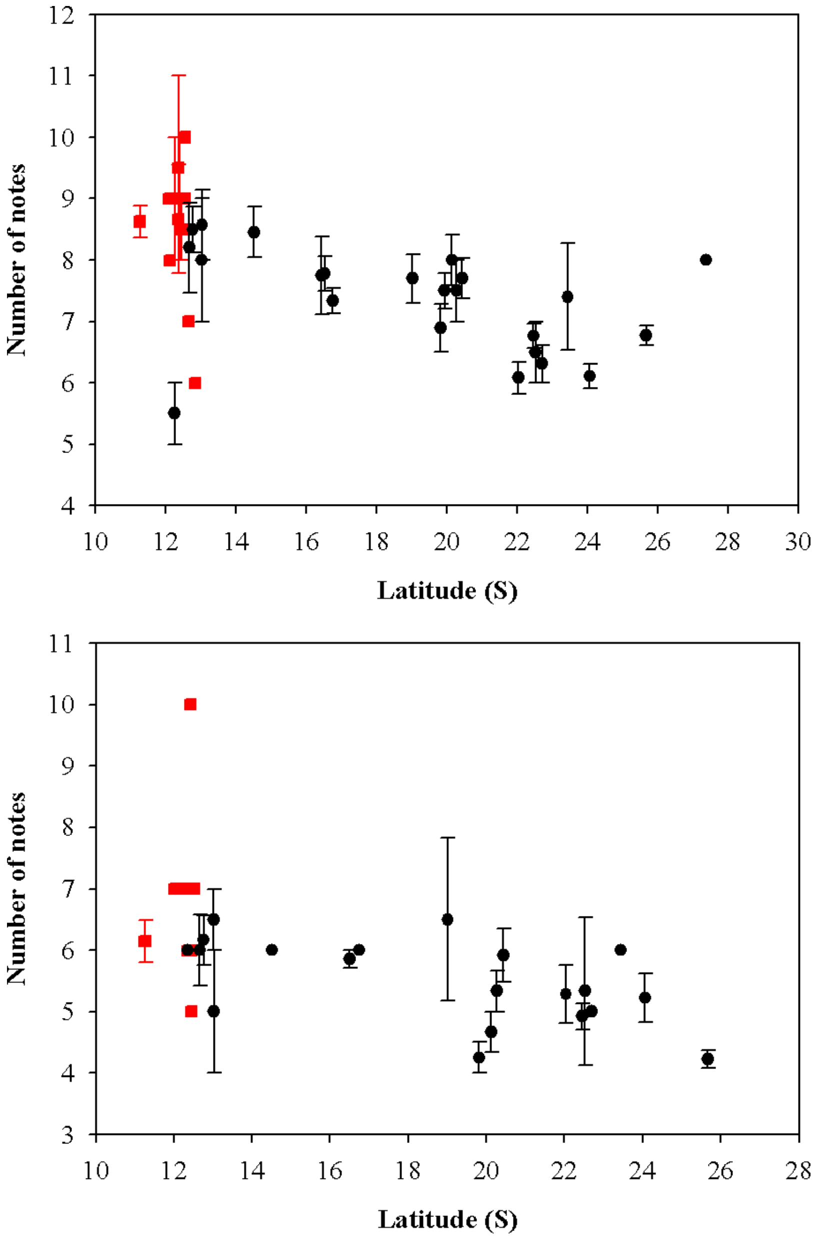


Figure 6. Latitudinal variation in number of notes of loudsongs for male (top figure) and for female (bottom figure) fire-eyes in Atlantic Forest. Error bars represent standard error of the mean, using individuals as replicates. Localities without error bars had only one individual sampled. Red squares show populations of *Pyriglena atra* and black dots show populations of *P. leucoptera*. See Figure 1 for location of the parapatric area between the two species.

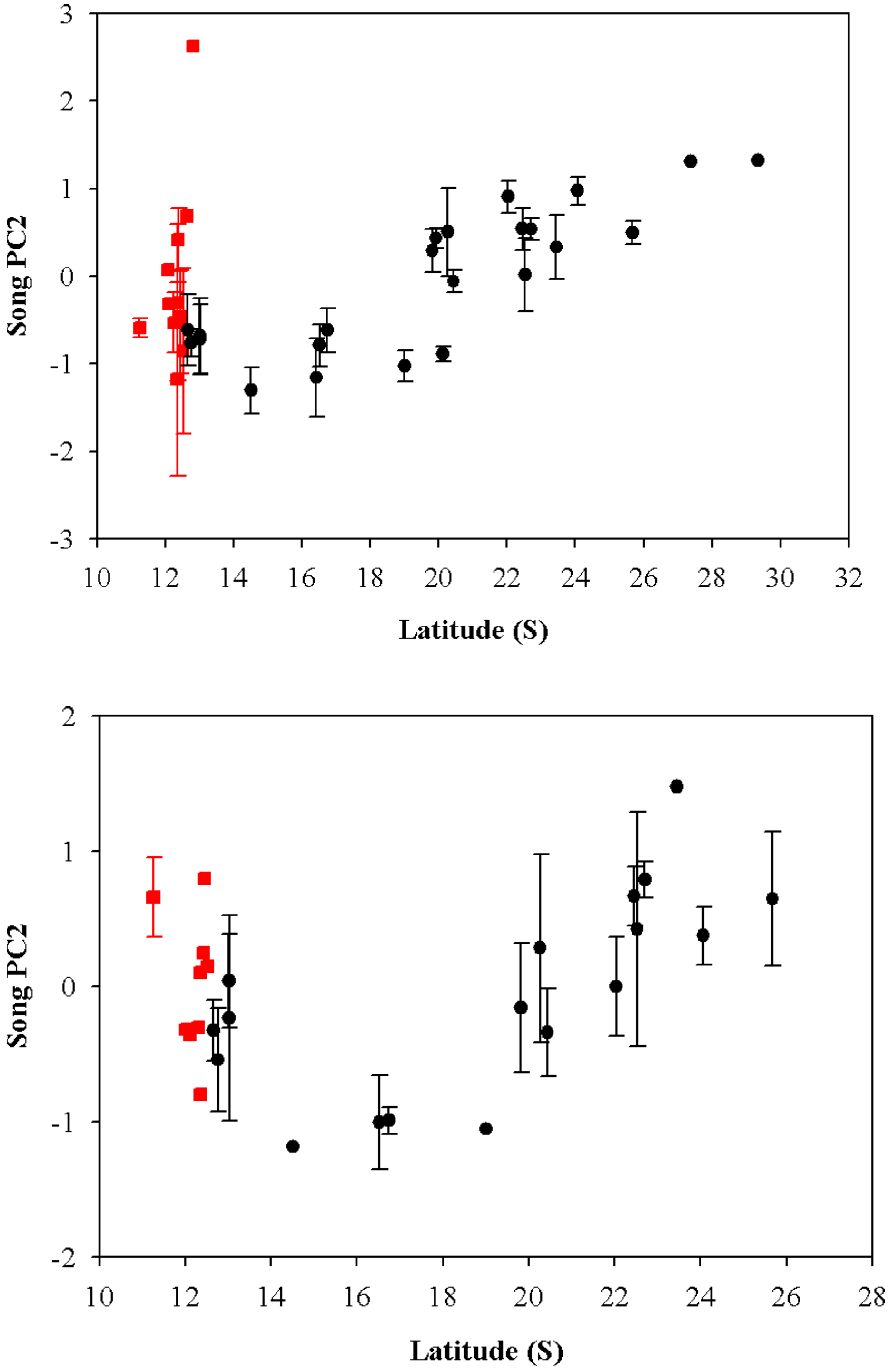
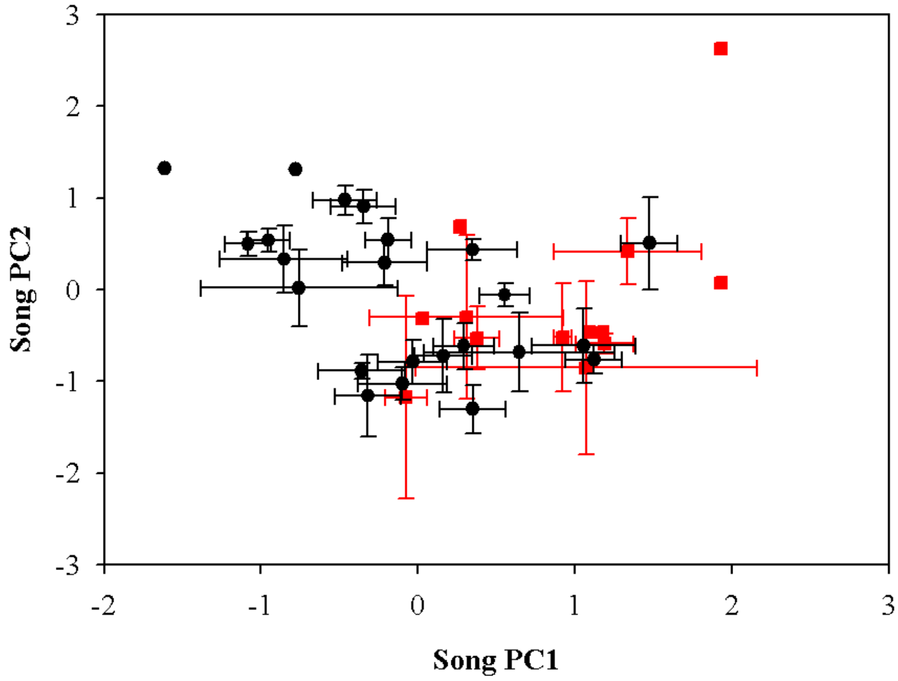


Figure 7. Latitudinal variation among males (top figure) and female (bottom figure) fire-eye populations in loudsong PC2 scores. Error bars represent standard error of the mean, using individuals as replicates. Localities without error bars had only one individual sampled. Red squares show populations of *Pyriglena atra* and black dots show populations of *P. leucoptera*. See Figure 1 for location of the parapatric area between the two species.



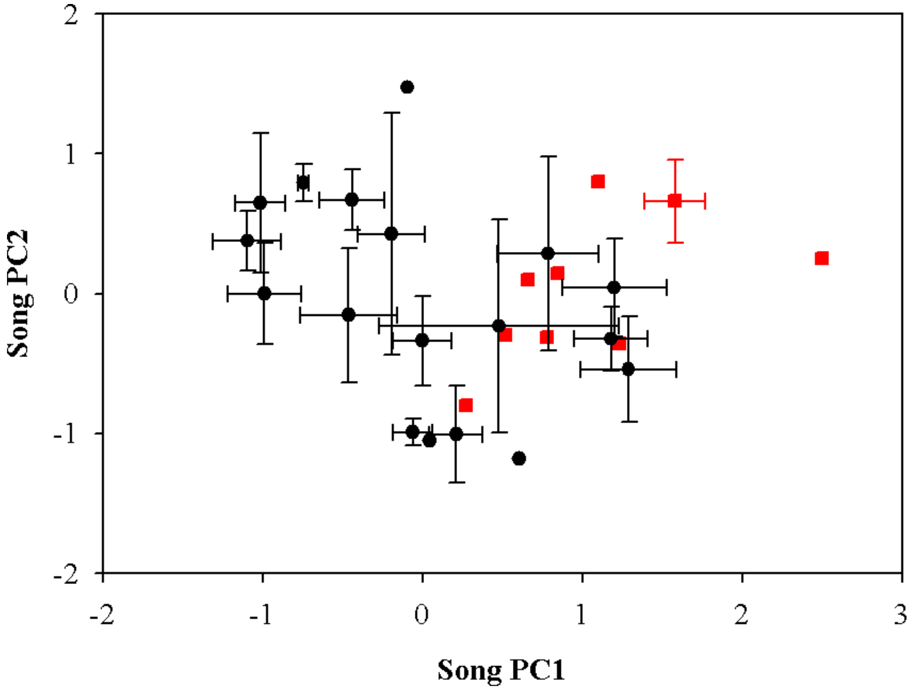


Figure 8. Principal components 1 and 2 depicting geographic variation in loudsongs of males (top figure) and females (bottom figure) in Atlantic Forest. Some localities have no error bars because only one individual was sampled. Red squares show populations of *Pyriglena atra* and black dots show populations of *P. leucoptera*.

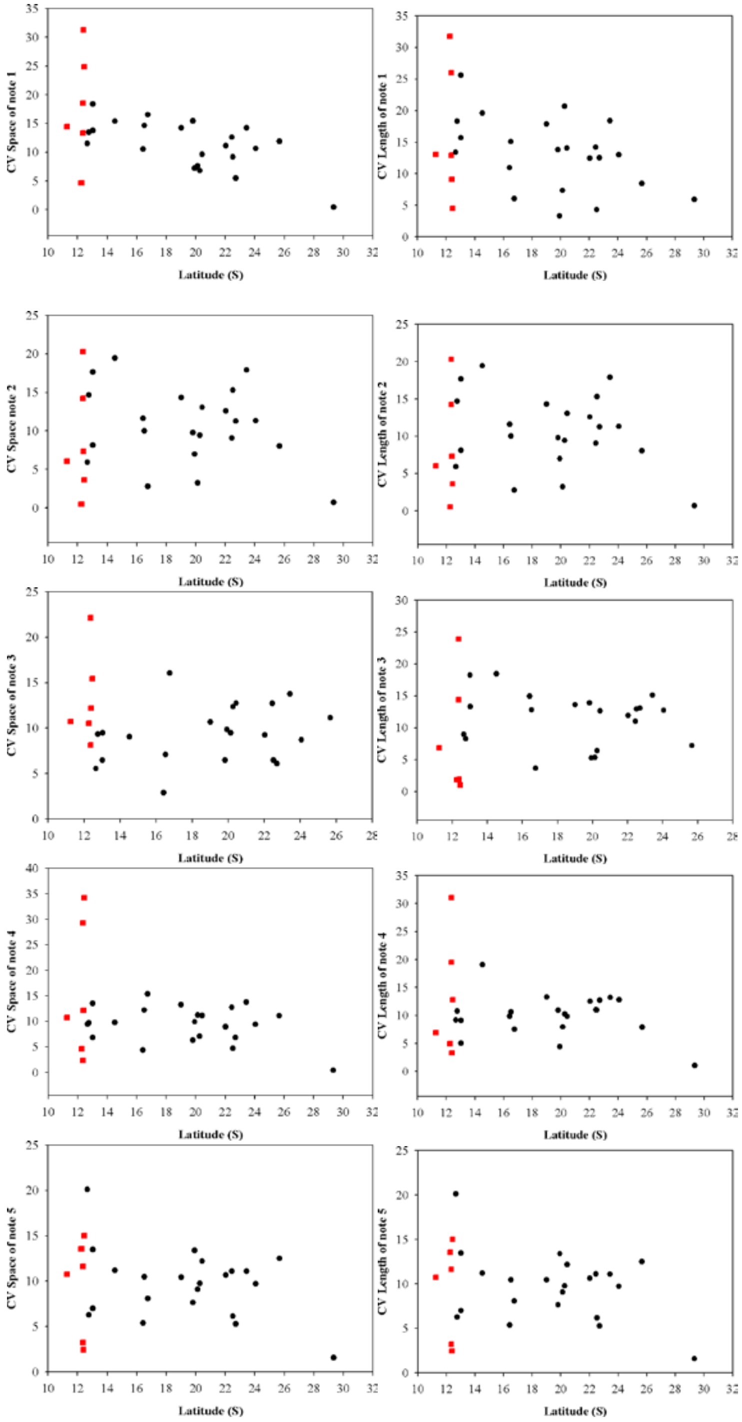


Figure 9. Geographic variation in coefficients of variation (CVs) for space of notes (left column) and length of notes (right column) of fire-eye loudsongs in Atlantic Forest. See Figure 1 for location of the parapatric area between the two species.

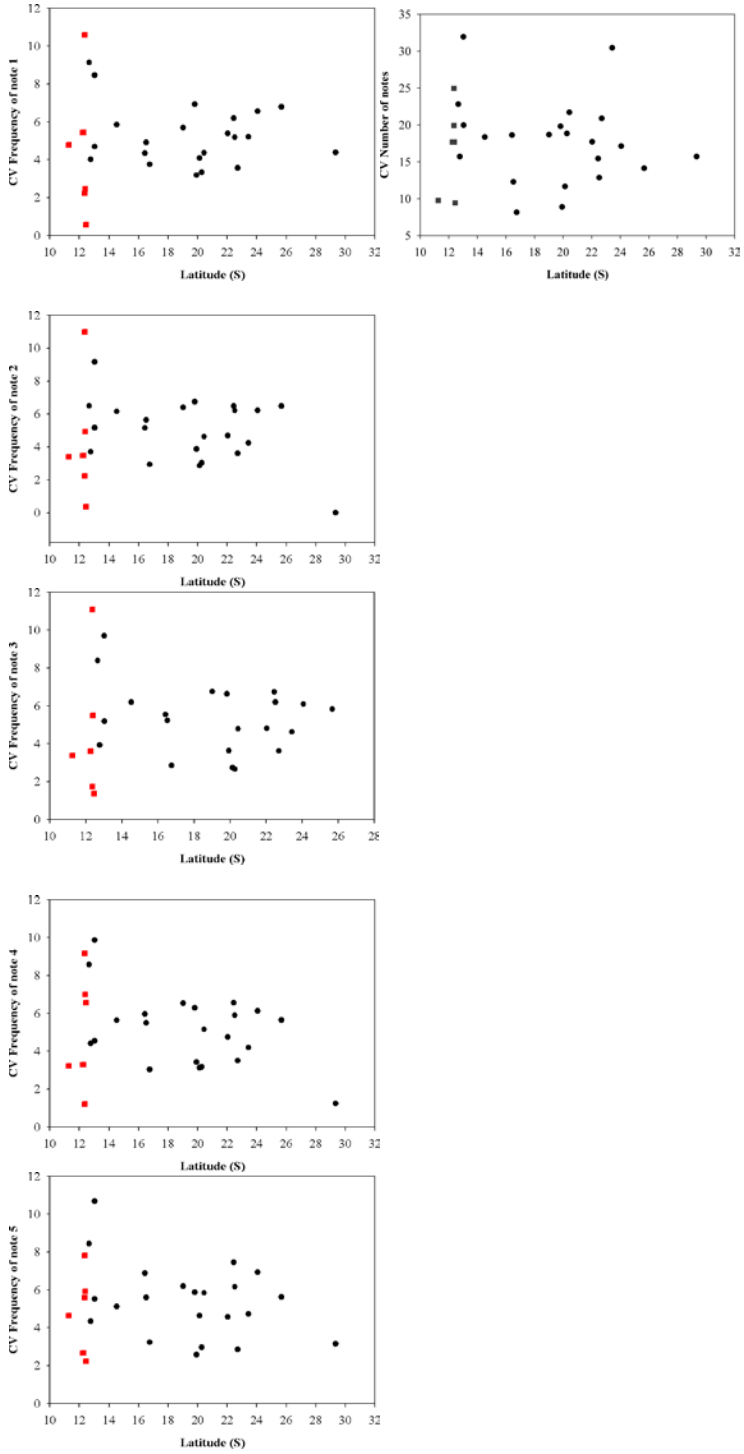


Figure 10. Geographic variation in coefficients of variation (CVs) for frequency of notes (left column) and pace notes (number of notes, right column) of fire-eye loudsongs in Atlantic Forest. See Figure 1 for location of the parapatric area between the two species.

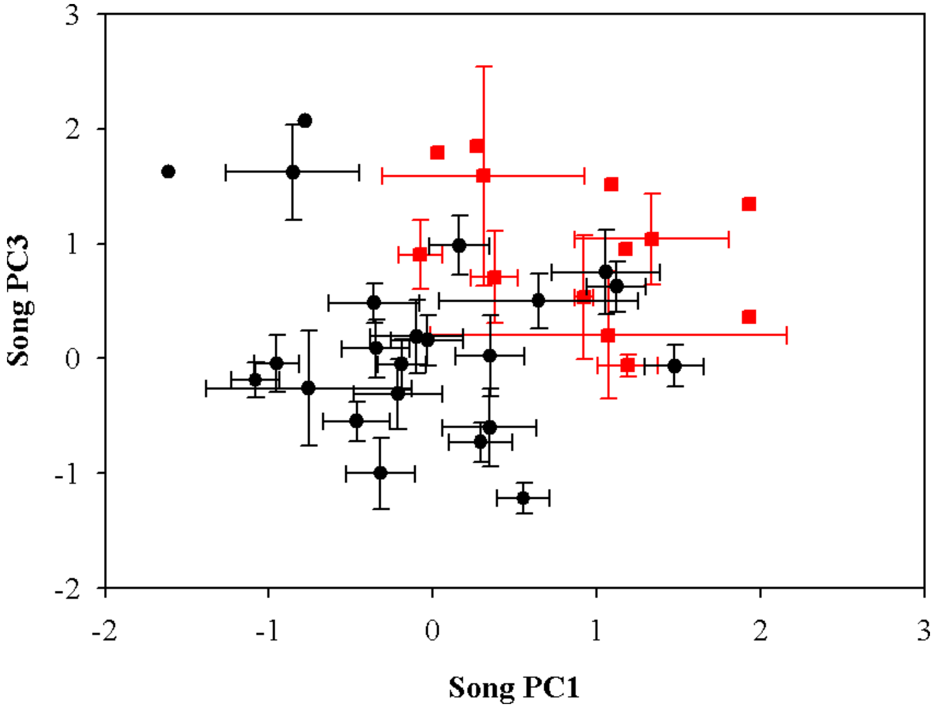
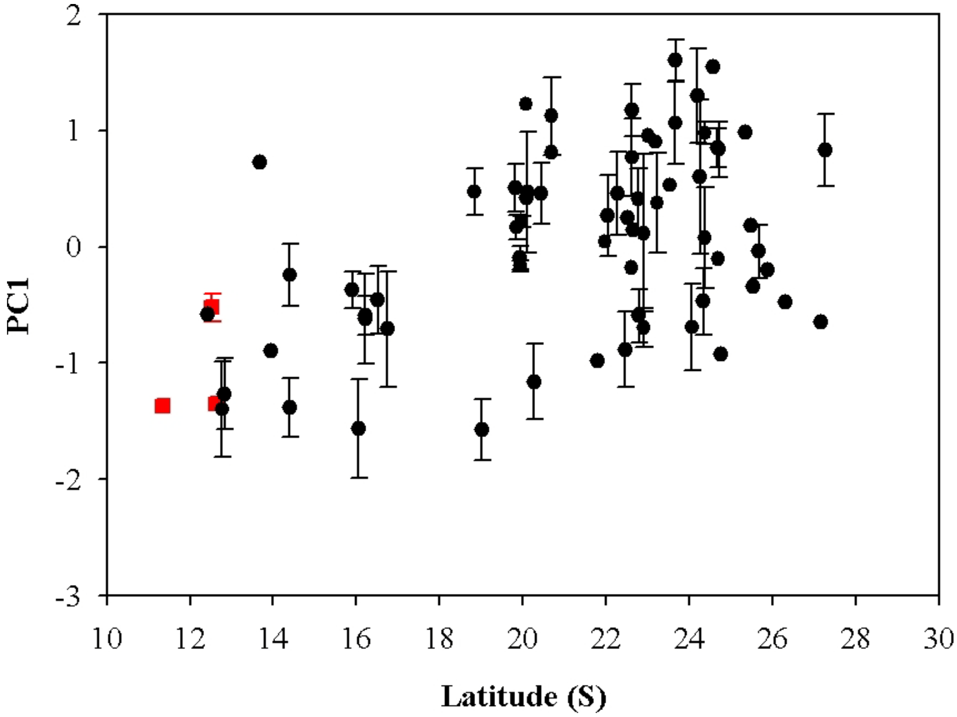


Figure 11. Principal components 1 and 3 depicting geographic variation in loudsongs of fire-eye males in Atlantic Forest. Some localities have no error bars because only one individual was sampled. Red squares show populations of *Pyriglena atra* and black dots show populations of *P. leucoptera*.



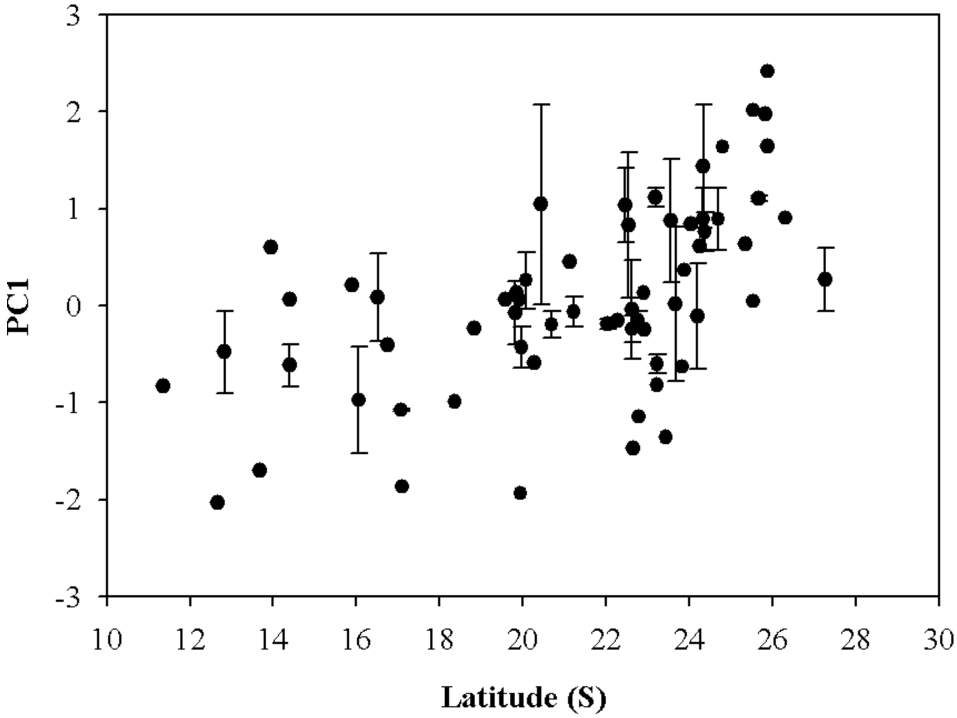


Figure 12. Latitudinal variation among male (top figure) and female (bottom figure) fire-eye populations in morphometric PC1 scores. Error bars represent standard error of the mean, using individuals as replicates. Localities without error bars had only one individual sampled. Red squares show populations of *Pyriglena atra* (not available for females) and black dots show populations of *P. leucoptera*.

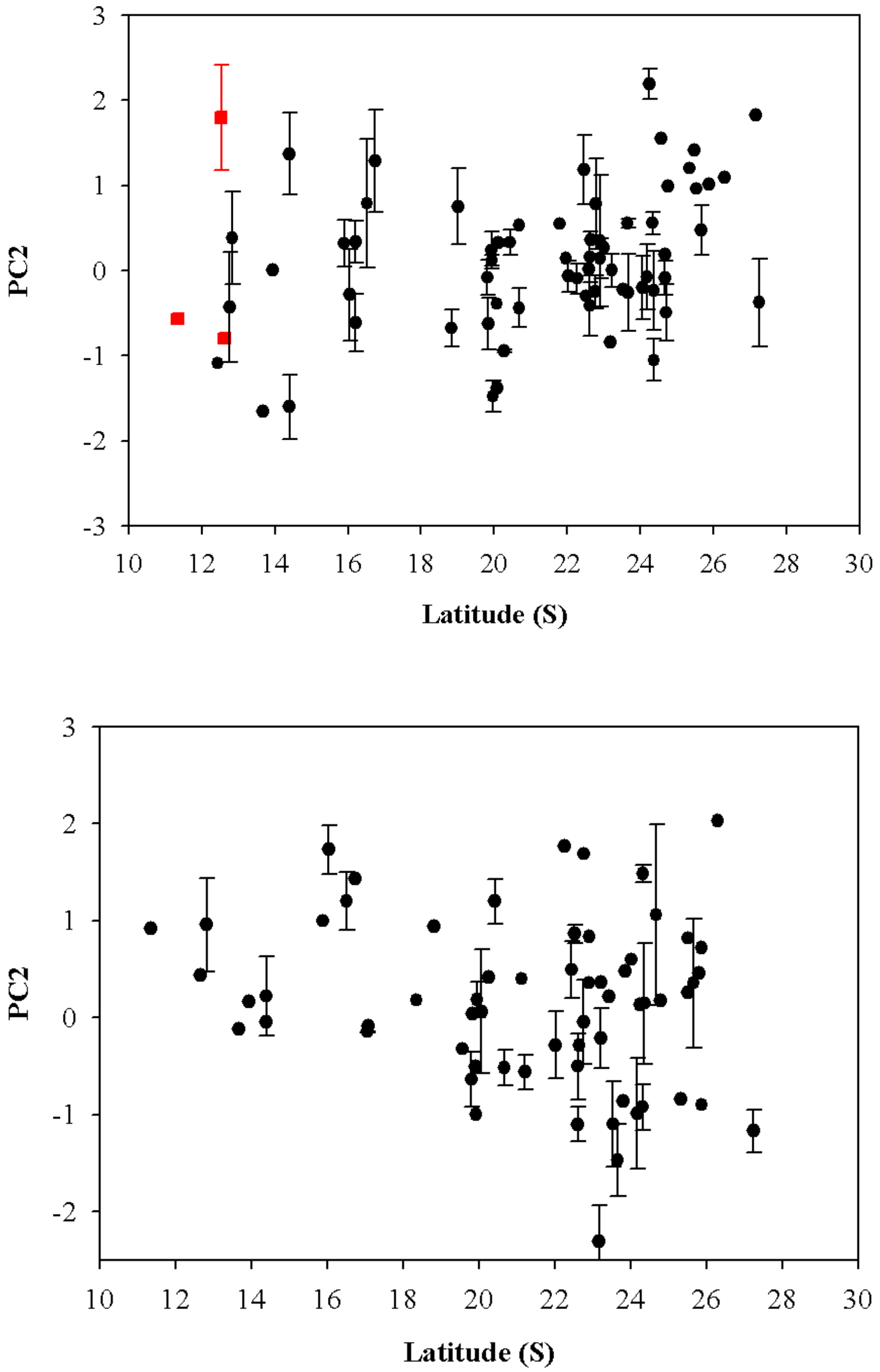


Figure 13. Latitudinal variation among male (top figure) and female (bottom figure) fire-eye populations in morphometric PC2 scores. Error bars represent standard error of the mean, using individuals as replicates. Localities without error bars had only one individual sampled. Red squares show populations of *Pyriglena atra* (not available for females) and black dots show populations of *P. leucoptera*.

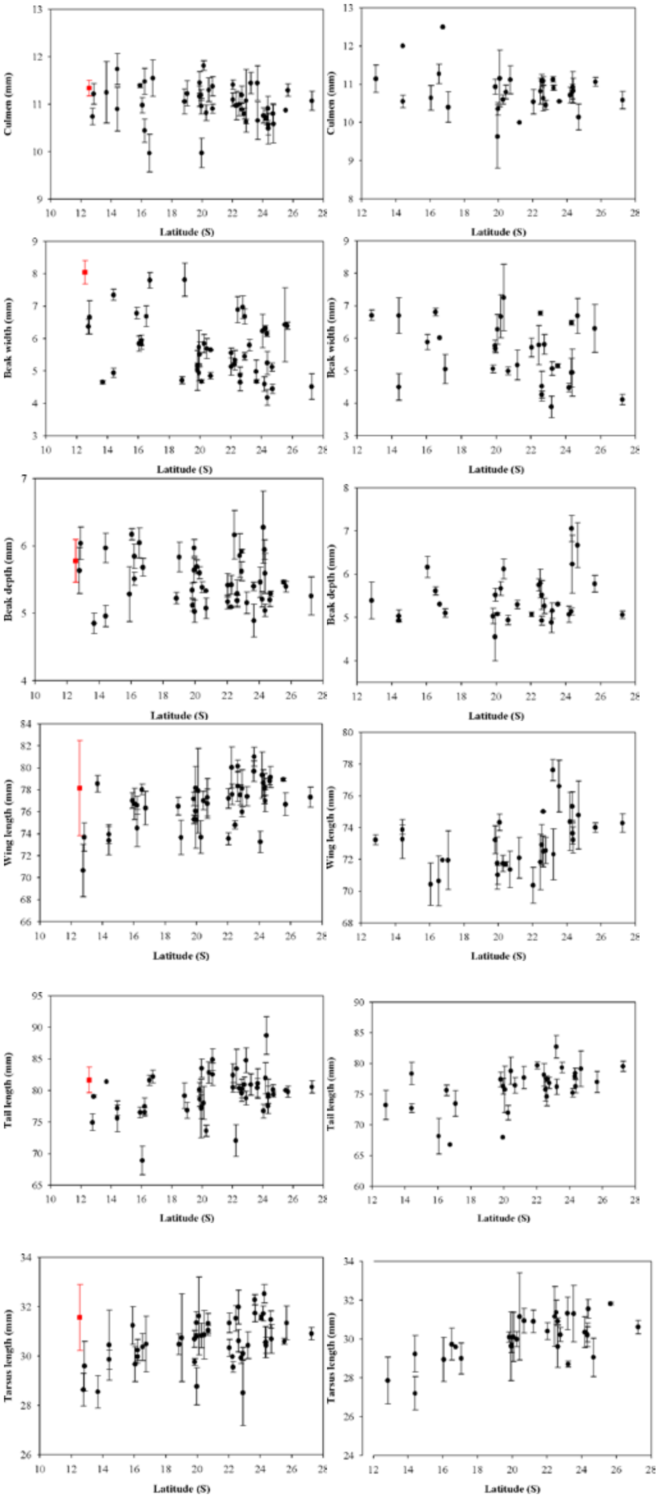


Figure 14. Latitudinal variation in the six morphometric characters for male (left column) and female (right column) throughout the fire-eye range in Atlantic Forest. Error bars represent standard error of the mean, using individuals as replicates. Localities without error bars had only one individual sampled. Red squares show populations of *Pyriglena atra* (not available for females) and black dots show populations of *P. leucoptera.*